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Divergent trajectories of antiviral memory after SARS-Cov-2 infection

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Research Article

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Divergent trajectories of antiviral memory after SARS-Cov-2 infection

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Contributions

EB, PK, CD and SJD conceptualised the study. SJD, PK, EB, CPC, DTS and LS designed and oversaw the clinical study. LS, DE, KJ, PM, AJM, PG, SAJ, ST, the OPTIC Study Group and the C-MORE Group contributed to the implementation of the clinical study. AO, AT, CD, DOC, DTS, MP, MWC, WSJ, EB, PK, and SJD designed and oversaw the laboratory studies. AJP, AO, CD, EA, JF, PJG, MWC, WSJ, EB, PK, SJD and TL were responsible for the implementation of the laboratory testing, while AB, ACH, AF, AL, BC, BK, CH, CJ, CP, CS, EAC, FA, HB, HS, JG, JG-J, JH, JM, KA, LB, LS-R, LSt, LT, MAA, MA, MLK, PR, RH, SB, SB-R, SL, SM, ST, STh, TJ, TT, TW, VAV, YL were responsible for laboratory testing and assay development. AT and DOC undertook the advanced data analysis. AO, AT, CD, DOC, DTS, EB, PK, and SJD prepared the manuscript, which was reviewed by all contributing authors. All other authors contributed to the implementation of the study and data collection.

Competing Interests

DWE declares lecture fees from Gilead, outside the submitted work. No other competing interests declared.

Key words: SARS-CoV-2, T cells, B cells, Antibodies, Durability, Healthcare workers, Longevity, variants of concern, COVID-19

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is normally controlled by effective host immunity including innate, humoral and cellular responses. However, the trajectories and correlates of acquired immunity, and the capacity of memory responses months after infection to neutralise variants of concern - which has important public health implications - is not fully understood. To address this, we studied a cohort of 78 UK healthcare workers who presented in April to June 2020 with symptomatic PCR-confirmed infection or who tested positive during an asymptomatic screening programme and tracked virus-specific B and T cell responses longitudinally at 5-6 time points each over 6 months, prior to vaccination. We observed a highly variable range of responses, some of which - T cell interferon-gamma (IFN-γ) ELISpot, N-specific antibody waned over time across the cohort, while others (spike-specific antibody, B cell memory ELISpot) were stable. In such cohorts, antiviral antibody has been linked to protection against re-infection. We used integrative analysis and a machine-learning approach (SIMON - Sequential Iterative Modeling Over Night) to explore this heterogeneity and to identify predictors of sustained immune responses. Hierarchical clustering defined a group of high and low antibody responders, which showed stability over time regardless of clinical presentation. These antibody responses correlated with IFN-γ ELISpot measures of T cell immunity and represent a subgroup of patients with a robust trajectory for longer term immunity. Importantly, this immune-phenotype associates with higher levels of neutralising antibodies not only against the infecting (Victoria) strain but also against variants B.1.1.7 (alpha) and B.1.351 (beta). Overall memory responses to SARS-CoV-2 show distinct trajectories following early priming, that may define subsequent protection against infection and severe disease from novel variants.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an RNA virus that causes coronavirus disease 2019 (COVID-19), first emerged in humans in December 2019 and has since spread globally, with more than 3.56 million deaths reported world-wide (June 2021 https://coronavirus.jhu.edu/map.html). Although the majority of infections cause asymptomatic or mild disease, a significant minority develop a severe illness, requiring hospitalisation, oxygen support, and invasive ventilation [1]. Healthcare workers (HCW) have been at the forefront of caring for patients with SARS-CoV-2 infection in community and hospital environments during the pandemic. High exposure rates have meant that a significant proportion of HCW have become infected and HCW most commonly infected are those working on the front line in patient facing roles, predominantly in acute medical specialities [2]. Older age, comorbidities and male sex remain the dominant factors that predispose to severe outcomes [3] – since HCW are predominantly younger and female [2], most have developed mild disease, although deaths are widely reported in this population.

Starting early in the pandemic, we and others have sought to characterise the immune responses during SARS-CoV-2 infection that are associated with viral clearance and disease severity. SARS-CoV-2 infection has been associated with the generation of high magnitude, broad T cell responses and high titres of immunoglobulin G (IgG) targeting SARS-CoV-2 spike and nucleoprotein (NP) antigens, particularly in severe COVID-19 [4]. Asymptomatic infection, that appears more common in younger people, may be associated with discordant T cell and humoral immunity with both the absence of IgG seroconversion in the presence of detectable T cell responses [5, 6] or conversely the presence of IgG in the absence of T cell immune responses [7]. However, more recently critical questions have emerged that include the durability of immune responses following initial infection, the quality of these responses, immune correlates of protection from re-infection, and the capacity of these responses to neutralise new variants of concern (VOC) that have emerged globally. These questions have become paramount following the development of effective vaccines for COVID-19, since deployment of these has been limited by vaccine supply, concerns around adverse events and vaccine hesitancy. Furthermore, to manage limited vaccine resource, people with previous infection are now being offered a single vaccine dose 6 months after infection in many European countries (France, Germany, Spain, and Italy) [8], on the assumption that natural immunity will protect from re-infection.

An in depth understanding of immune responses after SARS-CoV-2 infection, and how these change over time, will be critical to understanding who is susceptible to re-infection and to inform vaccine strategies. Currently, the precise correlates of immune protection from subsequent infection after primary disease, or after vaccination, are unknown. Previous reports suggest SARS-CoV-2 IgG antibodies [9] and previous exposure to seasonal coronaviruses (CoV) [10] are protective against subsequent SARS-CoV-2 infection. However, since the magnitude of T and B cell responses correlate with each other [11], dissecting the role of these immune subsets in protection from re-infection or severe disease on re-exposure is challenging. Several groups have now reported that SARS-CoV-2 specific T and B cells decline after acute disease [12, 13, 14, 15, 16], but there is high heterogeneity between individuals

in the levels of measurable immunity in different compartments it is unclear how or if the kinetics of this decline correlate with protection from subsequent infection. Concerns have been raised that SARS-CoV-2 re-infection associated with waning immunity is plausible, particularly since the seasonal coronaviruses, closely related to SARS-CoV-2, commonly re-infect the same host [17, 18]. However, waning of immune responses following acute infection, or vaccination is well recognised as part of the normal evolution of memory responses, and reports describing decline in immune responses have focused on ex vivo responses that may not reflect the memory recall potential of viral specific T and B cells responses. A particular concern is the identification of SARS-CoV-2 variants of concern (VOC) (B.1.1.7 - alpha, B.1.351 - beta, P.1 - gamma and B.1.617.2 - delta), with mutations which are associated with an increase in transmissibility, severity or escape from vaccine or SARS-CoV-2-induced immunity [19, 20, 21, 22, 23, 24, 25, 26, 27]. Immune escape, with a failure to neutralise the VOC, in live viral assays in vitro, appear following vaccination and after SARS-CoV-2 infection, and is pronounced in the context of lower antibody titres measured against the initial pandemic strain (B/Victoria).

