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Robust SARS-CoV-2-specific T-cell immunity is maintained at 6 months following primary infection

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Abstract

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#These authors jointly supervised this work

Author Contribution Statement

JZ, AD, HP, KV, HL and JB performed cellular analyses and data analysis. FA, ZA-C, LS, RB, EL, SA, BP, AH, GA, KB, MR and SL devised, set up and recruited the ESCAPE cohort. BH performed humoral analyses. SL, JZ and PM led on data interpretation and writing of the manuscript. All authors discussed the results and commented on the manuscript.

Competing Interests statement

The authors declare no competing interests.

The immune response to SARS-CoV-2 is critical in controlling disease but there is concern that waning immunity may predispose to re-infection. We analysed the magnitude and phenotype of the SARS-CoV-2-specific T cell response in 100 donors at six months following infection. T-cell responses were present by ELISPOT and/or ICS analysis in all donors and characterised by predominant CD4⁺ T cell responses with strong IL-2 cytokine expression. Median T-cell responses were 50% higher in donors who had experienced a symptomatic infection indicating that the severity of primary infection establishes a ‘setpoint’ for cellular immunity. T-cell responses to spike and nucleoprotein/membrane proteins were correlated with peak antibody levels. Furthermore, higher levels of nucleoprotein-specific T cells were associated with preservation of NP-specific antibody level although no such correlation was observed in relation to spike-specific responses. In conclusion, our data are reassuring that functional SARS-CoV-2-specific T-cell responses are retained at six months following infection.

Introduction

The SARS-CoV-2 pandemic has led to over 2 million deaths to date and there is an urgent need for an effective vaccine¹. There is considerable interest in understanding how adaptive immune responses act to control acute infection and provide protection from reinfection. Antibody responses against SARS-CoV-2 are characterised by responses against a range of viral proteins, including the spike, nucleoprotein and membrane proteins. Antibody levels do decline after clearance of primary infection and there is currently considerable interest in the relative maintenance of humoral responses over the longer term. Although initial analyses had shown loss of detectable virus-specific antibodies in a proportion of individuals, highly sensitive ELISAs can demonstrate detectable antibodies for at least 6-7 months in most people²⁻⁸. Information derived from study of immunity to related viruses such as SARS-CoV-1 and MERS⁹ has shown that cellular immune responses against these viruses are maintained for longer periods of time compared to antibody responses^{10,11}. This has led to the hope that cellular responses to SARS-CoV-2 will similarly be of more prolonged duration^{12,13}.

Studies to date have shown that virus-specific cellular responses develop in virtually all patients with confirmed SARS-CoV-2 infection¹⁴. These responses remain detectable for several weeks following infection but it is currently unknown how they are maintained thereafter¹⁵. In this study we characterised SARS-CoV-2-specific T cell immune responses in a cohort of 100 donors at 6-months post-infection.

Results

Characteristics of enrolled donors in the study

Blood samples were obtained from 100 convalescent donors at 6 months following initial SARS-CoV-2 infection in March-April 2020. Among the 100 donors, 77 (77%) were female and 23 (23%) were male with a median age of 41.5 years (22–65 years). None of the donors required hospitalisation at any time during the course of the study. Fifty-six (45 female and 11 male) of the 100 donors who experienced clinical symptoms of respiratory illness were grouped as “symptomatic” and 44 (32 female and 12 male) who did not experience any

respiratory illness were grouped as “asymptomatic”. There was no significant difference between the median age of the symptomatic (42.5 (23-62) years) and asymptomatic donors (40 (22-65) years). The characteristics of all the patients was summarised in Supplementary Table.

T-cell responses against SARS-CoV-2 are present in all donors

Interferon gamma (IFN- γ) ELISPOT analysis was used to determine the magnitude of the global SARS-CoV-2-specific T cell response. Peptide pools from a range of viral proteins, including spike, nucleoprotein and membrane protein, were used to stimulate fresh PBMC and the magnitude of the global SARS-CoV-2-specific T-cell response was determined. Median ELISPOT responses against the Spike glycoprotein (Spike); Nucleoprotein and Membrane (N/M); and ORF3a, ORF10, NSP8, NSP7A/b (Accessory) peptide pools were measured at 1 in 10,000 (0.010%), 12,500 (0.008%) and 66,666 (0.0015%) PBMC respectively (Figure 1A). Using the pre-2020 healthy donor PBMCs (Extended Data Figure 1) to set the cut-off point, 90 of 95 donors (95%) demonstrated a SARS-CoV-2-specific T-cell response to at least one protein with a median total value of 200 cells per million PBMC (1 in 5000) (Figure 1A). Eighteen donors did not have a demonstrable cellular response to Spike and no response to the N/M pool was seen in 8 individuals. No detectable response to any protein tested was seen in 5 donors by ELISPOT assay although all these donors responded by parallel intracellular cytokine analysis (Figure 1B).

Considerable heterogeneity was observed in relation to the magnitude of this response. The global and peptide-specific responses were then assessed in relation to the clinical features at the time of primary infection. Importantly, median aggregate ELISPOT responses were 50% higher in donors who had initially demonstrated symptomatic disease compared to those with asymptomatic infection (Figure 2A). This profile was consistent against both spike and aggregate N/M proteins where values were 42% and 55% higher respectively in donors with initial symptomatic infection (Figure 2B). No association was seen between ELISPOT response and donor age. These data collectively demonstrated that T-cell responses against SARS-CoV-2 can be detected in all donors and are 50% higher in donors with an initial symptomatic infection.

IL-2 production dominates SARS-CoV-2-specific T cell responses

Intracellular cytokine analysis (Gating strategy in Extended Data Figure 2) was then utilised to assess the specificity and pattern of cytokine production from SARS-CoV-2-specific CD4⁺ and CD8⁺ T-cells in 100 donors. Virus-specific cytokine responses were seen in 96 people, including the 5 individuals that had been negative by ELISPOT analysis (Figure 3A). Interestingly, CD4⁺ virus-specific T cell responses were twice as frequent as CD8⁺ responses at this six-month time point (0.025% of CD4⁺ pool vs 0.012% of CD8⁺ pool respectively) (Figure 3B). In particular, mean CD4⁺ responses against spike and non-spike (N/M/Accessory) proteins were measured at 0.009% and 0.015% of the CD4⁺ repertoire respectively whereas corresponding values for CD8⁺ cells were 0.0050% and 0.0078% (Figure 3B). No differences were observed in the virus-specific CD4:CD8 ratio in relation to demographic factors such as age, symptomatic disease or gender.

As expected, the profile of cytokine production differed between the CD4⁺ and CD8⁺ subsets (Figure 4A, B). IL-2 responses were dominant within CD4⁺ subsets (Figure 4B) and the pattern of cytokine production by virus-specific CD4⁺ T cells was dependent on antigenic specificity. Single IFN- γ , single IL-2 and dual positive IL-2+IFN- γ T cells comprised 0.0016%, 0.0052% and 0.0026% of the spike-specific CD4⁺ T cell response respectively, compared to 0.0017%, 0.0105% and 0.0031% of the non-spike-specific repertoire (Figure 4C).

Analysis of the Th cytokine profile in the supernatants of overnight *ex vivo* peptide-stimulated ELISPOT cultures confirmed IL-2 to be the dominant cytokine released by SARS-CoV-2 specific T cells with variable tumor necrosis factor (TNF) release, alongside IFN- γ as detected by ELISPOT. There was no release of cytokines indicative of other Th subsets including Th2 and Th17 (Extended Data Figure 3).

The magnitude of CD4⁺ T cell responses against spike and non-spike proteins within each individual was strongly correlated (Figure 4D). However, this association was less marked for the CD8⁺ subset where responses were dominant against non-spike proteins (Figure 4D). Altogether, these results suggest that SARS-CoV-2-specific T cell responses are characterised by a predominant profile of IL-2 production.

The T-cell response at six months correlates with peak antibody level

We next assessed how the magnitude, phenotype and cytokine profile of the virus-specific cellular immune response at six months correlated with the prospective profile of antibody production in the six months since infection. Antibody levels against both the Spike glycoprotein and nucleoprotein were available at serial time points from all donors (Figure 5A). These were used to define both the peak value of antibody level against each protein and the rate of decline in antibody level over the subsequent two months. Antibody levels fell by approximately 50% during the two months after peak level but stabilised somewhat thereafter although spike-specific responses continued to decline (Figure 5A).

The magnitude of the T cell ELISPOT response at 6 months against the spike protein was strongly correlated with magnitude of the peak antibody level against both spike protein and the RBD domain (Figure 5B). A similar correlation was observed between the cellular response to the N/M pool and the peak level of N-specific antibody (Figure 5B).

The rate of antibody decline was then assessed in relation to the profile of the cellular immune response at 6 months. Relative preservation of the N-specific antibody response was seen in donors with stronger N and M-specific T cell responses at six months suggesting the cellular responses may act to support antibody production (Figure 5C). However, no such association was observed in relation to spike-specific responses.

Finally, we also assessed expression of the chemokine receptor CXCR5, a marker for T follicular helper (T_{fh}) cells, on virus-specific T cells and related this to the pattern of stability of the virus-specific antibody response as positive correlations have been observed previously in HIV infection¹⁶. High numbers of circulating T_{fh} CD4⁺ T cells have been seen in severe acute infection¹⁷ but at 6 months CXCR5 was expressed on only 7% of virus-

specific CD4⁺ T cells and no correlation was observed with the profile of Ab level following infection. The above data demonstrate that the magnitude of the T-cell response at six months correlates both with the peak antibody level and a reduced rate of antibody waning against nucleoprotein.

Discussion

The magnitude and quality of the immune memory response to SARS-CoV-2 will be critical in preventing reinfection. Here we undertook, to our knowledge, the first assessment of the SARS-CoV-2-specific T cell immune response at six months following primary infection in a unique cohort of healthy adults with asymptomatic or mild-to-moderate COVID-19. This clinical group represents the great majority of infections and patients who had been hospitalised with severe disease were not included as they would have been treated with medication such as dexamethasone at the time of acute infection. The major finding was that virus-specific T cells were detectable in all donors at this extended follow-up period. Approximately 1 in 5000 PBMC were SARS-CoV-2-specific which is broadly comparable to findings within the first three months after infection. These values are lower than typical responses against persistent herpesviruses¹⁸ but comparable to those against acute respiratory viruses, including SARS-CoV-1^{19,20}.

The magnitude of the T cell response was heterogeneous and may reflect diversity in the profile of T cell immunity during acute infection²¹. A striking feature was that the magnitude of cellular immunity by ELISPOT was 50% higher in donors who had experienced symptomatic infection. This demonstrates that the initial 'setpoint' of cellular immunity established following acute infection is maintained for at least 6 months. A similar pattern has been observed early after acute SARS-CoV-2 infection²² and also in patients after SARS-CoV-1 infection (30). This is likely to reflect a response to higher viral loads and inflammatory mediators during acute infection^{23,24} although it is also possible that an elevated adaptive immune response during primary infection can itself act as a determinant of clinical phenotype²⁵. Cellular responses have a direct protective effect against severe coronavirus infection²⁶ and also support antibody production. Indeed, cytokine analysis showed that the CD4⁺IL-2⁺ subset was most significantly elevated in the symptomatic group.

It was noteworthy that CD4⁺ T cells responses against SARS-CoV-2 outnumbered CD8⁺ effector cells by ratio of 2 to 1. Again, a similar pattern has been demonstrated at earlier time points after SARS-CoV-2 infection and may reflect high levels of viral protein uptake by antigen-presenting cells and cross presentation to the CD4⁺ positive T-cell pool or preferential expansion of CD4⁺ T cells²⁷. Furthermore, cytokine analysis showed that IL-2 was the major cytokine produced by virus-specific CD4⁺ cells, indicating a proliferative potential which may auger well for long-term immune memory²⁸. IFN- γ responses are broadly equivalent to IL-2 at early time points after infection²⁹ but the profile at 6 months suggests that the relative proportion of Th1 effector cells decreases over time or they revert to central memory state³⁰. Polyfunctional T cells are typically associated with superior pathogen control³¹ and studies on SARS-CoV-2 infections have revealed decreased cytokine functionality in patients with severe disease²². The majority of CD4⁺ T cells at six months

expressed only a single cytokine and production of three or four cytokines was observed in <15% of cells. Of note, the pattern of cytokine production by CD4+ T cells varies with protein specificity, as seen in earlier reports²². Single IL-2 or IFN- γ producing cells were predominant against both spike and structural proteins but the former population was significantly greater in the CD4+ response against non-spike proteins, indicating that a retained Th1 effector profile is more common within the spike-specific pool. The expression of CXCR5 on CD4+ T cells has been correlated with the magnitude and persistence of humoral immunity in the setting of HIV infection³² but low expression levels on virus-specific CD4+ T cells in our study suggest that circulating virus-specific follicular helper cells are not sustained at high level after infection. Findings in acute infection have also failed to correlate circulating Tfh frequencies with the plasmablast response and suggest that non-CXCR5+ CD4+ T cell help may also operate²¹. The cytokine profile released by SARS-CoV-2-specific T cells in ELISPOT supernatants also showed that IL-2 was the dominant cytokine. Interestingly, low levels of IL-10, IL-4 and TNF were also seen and as IL-10 production is observed within subsets of virus-specific T cells^{33,34} these represent an interesting population of cells for future investigation.