Since April 2020, we have followed a cohort of SARS-CoV-2 infected HCW prospectively over time at Oxford University Hospital NHS Foundation Trust. Seventy-eight HCW infected during the UK's "first wave" (defined by positive PCR and seropositive for anti-spike antibodies) were assessed at up to six timepoints and followed for six months in 2020, pre-vaccination, with multiple immune parameters evaluated in more than 430 blood draws. Our aims are to characterise memory T and B cell responses following infection, and to determine the interactions between clinical presentation and the generation and maintenance of T and B cell responses over time. We assess the association of exposure to seasonal coronaviruses and symptomatic SARS-CoV-2 disease with the durability of SARS-CoV-2 specific responses. We evaluate the predictive value of clinical and immune parameters measured early after infection on the durability of immune responses using an integrative analysis with a machine learning platform (SIMON) [28, 29]. Using this approach, we define a group of high and low antibody responders with a differential capacity to neutralise the VOC.

Methods and materials

Detailed description of methods are included in the Appendix.

HCW volunteer recruitment and ethics

We sampled seventy-eight HCW at five or six time points each, over six months. HCWs were recruited from Oxford University Hospitals NHS Foundation Trust after a positive SARS-CoV-2 PCR test [2] in April-May 2020, including 66 volunteers with symptomatic disease (fever, shortness of breath, cough, loss of taste or smell, sore throat, coryza or diarrhoea) and 12 asymptomatic HCW who did not report any symptoms of COVID-19 in 2020 prior to staff screening or in the seven days following testing positive. The age, sex and ethnicity of the HCW are shown in **Supplementary Table 1**. Blood samples were acquired at multiple timepoints over 6 months (acute[range:1-20], 28 days [21-41], 56 days [42-73], 90 days [74-104], 120 days [110-140], and 180[160-200]) from onset of symptoms in the symptomatic group and from the date of positive PCR test for asymptomatic people diagnosed on screening. Nine hospitalised patients with severe disease were included for comparative analysis. All subjects were seropositive for anti-spike IgG antibodies by ELISA. Mild and asymptomatic participants were recruited under ethics approved by the research ethics committee (REC) at Yorkshire & The Humber - Sheffield (GI Biobank Study 16/YH/0247). Participants with severe disease were recruited after consenting into either the CMORE study protocol (research ethics committee (REC): Northwest – Preston, REC reference 20/NW/0235) and / or Sepsis Immunomics protocol [Oxford Research Ethics Committee C, reference 19/SC/0296]). The study was conducted according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines. Written informed consent was obtained for all participants enrolled in the study.

Isolation of peripheral blood mononuclear cells (PBMC), plasma and serum

PBMCs and plasma were isolated by density gradient centrifugation from blood collected in EDTA tubes, and serum was collected in a serum-separating tube (SST, Becton Dickinson) as previously described[5] and detailed in the Appendix.

T cell assays

T cell assays including interferon-gamma (IFN-γ) Enzyme-Linked immunospot (ELISpot) assay, 7-day proliferation assay and intracellular staining were performed [5]. For IFN-γ ELISpot assay we used SARS-CoV-2 peptide pools panning Spike (S1 and S2), membrane (M), nucleocapsid protein (NP), the X-domain of non-structural protein 3 (NSP3B), open reading frames 3 and 8 (ORF3 and ORF8), and cytomegalovirus, Epstein-Barr virus and Flu peptide pools (CEF) (2ug/ml per peptide) in a 16-18hour incubation at 37ºC. ELISpot plates were read using an AID ELISpot Reader (v.4.0) and results were reported as spot-forming units (SFU)/10^6 PBMC. T cell proliferation assay was performed using fresh or cryopreserved PBMC and CellTrace® Violet (CTV, Life Technologies) labelling and stimulated with peptide pools from SARS-CoV-2 spanning Spike (S1 and S2), M, NP, ORF3 and ORF8, and FEC-T (1µg/ml per peptide). On day 7, cells were stained with fluorochrome-conjugated primary human-

specific antibodies for CD3, CD4 and CD8 for analysis on a MACSQuant 10 flow cytometer. For Intracellular cytokine staining, PBMC were stained for CD3, CD4, CD8, CD154, IFN-γ, IL-2 and TNF-α then analysed on a BD LSR II.

Antibody and B cell assays

Standardised total anti-spike IgG ELISA [30] and anti-spike subclass and isotype ELISAs [31, 32] were performed. A multiplexed MSD immunoassay (MSD, Rockville, MD) was used to measure the IgG responses to SARS-CoV-2, severe acute respiratory syndrome coronavirus-1 (SARS-CoV-1), MERS-CoV and seasonal CoVs (human coronavirus (HCoV)-OC43, HcoV-HKU1, HcoV-229E, HcoV-NL63). For Microneutralisation Assay (MNA), the viral isolates used are described in the Appendix, and the assay was performed to determine the concentration of antibody that produces a 50% reduction in infectious focus-forming units of authentic SARS-CoV-2 in Vero CCL81 cells. Infectious foci were enumerated by ELISpot reader and data were analysed using four-parameter logistic regression (Hill equation) in GraphPad Prism 8.3. The Monogram Bioscience pseudotype neutralisation assay (PseudoNA) was performed [30].