One of the valuable features of our cohort was the availability of monthly antibody levels against the spike and nucleoprotein in the first six months after infection. Serological assays available at the early phase of the pandemic were 'semi-quantitative' and the more recent quantitative methodologies to determine antibody titre will be of interest to further improve data interpretation⁷. Higher T cell responses at 6 months against N/M proteins correlated with slower decline in N-specific antibody levels and indicate that these antibody responses may be highly T cell-dependent. In contrast, T-cell responses against spike were not related to the rate of decline of antibodies against that protein, potentially reflecting extracellular protein availability and the strong memory B cell response against spike epitopes³⁵. Nevertheless, spike protein-specific cellular responses were present in >80% of individuals at 6 months after mild to moderate infection and are also recognised as an immunodominant protein following SAR-CoV-1 infection³⁶. Spike glycoprotein is the major immunogen used in current vaccine trials and these findings indicate that strong and sustained spike-specific T-cell immunity is likely to be required to sustain immune protection and should be assessed in analysis of optimal vaccine strategies. Our finding that T cell responses against M/N proteins are equally as high as spike responses at 6-months after natural infection suggest that these proteins could also represent valuable components of future vaccine strategies.

Our findings demonstrate that robust cellular immunity against SARS-CoV-2 is likely to be present within the great majority of adults at six months following asymptomatic and mild-to-moderate infection. These features are encouraging in relation to the longevity of cellular immunity against this novel virus and are likely to contribute to the relatively low rates of reinfection that have been observed to date³⁷. Further studies will be required to assess how these immune responses are maintained over the longer term.

Materials and Methods

Ethical Statement and Clinical definitions

This study was approved by PHE Research Support and Governance Office (R&D REGG Ref NR 0190). Donors were recruited from a cohort of staff at Public Health England (PHE) that has been monitored for acute infection in March-April 2020. Written informed consent was obtained from all donors. The majority of donors were asymptomatic at the time of initial infection and none were admitted to hospital. All donors were SARS-CoV-2 seropositive using either the Euroimmun, RBD or Abbott test. Serum samples were taken at monthly intervals and assessed by the Euroimmun anti-spike ELISA or the Abbott anti-N assay system. Mean log values were used to determine antibody levels. Cut off levels for positivity were set at >0.8 for the Abbott (N), >5 for RBD (S) and >1.1 for EuroImmune (S) assay as described earlier³⁸. Blood samples for cellular analysis were taken at 6 months from the initial PCR-positive test and SARS-CoV-2 sero-negative and pre-2020 healthy donor samples were used as controls.

Synthetic peptides

Pepmixes of 15-mer peptides overlapping by 11 amino acid residues covering the major proteins of SARS-CoV-2 (Spike Glycoprotein (PM-WCPV-S), Membrane protein (PM-WCPV-VME), nucleoprotein (PM-WCPV-NCAP), ORF3A (PM-WCPV-ORF3A), ORF 7A/B (PM-WCPV-NS7A/7B), ORF10 (PM-WCPV-ORF10) and Non-Structural protein 8 (PM-WCPV-NS8) (JPT Peptide Technologies, Berlin, Germany). A pool of immuno-dominant epitopes from common viruses including Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and influenza virus (PM-CEFX) was included as positive control (JPT Peptide Technologies).

ELISPOT assay

T cell responses were assessed by ELISPOT assay using a Human IFN γ ELISPOTPro kit (Mabtech, NS, Sweden) following the manufacturer's instructions. Briefly, freshly isolated PBMC were rested overnight prior to assay. Plates were washed with filtered PBS (Sigma Aldrich, Missouri, US), and blocked with culture media containing 10% batch tested FBS (Gibco, Thermo Fisher Scientific, Massachusetts, US). As standard 3×10^5 PBMC per well were stimulated in triplicate with overlapping peptide pools (JPT Peptide Technologies) at a concentration of $1 \mu\text{g/ml}$ of individual peptide for 18hrs. In some cases, assays were run in duplicate or with 2.5×10^6 PBMC as a minimum. Negative controls comprising DMSO and positive controls, anti-CD3 and CEFX pepmix (JPT Peptide Technologies), were also included. Spots were counted using an AID ELISPOT Reader System (AID GmbH, Strasberg, Germany). Mean spot counts for negative control wells were subtracted from the mean of test wells to generate normalised readings, these are presented as Spot Forming Cells per million input PBMC (SFC/ 10^6 PBMC). Background levels of response were determined using 12 banked PBMC taken from healthy donors prior to 2020 and 6 seronegative PBMC post-2020. The value of (median + 2xStandard deviation) was used as the lower limit to indicate a positive response in the test cohort. This was measured as 32 and 19 spots/million PBMC for Spike and Nucleoprotein/Membrane respectively (Extended Data Figure 1).

Intracellular cytokine staining (ICS)

Freshly isolated PBMC were rested overnight prior to the assay. 1.5×10^6 PBMCs were stimulated with peptide pools for Spike, or a combination of NCAP, VME1, ORF10, NS7A, NS7B, AP3A, and NS8 at a concentration of $1 \mu\text{g/ml}$ of individual peptide for 6hrs in the presence of protein transport inhibitor cocktail (Ebioscience, San Diego, CA, US). After incubation, PBMCs were harvested and washed before adding fixable red viability dye (Thermo Fisher Scientific) and cell surface antibodies anti-CD3-PerCP5.5 (Biolegend, San Diego, CA, US) (1:100), anti-CD4-APC-Cy7 (Biolegend) (1:40), anti-CD8-BV510 (Biolegend) (1:40), anti-PD-1- Pacific Blue (Biolegend) (1:20) and anti-CXCR5-APC (Biolegend) (1:20). Staining was performed at 4°C for 30 mins. Then the PBMCs were washed and fixed with Ebioscience IC Fixation buffer at 4°C overnight. Following incubation, the fixed cells were permeabilized with 0.1% Triton-X 100 at (Sigma Aldrich) on ice for 30min, washed with PBS and stained at 4°C for 50 mins with the intracellular antibodies, anti-TNF-AF488 (Biolegend) (1:20), anti-IFN- γ -AF700 (Biolegend) (1:20), anti-IL-2-PE-Cy7 (Biolegend) (1:20) and anti-IL-4-PE (Biolegend) (1:20). All antibodies were purchased from Biolegend. Finally, the cells were washed in PBS before analysis on Beckman Coulter Gallios Flow cytometer (Beckman Coulter, High Wycombe, US). Negative controls without peptide-stimulation were also included for each donor sample. Flow cytometry data was analysed using FlowJoTM v.10.7.1 software (FlowJo LLC, Ashland, Oregon, US). Pestle and SPICE software (version 6) was used to determine the frequency of different cytokine response patterns based on all possible combinations³⁹.

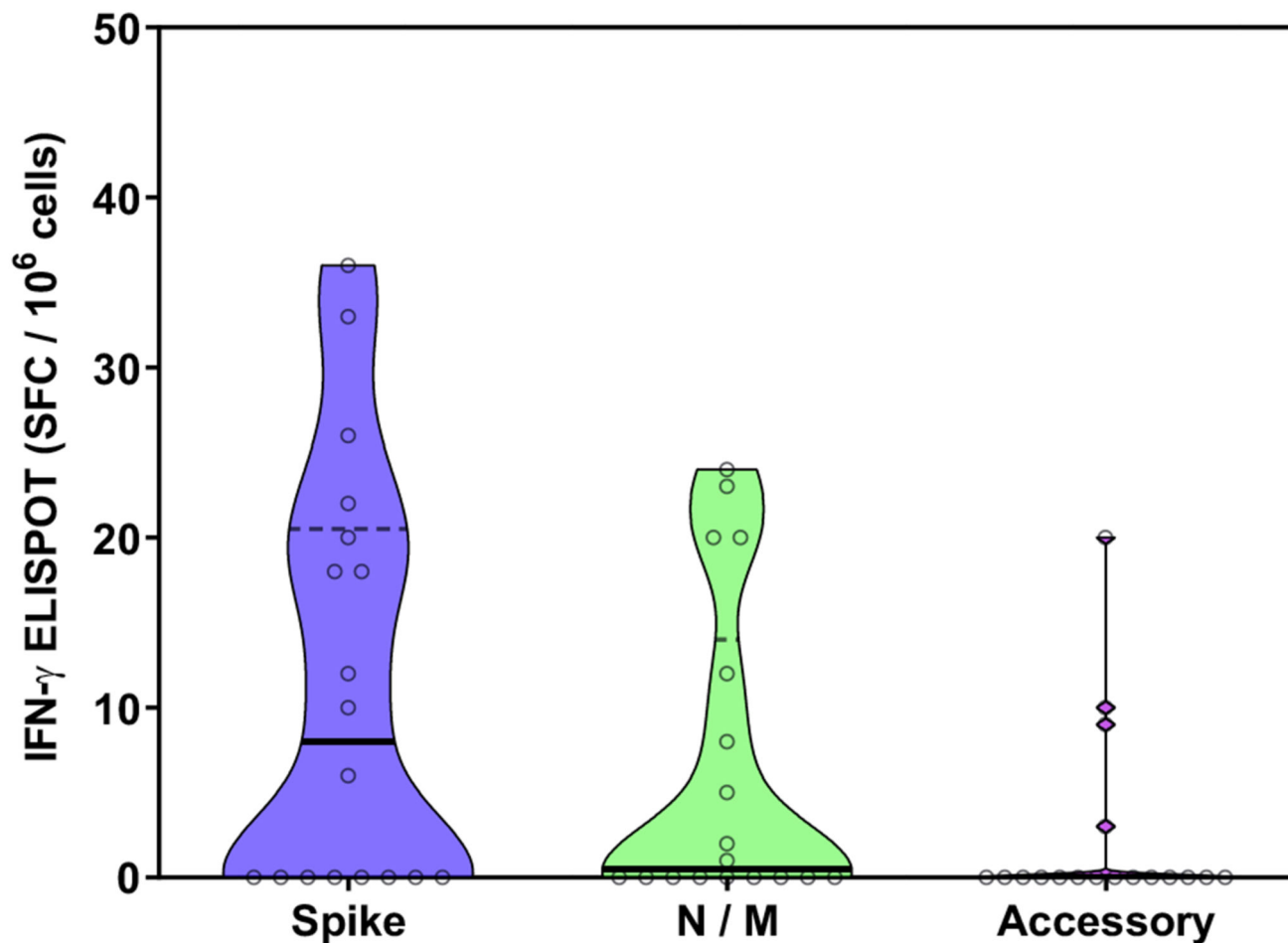
Supernatant Cytokine Profile

Following overnight peptide stimulation in ELISPOT assays 50ul of supernatant was removed and combined from two duplicate wells and cryopreserved at -80°C . Supernatant from eleven donors responding in the ELISPOT assay were profiled using a 12-plex Legendplex T Helper Cytokine Panel Version 2 (Biolegend) following the manufactures instructions. Cytokine beads were analysed on a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, US). Data was analysed with Legendplex Software (Biolegend) and the average cytokine level determined from two duplicate samples.

Statistical analysis

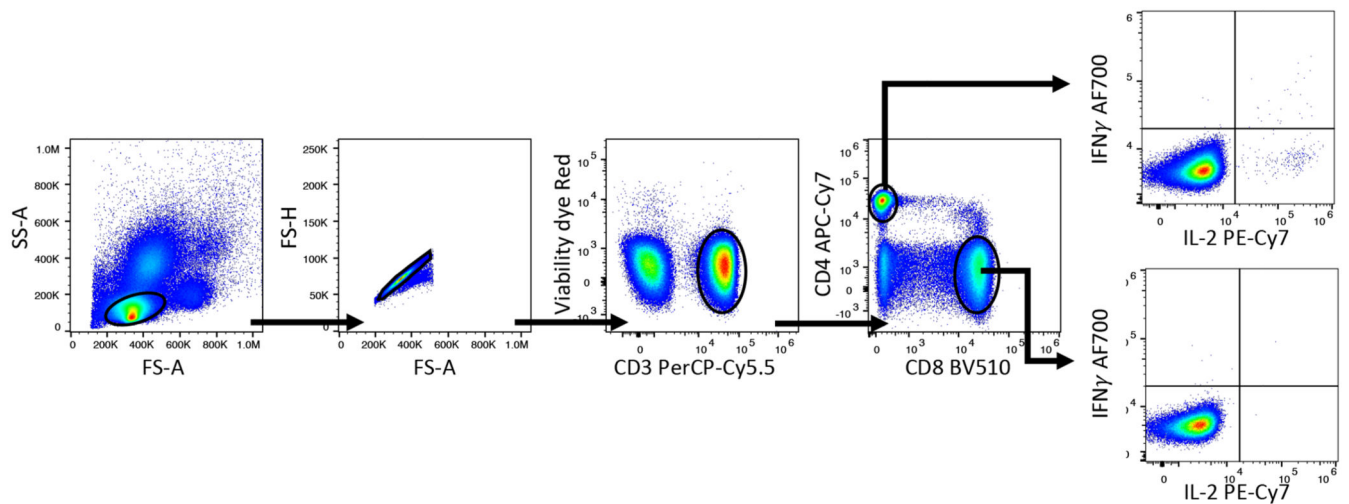
Statistical analysis was performed with GraphPad Prism 8. A Mann-Whitney 2-tailed U test was used to compare variables between two groups, a Wilcoxon matched-pairs signed rank test was used to compare paired non-parametric data, and a Friedman test with Dunn's multiple comparisons test was used to compare non-parametric data between more than 2 groups. Correlations were performed via Spearman's rank correlation coefficient. Two-way ANOVA with Dunnett multiple comparisons test was used to determine significance of cytokine profile data. Statistical significance was determined as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Extended Data