For the Spike-specific SARS-CoV-2, OC43, HKU1, 229E and NL63 IgG^+ and IgA^+ B cell memory ELISpot assays, PBMCs were cultured for 3-3.5 days with polyclonal stimulation, and added to Mabtech flurospot plates coated with the relevant spike glycoprotein (SARS-CoV-2 at 10µg/ml, OC43 at 10µg/ml, NL63 at 15µg/ml, HKU1 at 5µg/ml and 229E at 10µg/ml, all diluted in PBS). All cells were incubated for ≥16 hours at 37°C, and following development Spot forming units were enumerated using AID ELISpot 8.0 software on the AID ELR08IFL reader. For antibody-dependent effector functions, the spike-specific antibody-dependent effector functions, natural killer cell activity (ADNKA), neutrophil phagocytosis (ADNP) and monocyte phagocytosis (ADMP) were performed [31], and are detailed in the Appendix alongside the Antibody-dependent complement deposition (ADCD) assay.

Integrative analysis using unsupervised and supervised machine learning in SIMON

The integrative analysis was performed using SIMON (Sequential Iterative Modeling "Over Night") software [28, 29] as detailed in the Appendix. The integrated dataset was generated using the standard extract-transform-load (ETL) procedure to merge total of 29 csv files across 14 assays and clinical data via donor-specific variable (Donor ID) according to the SIMON method. The outcome of immune response durability was calculated based on the titre of the anti-N specific antibodies measured 6 months post symptoms onset (pso), and individuals with anti-N antibody titre ≥ 1.4 were labelled as high responders, while individuals having anti-N antibody titre below 1.4 were low responders. Before integrative analyses, data was pre-processed (centre/scale), missing values were median imputed, features with zero-variance, near-zero-variance and with correlation (cut-off 0.85) were removed using SIMON software. The t-distributed stochastic neighbour embedding (t-SNE) (2,000 iterations, perplexity 30, and theta 0.5) followed by clustering (seed number 1337, number of clusters 3) was performed to analyse the pre-processed integrated dataset (excluding disease severity and timepoint which are used as grouping variables). Principal component analysis (PCA) was performed on multivariate

immunological parameters (continuous variables, excluding features with less than 10% of unique values and grouping variable - disease severity). Pairwise correlations of immunological parameters in the integrated dataset were visualized as a correlogram and Spearman's rank correlation coefficient was computed. Values shown on the correlogram were adjusted for multiple testing using Benjamini-Hochberg correction at the significance threshold (False discover rate, FDR < 0.05). Agglomerative hierarchical clustering was performed on the samples with immunological parameters analysed on day 28 pso and visualized as the dendrogram on heatmap (tightest cluster ordered first). To identify early immunological signature at day 28 pso that can predict if the individual will be high or low responder 6 months pso, we performed SIMON analysis on all immunological parameters (day 28 pso) using 172 ML algorithms . Missing values (29% missingness) were removed using multi-set interaction function ('mulset', SIMON software), resulting in 30 resamples. Each resample was split into train/test partition (75%/25%) preserving the balanced distribution of the outcome class (seed number 1337). The models were evaluated using 10-fold cross-validation on training sets (train AUROC), and additionally on the held-out test sets (test AUROC). The best performing model was built using the Sparse Partial Least Squares (sPLS) algorithm (train AUROC: 0.95 (CI 0.5-1) and test AUROC: 1). In the final step, SIMON calculated the contribution of each feature to the model as variable importance score (scaled to maximum value of 100).

Statistical analyses

Statistical analysis was performed using R (https://www.r-project.org/), integrative analysis was performed using SIMON software [28, 29], figures were made with R using R package ggplot2 [33] and GraphPad Prism 8. Kruskal-Wallis test —unless otherwise specified — was used for comparison of the disease severity groups. Wilcoxon rank-sum test —unless otherwise specified — was employed to compare between study time points. A generalised additive mixed model (GAMM) by restricted maximum likelihood (REML) was used to fit the immunological measures (log10 transformed) using Gaussian process smooth term (R package *gamm4* [34]). ICS cytokine expression analyses was performed using PESTEL v2.0 and SPICE v6.0. Statistical significance was set at $P<0.05$ and all tests were 2-tailed. Machine learning analysis was performed using SIMON software (https://genular.org).

Data Availability

Data relating to the findings of this study are available from a research data repository Zenodo (https://zenodo.org/record/4905965).

Results

Anti-N IgG decline over time and stratify by disease severity, whilst Anti Spike IgG and memory responses are maintained

Anti-nucleocapsid (NP) and spike (S) total IgG (tIgG) responses were assessed by ELISA in both symptomatic and asymptomatic individuals (**Fig. 1A**). The magnitude of the IgG response varied markedly between people in both cohorts, with a proportion of individuals' anti-nucleocapsid tIgG level recorded in the negative or indeterminate range of the assay at all time-points.

Asymptomatic and mild infection induces similar anti-NP responses in the early phase (<20 days post PCR positivity/symptom onset) of observed infection (P=0.6125, **Supplementary Fig. 1A**). However, anti-NP tIgG levels in the two disease cohorts separated as higher levels were observed in those with mild infection from the day 28 timepoint onwards (P =0.0015 for day 28 comparison, **Supplementary Fig. 1A**). Anti-NP IgG responses waned over time with a significant decrease from approximately day 28 to day 180 timepoints (P=0.00071 for asymptomatic and P=7.2x10^{-9} for mild symptomatic individuals, **Fig. 1A**). Most (91.7%) asymptomatic individuals have an indeterminate or negative anti-NP tIgG response to the nucleocapsid antigen at the day 180 timepoint.

Over the time course of observation, anti-spike IgG antibody levels (**Fig. 1B**) in individuals remained consistent in individuals with asymptomatic (P=0.35) and severe (P=0.44) COVID-19 disease. Similarly, the initial anti-spike tIgG responses increased in individuals with mild disease and remained consistent from day 28 to the 6-month timepoint (P=0.12). Furthermore, disease severity was not a significant predictor of anti-spike tIgG levels in those with asymptomatic and mild SARS-CoV-2 infection throughout the 6-month observation (P =0.632, GAMM, **Fig. 1B**).