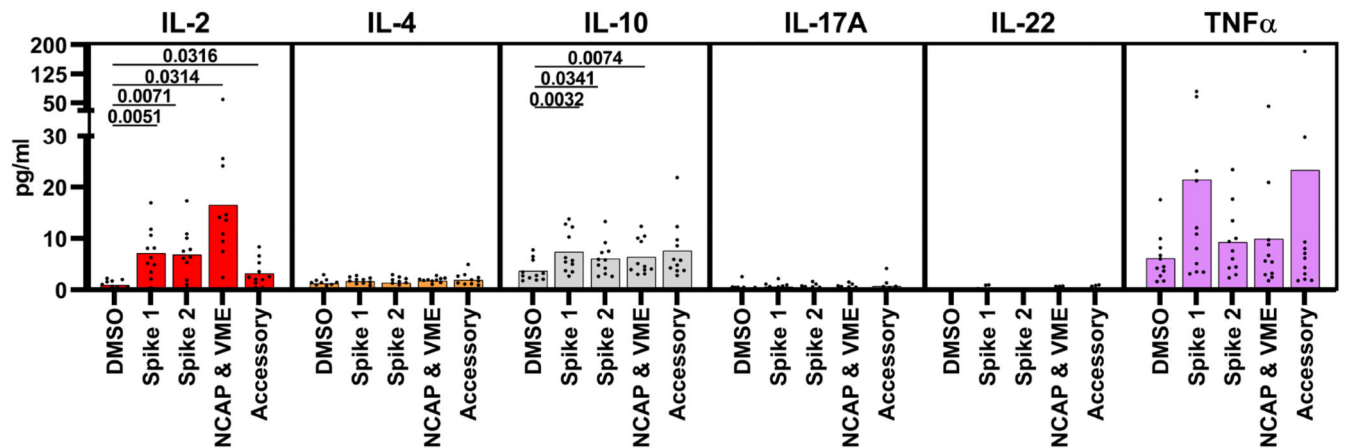
Extended Data Figure 1. Set-up of cut-off point for ELISPOT responses against SARS-CoV-2 protein pools using pre-2020 healthy donors PBMC.

ELISPOT responses against SARS-CoV-2 protein pools from 17 pre-2020 healthy donors were performed using Spike (pools 1 and 2), N/M and Accessory proteins (ORF3a, ORF10, NSP8, NSP7A/b), with DMSO as negative control. Data in graph represented as SFC per million PBMC. Each point on violin plot represents a single donor. Bold black line represents median.



Extended Data Figure 2. Gating strategy for Intracellular cytokine staining.

First, lymphocytes were gated according to FS-A and SS-A before removing doublets according to FS-A and FS-H. Live T cells were then gated using an amine reactive red fixable viability dye and anti-CD3. Finally, CD4⁺ and CD8⁺ T cells were gated according to positive staining with anti-CD4 and anti-CD8, respectively.



Extended Data Figure 3. Characterisation of the Th cytokines released by SARS-CoV-2 specific cells after peptide stimulation.

Characterisation of the Th cytokines released by SARS-CoV-2 specific cells during peptide stimulation shows IL-2 is consistently the dominant cytokine released. Supernatant from the wells of ELISPOT assays from eleven responding donors was analysed to assess the release of cytokines representative of classical Th subsets. In addition to the shown, IL-5, -9, -13 and IL-17A/F were not detected. The significance was determined using RM Two-way ANOVA with Geisser-Greenhouse correction, Dunnett multiple comparisons test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

All raw datasets corresponding to Figs. 1–5 have been uploaded to <http://doi.org/10.5281/zenodo.4498304>. The processed datasets are available from the corresponding author on reasonable request.

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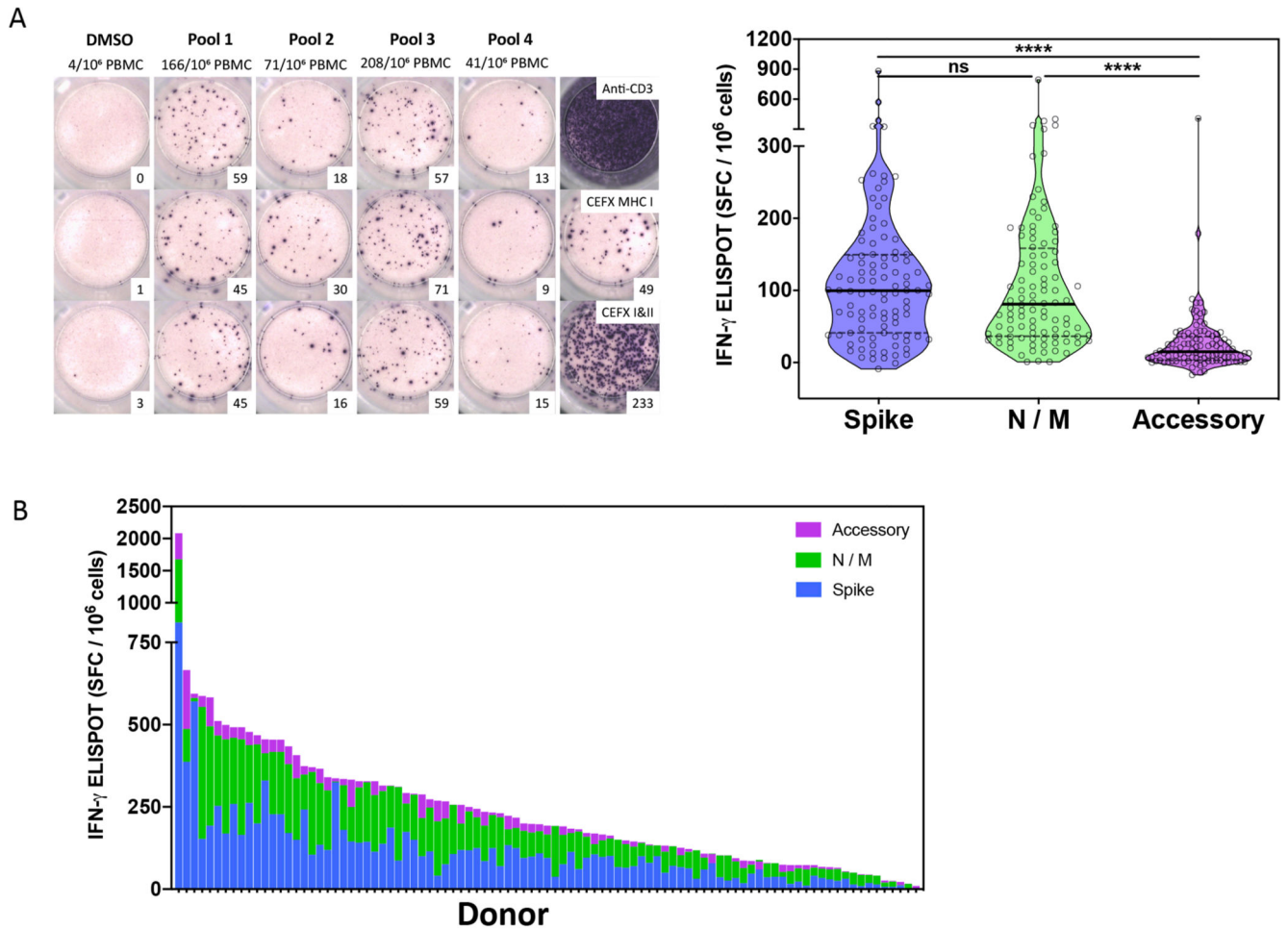