In line with the tIgG antibody binding to spike remaining consistent, we observed a steady number of IgG+ memory B cells following an initial increase (**Fig. 1C**). Anti-SARS-CoV-2 spike-specific IgG+ memory B cells at 6 months following symptom onset were higher than observed during early infection in mild (P=0.00042, **Fig. 1C**) and severe (P=0.0027, **Fig. 1C**) individuals. For asymptomatic individuals, no change was observed in cell frequencies when comparing the earliest samples collected and 6-month timepoints (P=0.54), although we note that the timing of infection onset for asymptomatic individuals cannot be precisely determined. Asymptomatic and mild disease did not predict different kinetics for the IgG memory response (P=0.284, GAMM, **Fig. 1C**).

Pseudo-neutralisating antibodies decreased in all disease severities over time

Pseudo-neutralisating antibodies (pseudoNA) were measured in all individuals (**Fig. 1D**) using an assay that incorporates the spike glycoprotein. Disease severity was a significant predictor of pseudoNA (P=0.00073, GAMM, **Fig. 1D**) – with higher pseudoNA levels with increasing disease severity at all time points measured (**Fig. 1D and Supplementary Fig. 1D**). Regardless of disease severity, the pseudo-neutralising capacity of circulating antibodies to the Wuhan/B lineage virus decreased over 6 months following the detection of SARS-CoV-2 infection (asymptomatic P=0.023; mild P=4.2 x10^{-9}; severe P=0.01, **Fig. 1D**). People with severe infection maintained pseudoNA 6 months post symptom onset, and at higher levels than in those with mild or asymptomatic infection (P=0.00022, Kruskal-Wallis test, **Supplementary Fig. 1D**). The decline was less marked in asymptomatic individuals with no decrease observed from day 28 to day 180 (P=0.41, **Fig. 1D**); however, the difference in the pseudoNA titres in the mild vs asymptomatic groups remained until day 180 (P=0.0148). At day 180 post symptom onset or PCR confirmation, one asymptomatic and four symptomatic individuals no longer mounted a positive

result in the pseudoNA assay, one of whom consistently did not mount pseudoNA capacity at all time points measured.

Mild infection induces a more multifunctional antibody profile

A cohort of 30 individuals with mild infection, along with the 9 and 12 participants with severe and asymptomatic infection respectively were selected to comprehensively characterise antibody profiles.

Circulating isotypes and subclasses

Circulating IgM levels decreased over time in those with asymptomatic (P=0.021, day <20 vs day 180), mild (P=0.0004, day <20 vs day 180) and severe (P=0.007, day <20 vs day 180) infection, while IgA levels in participants remained constant in all disease cohorts (asymptomatic: P=0.65; mild: P=0.59; severe: P=0.065), throughout the observed 6-month time course (**Fig. 2A and 2B**) as previously reported[12]. The quantified amounts of IgG1 were consistent over time in asymptomatic (P=0.86, day <20 vs day 180) and severe (P=0.92, day <20 vs day 180) infection. Despite initial low titres of IgG1 in participants with mild infection, IgG1 circulating antibody titres were maintained from day 28 to 6 months post symptom onset (P=0.89, **Fig. 2C**). While circulating IgG3 antibodies in participants with mild infection were maintained at consistent levels throughout the 6-month period (P=0.062), levels decreased over this time in asymptomatic (P=0.0022, day <20 vs day 180) and severe (P=0.021, day <20 vs day 180) individuals (**Fig. 2D**). Notable SARS-CoV-2 spike-specific IgG2 responses were only detected at one or more time-points in a small number of individuals tested (asymptomatic: 3/12; mild: 3/30; severe: 1/8) (**Supplementary Fig. 2B**), while there was no spike-specific IgG4 detected above the LLOQ of the ELISA (data not shown). For all IgG subclasses detected, asymptomatic or mild disease severity were not significant predictors of responses over time (IgG1: P=0.36; IgG2: P=0.92; IgG3: P=0.0519, GAMM, **Figs. 2C–D**). All paired analysis was by Wilcoxon rank sum test.

Diversity of antibody responses

We measured the ability of the anti-spike antibodies in those with severe or asymptomatic infection as well as a selection of individuals with mild infection, to induce innate effector functions: ADNP, ADMP, ADNKA and ADCD.

Asymptomatic and mild disease severity was not a significant predictor of Fc-mediated effector functional responses (ADNKA P=0.798; ADMP P=0.117; ADNP P=0.206) except for ADCD (P=0.00314) (**Fig. 2E–H**). Furthermore, normalised ADMP and ADNP scores, as well as the percentage of CD107a-expressing NK cells were stable over time, between 28 days and 180 days post symptom onset or PCR confirmation for those with asymptomatic (ADMP: P=0.96; ADNP: P=0.48; ADNKA: P=0.2) and mild (ADMP: P=0.64; ADNP: P=0.75; ADNKA: P=0.8) infection (**Fig. 2E–H**). Similarly, no decline was observed for these Fc-mediated functions from the acute sampling to 6 months post symptom onset in the severe cohort (ADMP: P=0.89; ADNP: increase P=0.021; ADNKA: P=0.075) with the ADNP increasing over time (P=0.021) (**Fig. 2E–H**). ADCD waned dramatically in those with severe disease over the 6-month period (P=0.00031) but similarly to the other Fc-mediated functions,

ADCD remained consistent from day 28 to day 180 in asymptomatic (*P*=0.34) and mild (*P*=0.1) infection (**Fig. 2E–H**). Despite waning over time, ADCD responses differed amongst the disease severity groups out until day 180 (*P*=0.0032, Kruskal-Wallis test, **Supplementary Fig. 1L**). All paired analysis were by Wilcoxon rank sum test.

We visualised the relative contribution of each of the anti-SARS-CoV-2 spike antibody feature in **Fig. 2I**. The polar plots demonstrate the diversity of asymptomatic and mild infection-induced antibody characteristics and functions on day 28 and day 180. Each wedge represents an antibody feature, and the size of each wedge is indicative of the magnitude of the response. The consistently high spike-specific IgG and spike-specific IgG+ memory B cells is clearly reflected in these plots for both mild and asymptomatic individuals. For both day 28 and day 180, a more multifunctional response was observed in individuals with mild infection, particularly for the antibody-dependent phagocytosis effector functions, which contribute markedly less to the antibody profile of asymptomatic individuals. Over time, few marked changes were observed in the relative contribution of the SARS-COV-2-specific antibody features in asymptomatic individuals, apart from an increased contribution of IgG1 and ADNKA, and decreased IgG3. Similarly, for individuals with mild infection, substantial relative decreases in IgM, pseudo-neutralising antibodies, IgA and IgG3 were noted, as well as relative increases in ADNKA and ADNP to the antibody profile.