Figure 1. Robust T-cell immunity against SARS-CoV-2 is present in all donors at 6 months following primary infection.

A. ELISpot responses against SARS-CoV-2 protein pools at 6 months following primary infection. Left panel: A representative ELISpot from 1 of 95 donors against Spike (pools 1 and 2), N/M and Accessory proteins (ORF3a, ORF10, NSP8, NSP7A/b), with DMSO as negative control and CEFX peptide pools and anti-CD3 as positive controls. Right panel: Summary data of all patients (N=95) studied according to Spike, N/M and Accessory peptide pools. Data in graph represented as SFC per million PBMC. Each point on violin plot represents a single donor. Bold black line represents median. The significance between pools was determined using a Friedman test (two sided) with Dunn's multiple comparison test, $p < 0.0001$ (****). **B.** Aggregate ELISpot response against SARS-CoV-2 proteins at 6 months following primary infection. The spot numbers were aggregated for individual donors and shown in a bar chart.

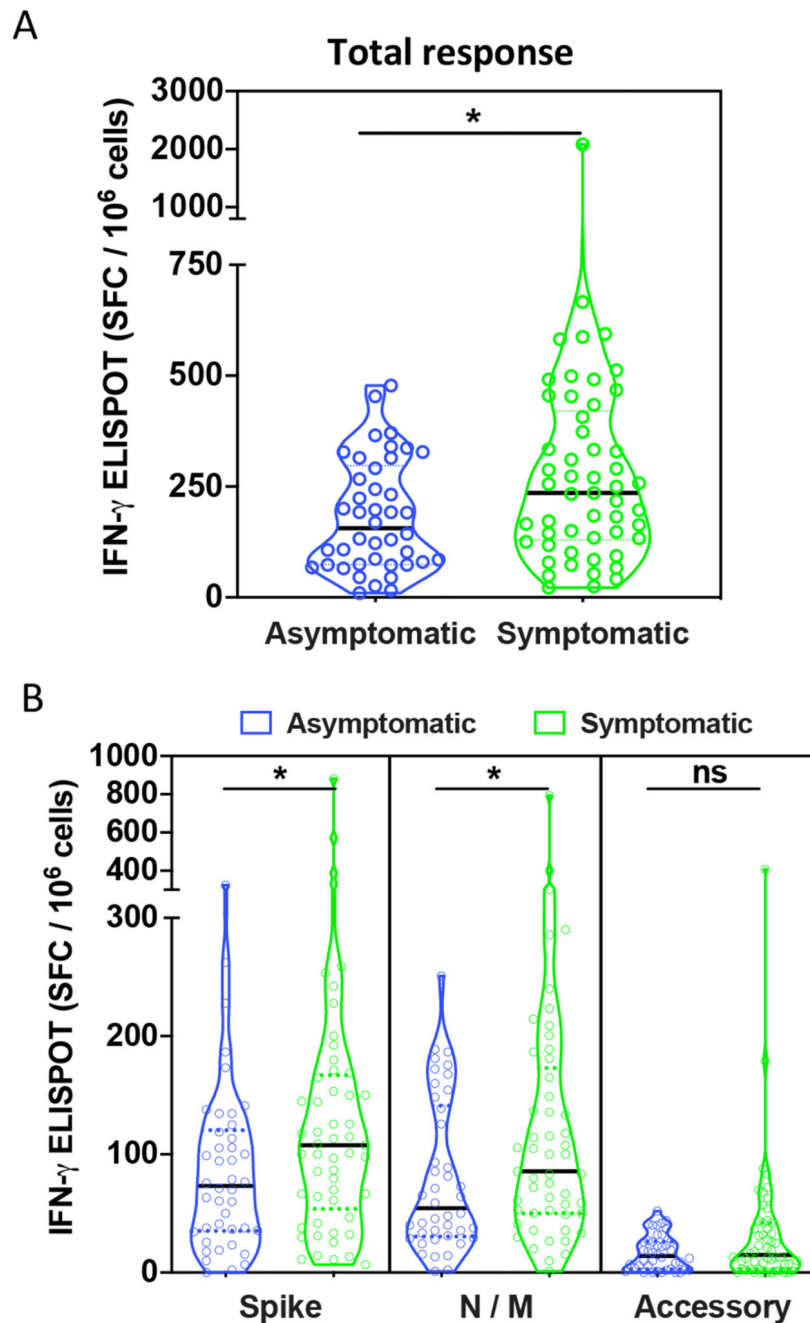


Figure 2. T-cell responses against SARS-CoV-2 are 50% higher in donors with an initial symptomatic infection.

The cohort was divided into two groups according to symptoms at initial infection. **A.** The aggregated T-cell response (as SFC per million PBMC) against all peptide pools was compared between patients with (N=52) and without (N=43) respiratory symptoms (P=0.0235). **B.** T-cell responses (as SFC per million PBMC) to Spike (pools 1 and 2) (P=0.0330), N/M (P=0.0330), and Accessory proteins (P=0.2647) were compared between patients with and without symptoms. Each point on violin plot represents a single donor.

Bold black line represents median. The significance was determined using Mann-Whitney testing (two sided), $p < 0.05$ (*).