SARS-CoV-2 infection elicits transient cross-reactive antibodies and memory B cells specific for other circulating coronaviruses.

Next, we evaluated the IgG responses to seasonal coronaviruses (229E, HKU-1, NL63-S and OC43-S) severe acute respiratory syndrome (SARS-CoV-1) spike protein and Middle East Respiratory Syndrome (MERS) virus spike protein using the MSD assay (**Fig. 3A**). IgG responses to these viral antigens were detected at the earliest time points. The kinetics of these IgG responses followed those seen to SARS-CoV-2 spike, suggesting that seasonal coronavirus cross-reactive responses were enhanced by SARS-CoV-2 infection. Responses to OC43-S, 229-E and HKU-1 were particularly high and correlated significantly with disease severity at day 180 and at the earliest time point assessed (day <20) **(Supplementary Fig. 2C).** The MSD assay also measured IgG responses against SARS-COV-2 Spike, NP and the RBD antigens, supporting our observations using the ELISA assay (**Supplementary Fig. 2D**).

IgG+ Memory B cells specific for the spike glycoprotein from seasonal coronaviruses (229E, HKU1, NL63 and OC43) were determined at the earliest timepoint available (acute <day 20 or day 28) and the 6-month final sampling (**Fig. 3B**). The lowest responses were observed in 229E and NL63 spike IgG+ ASCs following polyclonal stimulation, which also were consistent over time with the exception of the decreased number of NL63 spike-specific IgG+ memory B cells in individuals with mild infection (*P*=0.0046). Higher responses were detected when testing the specificity of cultured PBMCs to the beta-coronaviruses (HKU1 and OC43) spike glycoprotein. However, the boosted memory response

was transient, particularly in individuals with mild infection (HKU1: P=1x10^{-7}; OC43: P=1.5x10^{-7}) in which the decrease was more marked, which may be due to a higher sample number.

Effector poly-specific SARS-CoV-2 T cells are higher in those with mild symptoms and decline 6 months after infection

We examined the magnitude of the T cell response to SARS-CoV-2 using an ex vivo IFN-γ ELISpot assay at 28 days, 90-120 days and 180 days after SARS-CoV-2 infection (N=64-78 HCW/timepoint, 57 participants at all timepoints (including 12 with asymptomatic infection), and 6 volunteers with severe COVID-19 at day 180 (**Fig. 4A and 4B and Supplementary Table 3).** We have previously shown that this assay is specific for SARS-CoV-2, with negligible responses detected in SARS-CoV-2 pre-pandemic unexposed participants [5].

IFN-γ responses to at least one antigenic pool were seen in 67/70 (96%) volunteers tested 28 days after SARS-CoV-2, with a median total response across the pools of 373 (IQR 201–842) SFC/10^6 PBMC; here a response to spike (S1 and S2) was seen in 61/70 tested (87%) median 180 (IQR 71-364) SFC/10^6 PBMC, for M in 47/70 (67%) median 63 (IQR 25-160) SFC/10^6 PBMC and for NP in 62/70 (89%) median 121 (IQR 73-250) SFC/10^6 PBMC. However, total summed responses declined by a median of 60% after 90 days, and by 75% at 180 days (**Supplementary Table 3**). The majority (61/77 (79%)) of participants had detectable responses to at least one antigenic pool at 180 days, with responses to NP antigen most commonly observed 47/77 (61%) median 40 (IQR 23-73) SFC/10^6 PBMC. Responses to ORF3, ORF8 and NSP3B were less frequent than responses to S1, S2, M and NP at day 28 and lower at day 180.

IFN-γ ELISpot responses to SARS-CoV-2 antigens were higher in the mild symptomatic cohort (n=66), compared to the asymptomatic group (n=12) at 28 days, with median responses to all summed pools 455 (IQR 252-976) SFC/10^6 PBMC for mild disease compared to 196 (IQR 74-243) SFC/10^6 PBMC in the asymptomatic group (**Supplementary Fig. 3A**). There was no significant change in the magnitude of the T cell response in the asymptomatic group in the 6 months after infection (**Fig. 4A**).

We next used ICS to examine the duration of multiple T cell functions and the polyfunctionality of the T cell response over time at 28 and 180 days pso in individuals with ex vivo T cell ELISpot levels >100 SFC/10^6 PBMC for sensitivity reasons (n=18 with n=15 available at both timepoints for paired analysis (Gating strategy in **Supplementary Fig. 3D,** results in **Supplementary Fig. 4 and Supplementary Fig.5**). Similar to the ELISpot data, the majority of T cell responses decreased over time. In terms of functionality, we found that CD4+ T cells were polyfunctional, with the majority of cells expressing >1 and up to all 5 functional markers at both timepoints. Similarly, NSP3B-specific CD8+ T cells were also polyfunctional at both timepoints examined, with most cells expressing >1 functional marker (**Supplementary Fig. 4J**). There were no functional changes between the two timepoints.

T cell memory proliferative responses decline 6 months post SARS-CoV-2

We and others have found the assessment of T cell proliferation to be a sensitive method of detecting antigen-specific recall responses. We used this assay to evaluate the frequency of circulating SARS-CoV-2-specific CD4+ and CD8+ T cell in our longitudinal cohort (n = 54 – 57; gating strategy presented in **Supplementary Fig. 3B)**.

We did not observe any differences in the magnitude of circulating FEC-specific (control) CD4+ or CD8+ T cells within the 6 months period **(Supplementary Fig. 3C).** In the asymptomatic group, at 28 days pso 7/8 (87.5%) made a CD4+ T cell response to at least one SARS-CoV-2 protein (excluding S1 and S2 where have previously reported finding responses in the majority of unexposed volunteers [5]) while 5/8 (62.5%) of them had CD8 T cell response to at least one of M, NP, ORF3 or ORF8 proteins **(Fig. 5A-C Supplementary Table 4)**. Most of this response was targeted to M and NP **(Fig. 5A-C and Supplementary Table 4)**. At 180 days pso, 6/8 (75%) of recovered subjects had a CD4+ or CD8+ T cell response which was mostly focused on M, NP and ORF3. We observed no difference in the proliferative capacity of SARS-CoV-2-specific CD4 and CD8 T cells at 28- and 180-days post disease onset in the group with asymptomatic disease (n = 8) **(Fig. 5A-C and Supplementary table 4 and 5)**.