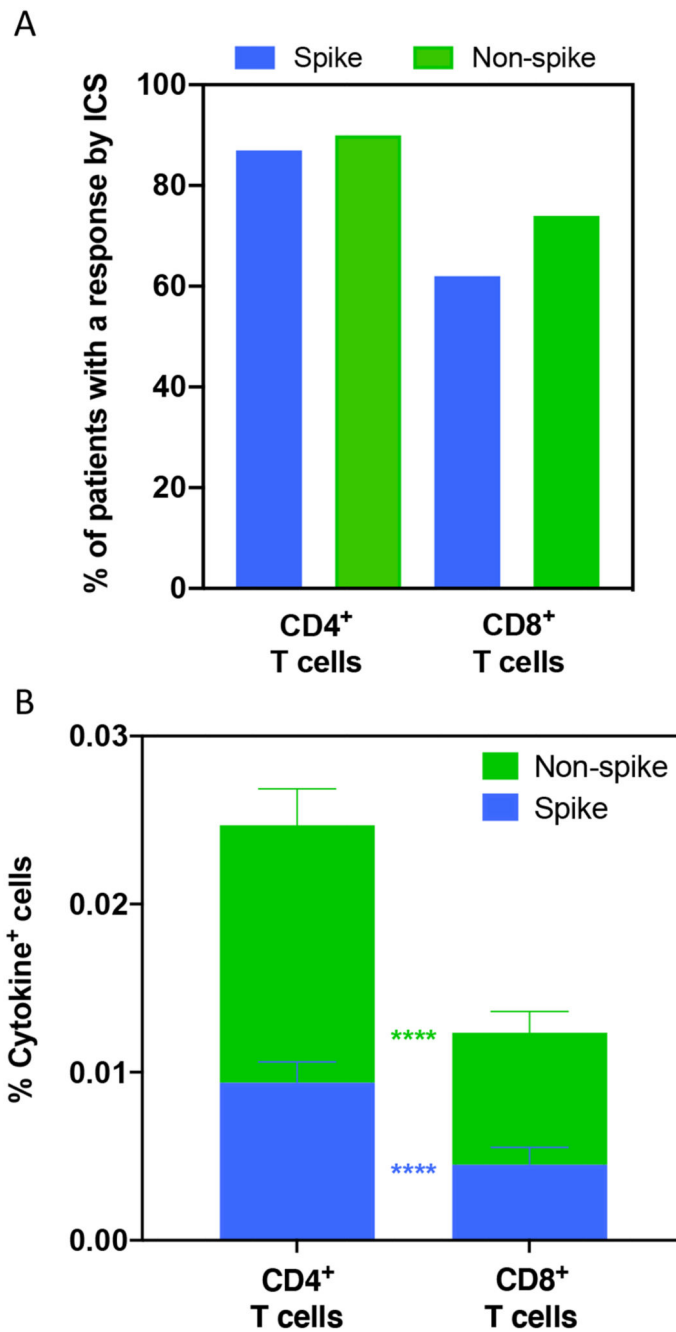


Figure 3. Overall detection of SARS-CoV-2-specific T cell responses by ICS.

A. Proportion of donors (N=100) with a detectable IFN- γ and/or IL-2 response by ICS for CD4⁺ T cells and CD8⁺ T cells against Spike and Non-spike proteins 6 months following primary infection. **B.** Aggregated IFN- γ and IL-2 ICS responses for CD4⁺ and CD8⁺ T cells against Spike and Non-spike proteins (N=100). The significance was determined using Wilcoxon matched-pairs signed rank test (two sided), $p < 0.0001$ (****). Error bars represent standard error of the mean.

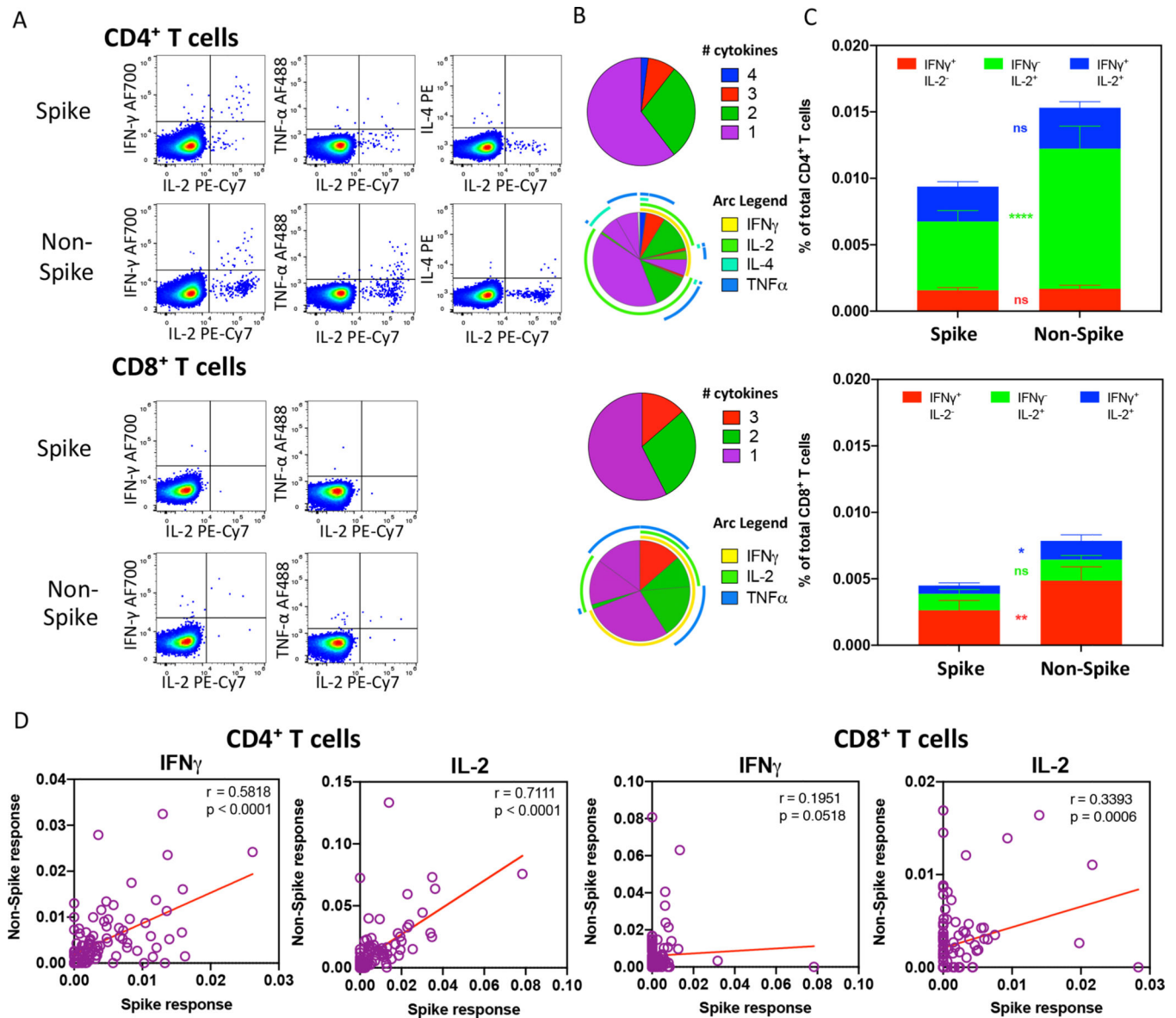


Figure 4. SARS-CoV-2-specific T-cell responses are characterised by a predominant profile of IL-2 production.