In the cohort with mild disease, at 28 days, T cell responses to at least one SARS-CoV-2 protein outside of spike region were observed in 42/49 (86%) for CD4+ T cells and 45/49 (91%) for CD8+ T cells. Similar to the asymptomatic cohort, these responses were focused on M, NP and ORF3 regions of SARS-CoV-2 **(Fig. 5A-C, Supplementary Table 4)**. At 180 days after symptom onset, this frequency of people responding to at least one protein as above reduced to 37/49 (75%) within CD4+ T cells and 35/49 (71%) for CD8+ T cells with a focus on M, NP and ORF3 similar to CD4+ T cells **(Fig. 5A-C and supplementary Table 4 and 5)**. In the volunteers with mild disease, we found a significant reduction in the circulating frequencies of SARS-CoV-2-specific CD4+ and CD8+ T cells to all proteins except NP and ORF8 for CD4+ and ORF3 and ORF8 for CD8+ T cells by day 180 **(Fig. 5A-C)**.

When we assessed the difference in the magnitude of the proliferative CD4+ and CD8+ T cell responses at 28- and 180 days pso in both asymptomatic and mild cases (analysed together as one group), we found significantly higher frequencies of SARS-CoV-2 specific CD4+ T cells compared to CD8+ responses at both timepoints in all proteins except NP and ORF8 for 28- and 180-days post symptom onset and ORF3 responses at 28 days post symptom onset only. Our data shows that the bias in antigen-specific responses to SARS-CoV-2 towards CD4+ T cells is maintained in the T cell memory compartment long after recovery from acute infection. Taken together, the results show that at 6 months post infection with SARS-CoV-2, convalescent subjects show diminished but detectable anti-SARS-CoV-2-specific memory T cells in both the CD4 and CD8 T cell compartments, with only 8/56 (14%) showing no proliferative response to any non-spike protein, suggesting durable immune response at least up to 6 months post initial infection.

Integrative analysis to Identify immune and clinical parameters associated with disease severity

To further investigate the trajectory of cellular and humoral adaptive immune responses during SARS-CoV-2 infection and relationship with disease severity, we performed integrative analysis on aggregated immunological and clinical data from 433 samples obtained from 86 donors (12 asymptomatic, 66 mild, 8 severe) on 6 different timepoints (**Fig. 6A)**. We investigated the trajectory of immune responses after SARS-CoV-2 infection and determined whether samples obtained from individuals with asymptomatic infection are more similar to samples obtained at later timepoints after infection in the individuals with mild, symptomatic disease. A t-distributed stochastic neighbour embedding (t-SNE) representation of integrated data revealed heterogeneity of immune responses in infected individuals, irrespective of days post symptom onset when these samples were collected (**Fig. 6B**, *left panel*). Majority of samples were separated between asymptomatic and mild individuals, while there was an overlap in similarity between individuals with mild and more severe disease (**Fig. 6B***, right panel*). To further delineate differences in clinical and immunological parameters of SARS-CoV-2 infected individuals, we performed clustering analysis on the resulting t-SNE representations (**Fig. 6C**) and compared expression of 16 clinical and 49 immunological parameters to identify each of three clusters (**Fig. 6D**). This approach identified heterogeneity within the SARS-CoV-2 positive individuals with mild disease clustered in two groups (**Fig. 6C and 6D**, *clusters 1 and 2*). In cluster 1, the majority of samples displayed increased antibody and T cell responses in comparison to other clusters, and some individuals with mild infection that showed clinical and immunological similarity to severe COVID-19 patients (**Fig. 6C and 6D**, *cluster 1*). In contrast, cluster 2 contained individuals with lower overall antibody and T cell responses and all were from individuals with mild disease (**Fig. 6C and 6D**, *cluster 2*). Clinical parameters were driving a major separation between asymptomatic SARS-CoV-2 positive individuals from those with mild or sever disease (**Fig. 6D**, *cluster 3*).

To gain an insight into immunological differences between individuals with asymptomatic and mild infection, we performed principal component analysis (PCA) on dataset containing only immunological parameters. The immunological parameters alone could explain 38.6% of variance between SARS-CoV-2 positive individuals, while separation was not driven by the disease severity (**Fig. 6E**). Comparable to t-SNE analysis, samples from individuals with mild disease were separated into three major groups having distinct immunophenotype (immunophenotypic group 1) (**Fig. 6E**, *lower right quadrant*) or sharing immunological similarity with samples from individuals with severe (immunophenotypic group 2) (**Fig. 6E**, *upper right quadrant*) or asymptomatic disease (immunophenotypic group 3) (**Fig. 6E**, *center*). To reveal which parameters are driving the separation, we visualized relationship between variables using correlation plot (**Fig. 6F**). T cell parameters were driving the separation of immunophenotypic group 1, while antibody responses separated immunophenotypic group 2 (**Fig. 6F**). The most important variables in explaining the variability between SARS-CoV-2 positive individuals in immunophenotypic group 1 were total IFN-γ ELISpot T cells, S1 and S2-stimulated IFN-γ ELISpot T cells, and anti-S IgG, anti-RBD IgG, ADCD, S-IgG from OC43 and HcoV-HKU1 in immunophenotypic group 2 that were correlated with principal components 1 and 2 (PC1-PC2) (**Fig. 6G and 6H**). The correlation plot revealed positive correlation between antibody responses, and negative correlation between T cell responses with the time when samples were

obtained (**Fig. 6F**). To further examine these associations between immunological parameters, we performed correlation analysis, which confirmed strong positive correlation between antibody and T cells responses (**Fig. 6I**). The antibodies directed against N, S and RBD from SARS-CoV-2, were positively correlated with antibody functionality, such as pseudoneutralising capacity and ADCD, ADNP and ADMP, and positively correlated with IFN-γ ELISpot T cell responses against S1, S2 and N (**Fig. 6I**). The antibody responses to S protein from other circulating coronaviruses, such as SARS-CoV-1, MERS, HcoV-HKU1, 229e and OC43 were also contained in this cluster being positively correlated with antibody and T cell responses (**Fig. 6I**). This cluster was negatively correlated with time, confirming the observations from primary analysis (**Fig. 6I**). Notably, there was a negative correlation between NL63 S antibodies and S and RBD SARS-CoV-2 specific antibodies (**Fig. 6I**). There were other apparent relationships in two other clusters identified, that were not associated with time, including positive correlation between proliferating T cells stimulated with different SARS-CoV-2-specific peptides, and positive correlation between ADNKA and S-IgA and S-IgG1, while negative correlation with S-IgM (**Fig. 6I**).