A. Features of CD4⁺ and CD8⁺ T-cell responses against SARS-CoV-2 proteins by intracellular cytokine staining at 6 months. Representative flow plots of CD4⁺ (Top panel) and CD8⁺ (Bottom panel) T-cell responses against peptide pools from Spike or non-spike (aggregate of N, M, ORF3a, ORF10, NSP8 and NSP7A/b) proteins. **B.** Polyfunctional analysis of SARS-CoV-2-specific CD4⁺ and CD8⁺ T-cells at 6 months. Relative distribution of single or multiple cytokine responses in CD4⁺ (Top panel) and CD8⁺ (Bottom panel) T-cells, and pattern of co-expression of IL-2, IFN- γ , TNF and IL-4 in SARS-CoV-2-specific T-cells. **C.** Aggregate ICS responses for CD4⁺ and CD8⁺ T-cells against Spike and Non-spike proteins according to IFN-g and/or IL-2 production (N=100). The significance was determined using Wilcoxon matched-pairs signed rank test (two sided), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.0001$ (****). The exact P values are: for CD4⁺ T cell and Spike vs Non-

spike, IFN γ +/IL-2-p=0.6629, IFN γ -/IL-2+ p<0.0001 and IFN γ +/IL-2+ p=0.1536; for CD8+ T cells Spike vs Non-spike, IFN γ +/IL-2- p=0.0036, IFN γ -/IL-2+ p=0.3071 and IFN γ +/IL-2+ p=0.0336. Error bars represent standard error of the mean.

D. Correlation of Spike and Non-spike responses according to IFN- γ and IL-2 production by CD4+ (left panel) and CD8+ (right panel) T-cells at 6 months (N=100). Spearman's Rank correlation (two sided) was used to test the significance, and p value and r value (correlation coefficient) are indicated for each panel.

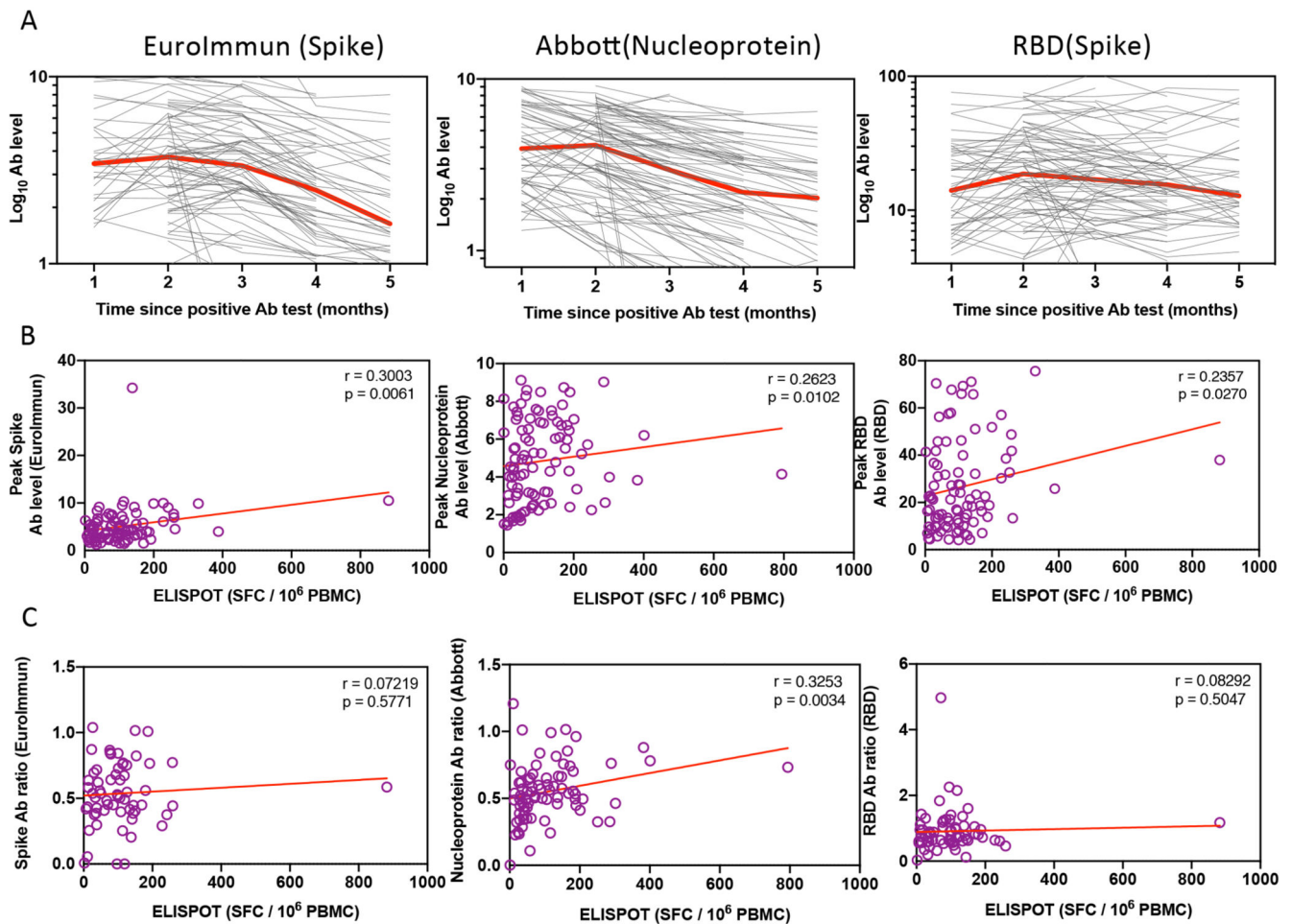


Figure 5. The magnitude of the T-cell response at six months correlates with peak antibody level.

A. Antibody levels against Spike (N=81), Nucleoprotein (N=94), and RBD (N=87) of all patients at each time point post infection were plotted. Each grey line represents an individual patient. The median antibody level over time is shown in red. **B.** The correlation of ELISOT responses at 6 months against peak antibody levels (Spike: N=82, Nucleoprotein: N=94, and RBD: N=87) were assessed for each antibody. **C.** Correlation of ELISOT responses at 6 months with rate of antibody decline (expressed as ratio of ‘antibody level at 2 months after peak level’: ‘antibody peak level’) (Spike: N=60, Nucleoprotein: N=79, and RBD: N=67). The line represents linear regression. Spearman’s Rank correlation (two sided) was used to test the significance, and p value and r value (correlation coefficient) are indicated in each panel.