The integrative analysis revealed three distinct immunophenotypic groups of SARS-CoV-2 infected individuals strongly connected to cellular and humoral immune profiling beyond the disease severity and clinical parameters.

Identifying an early immunological signature associated with a durable immune response to SARS-CoV-2

To elucidate an early immunological signature that could predict whether an individual will mount a durable and protective immunity against SARS-CoV-2 6 months after infection, we stratified SARS-CoV-2 infected individuals into high and low responders, based on the seropositivity status (N IgG titres >=1.4), which has recently been identified as a correlate of protection [35]. We then asked whether the components of cellular or humoral immunity within one month of infection (28 days pso) were predictive of the ability of individuals to develop protective immunity against SARS-CoV-2 (6 months pso). First, using an unsupervised machine learning approach, i.e., hierarchical clustering of integrated immunological data on day 28 pso, we identified two groups of SARS-CoV-2 infected individuals based on the response status 6 months pso (**Fig. 7A**). While the majority of SARS-CoV-2 infected individuals with mild disease would mount protective immunity 6 months pso and become high responders, there was a proportion of individuals with mild disease that failed to mount durable and protective immunity (low responders) (**Fig. 7A**). The majority of individuals with asymptomatic infection were low responders. High responders mounted stronger antibody responses, in particular N-IgG and pseudo-neutralising antibodies, and overall, stronger T cell responses, including IFN-γ-positive and proliferating T cells, than low responders 28 days pso (**Fig. 7A**). Antibody responses to spike protein from 229e and NL63, B cell ELISpot and ADNKA were increased in low responders early after SARS-CoV-2 infection in comparison to high responders (**Fig. 7A**).

To further define the immunological features that can distinguish individuals with durable and protective immunity and predict if the individual is on the trajectory to become a high or low responder, we used

584 the SIMON supervised machine learning approach [28, 29]. We generated 30 resamples and tested 3,565
585 models using 172 machine learning algorithms (*Materials and methods*). The best performing model
586 built using Sparse Partial Least Squares (sPLS) algorithm (train AUROC: 0.95 (CI 0.5-1) and test
587 AUROC: 1) used only 8 out of 49 measured parameters on day 28 pso to predict if the individual will
588 become high or low responder 6 months pso (**Fig. 7B**). The features that were contributing the most to
589 this model included antibody responses to N and S, ADCD and pseudo-neutralising antibodies to
590 SARS-CoV-2, and T cell IFN-γ ELISpot (S1/S2, M and total positive T cells) which were significantly
591 increased in high responders 28 days pso compared to low responders (**Fig. 7C and 7D**). Together,
592 these data indicate that early generation of antibodies with high binding, neutralising and effector
593 function, and functional T cell responses following infection can predict the responsiveness potential,
594 i.e., protection and duration of SARS-CoV-2 immunity of the individual. Additionally, these findings
595 suggest that a coordinated action of both T and B cells early after infection is required for establishment
596 of durable and protective immunity.
597
598 The generation of durable and functional humoral and cellular immunity in a proportion of SARS-CoV-
599 2 infected individuals (high responders) may provide protection against re-infection, including also
600 against variants of concern (VOCs). Thus, we assessed the neutralising antibody responses in high
601 and low responders against the infecting (Victoria) strain and against variants B.1.1.7 and B.1.351 (**Fig.**
602 **7E**). Individuals with durable and protective SARS-CoV2 immunity shown high neutralisation antibody
603 titres against wild-type circulating SARS-CoV-2 (Victoria) strain, and against two novel variants,
604 including B.1.1.7 (alpha) and B.1.351 (beta) (**Fig. 7E**). High responders had significantly higher
605 neutralising antibody titres against B.1.1.7 alpha variant one-month pso, and these higher neutralising
606 antibodies were preserved 6 months pso (**Fig. 7E**).
607
608 Altogether, these data suggest that generation of immunity to SARS-COV-2 shows distinct trajectories
609 following early priming, and early antibody responses are important to mediate protective and durable
610 immunity that can also provide protection against novel variants.
611
612 **Discussion**
613 Key questions on the trajectory of the SARS-CoV-2 specific immune response to natural infection, and
614 the maintenance of immune memory remain highly relevant even as highly effective vaccines are being
615 rolled out worldwide. Firstly, even with high availability of vaccines there will always be a pool of
616 unvaccinated people due to vaccine hesitancy or access difficulties, and this will include people who
617 have had natural infection. Secondly, as of June 2021 only 12% of the world's population is estimated
618 to have received at least one dose of vaccine [36], so for much of the immunity globally is from natural
619 infection, which remains a cornerstone of population-level immunity. Thirdly, measuring immune
620 responses to antigens not included in spike-containing vaccines are used as biomarkers of previous
621 SARS-CoV-2 infection and as such are widely used to stratify immune responses to vaccination, since
622 prior SARS-CoV-2 is known to enhance vaccine responsiveness [37, 38]. Finally, understanding how the
623 early immune response translates into lasting immunity towards emerging variants of concern is crucial

to accelerate predictions of population risk and to drive policy. In this manuscript, we characterise the magnitude, function and maintenance of humoral and cellular T and B cell immunity, and the relationship between clinical and multi parametric immune data. We then evaluate the ability of antibodies to neutralise live SARS-CoV-2 virus 6 months after primary infection to variants of concern and provide insight into the early predictors of durable neutralising antibody after natural infection.

Compatible with other studies [12, 39, 40, 41], our data shows a peak of anti-NP and anti-S binding antibody (IgG) magnitude 28 days after onset of symptoms, with anti-NP responses declining over the next five months, although these responses remain above the threshold of detection in the majority. In contrast, anti-S IgG responses were well maintained, in keeping with the reported longer half-life for decay of anti-S IgG responses compared with anti-NP IgG responses [12], along with maintenance of B cell memory. Neutralisation measured by a pseudo-neutralisation assay showed a decline over time but was generally maintained six months following infection. High levels of neutralisation were seen earlier post symptom onset (from 7 days) compared with the IgG binding assays, which may represent contributions from IgM [42] and IgA [43]. Some of the observed decline in neutralising antibodies over time may represent a threshold effect – NAb are a subset of total IgG such that gradual declines over time are first measurable in NAb, but biologically important neutralisation may still occur below the detection threshold. Fc-mediated functionality including antibody dependent NK activation, phagocytosis and complement deposition was maintained over the 6 months duration which may make an important contribution to protective immunity and was significantly associated with increasing disease severity.

Taken together, B cell polyfunctionality was lower in those with asymptomatic infection, compared with those with mild disease early after infection (day 28), though by 6 months the profiles between the cohorts looked similar. The most notable changes were a reduction in IgM spike responses but a relative maintenance of IgG3 spike responses in the mild cohort that was not seen in the asymptomatic cohort.

Previous studies have shown that early distinct antigenic targets and qualitative features of SARS-CoV-2-specific antibodies are associated with disease trajectory [44, 45], whilst multifunctional antibody responses, and particularly ADCD and ADNP, following adoptive transfer of IgG from convalescent rhesus macaques have been shown to contribute to protection from SARS-CoV-2 challenge [46]. Furthermore, vaccine-induced Fc-mediated polyfunctionality has been observed following administration of efficacious vaccines in both macaque and human studies [31, 47]. While the capacity of Fc receptor binding appears to be lower in convalescent individuals against VOCs, evidence is emerging of maintenance of vaccine-induced Fc-functional antibody properties against VOCs supporting resilience of humoral immunity against VOCs independent of neutralisation [48].

In evaluating SARS-CoV-2 specific effector T cell responses over six months in an IFN-γ ELISpot assay, we showed that there was significant heterogeneity in the magnitude of responses between individuals as previously reported [12, 49, 50]. The majority of people showed robust T cell responses in the first 28 days after infection, though these were significantly lower in the asymptomatic cohort. Within 3 months

of infection there was a marked decline in T cell responses and by 6 months, these were reduced by 75% and were undetectable in approximately 20%. We used a flow cytometry based 7-day proliferation assay to assess memory T responses of both CD4+ and CD8+ T cell subsets to show a dominant CD4+ T cell subset response. Although memory proliferative responses have been shown to "mature" over time, particularly following vaccination [51, 52], we show that proliferative responses (both CD4 and CD8), targeting Spike, M, and NP decline markedly between day 28 and day 180. ICS analysis showed that CD4+ T cells were the dominant subset targeting S1, S2 and M antigens, whilst NP were targeted by both CD4+ and CD8+ T cells, and NSP3B was targeted by CD8+ T cells. Polyfunctional T cells, producing multiple cytokines, were generated at day 28, and although the magnitude of the response declined, polyfunctionality was generally retained out to 6 months.

In our study we show that symptomatic infection is associated with more robust cellular and humoral immune responses compared to the asymptomatic group early after PCR+ confirmed infection. An association between asymptomatic infection and lower antibody responses has been previously reported [53], and we and others have shown a correlation between disease severity and higher levels of antibody and T cell responses in early disease [4, 54]. Similar results have been reported in other disease settings including robust immune responses associated with disease severity in H1N1/09 influenza A [55]. In contrast, a previous prospective SARS-CoV-2 screening study has observed that asymptomatic infection is associated with highly functional cellular immune responses [56]. Either way, humoral and cellular immune responses measured months after primary infection is found at low magnitude following asymptomatic infection. These findings raise the possibility that people with asymptomatic SARS-CoV-2 infection may have less protective immunity months after primary infection. A limitation to our study, is that the timing of infection onset in asymptomatic HCW, (even though PCR+) is not precisely defined. As such, it is theoretically possible that the asymptomatic individuals in our study are later in their disease course at detection, which was further explored by integrative analysis.

To elucidate the trajectory of the immune response of SARS-CoV-2 infected individuals over time and identify signatures associated with the maintenance of protective immunity, we performed an integrative analysis in the cohort of 86 individuals on all 433 samples. The results of the integrative analysis led to several key findings. First is the identification of immunophenotypic groups of SARS-CoV-2 infected individuals beyond disease time course and disease severity. By integrating over 70 immune parameters with clinical data, disease severity and temporal changes, we generated a computational model using t-SNE embedding algorithm that coupled immunological phenotypes of each individual with the disease severity and other clinical parameters. The t-SNE representation of integrated data revealed minimal clustering by time point, suggesting that heterogeneity of the immune response during the SARS-CoV-2 infection is independent of the time course during the infection. While some of the individuals with asymptomatic infection may be later in their disease course at detection, the majority did not cluster with the samples obtained from individuals with mild or severe infection at later timepoints after the infection. The major separation of individuals with asymptomatic disease was driven by clinical parameters, while the mild cohort clustered into 2 immunophenotypic groups (not driven by clinical

parameters), one of which shared phenotype with the severe disease cohort. The PCA analysis provided further support for the heterogeneity of the immune responses in the SARS-CoV-2 infected individuals with mild disease and separation into three immunophenotypic groups, confirming that 38.6% of variance between individuals was explained by the immunological data. The results suggested that immunophenotypic group 1, exhibiting robust binding (anti-N and anti-S) and functional (pseudoneutralising and ADCD/ADMP) antibody responses and memory B cell involvement, shared similarity with individuals with severe disease, while immunophenotypic group 2 composed of functional IFN-γ T cell responses represented an unique proportion of individuals with mild disease, early in the course of the disease (as indicated by negative correlation with time when samples were acquired). The third immunophenotypic group – defined by the lower overall antibody and T cell responses - shared similarities with the asymptomatic cohort, suggesting that some individuals may fail to develop robust antibody and T cell responses despite having mild infection. These results support the magnitude of the immune response being determined by factors beyond disease severity, including viral factors and the individual's immunocompetence. Using correlation analyses, we observed a positive association between spike and nucleocapsid T cell and antibody responses (both decreased with time, confirming the primary analysis) and cross-reactivity to other coronaviruses which correlated with spike and nucleocapsid T cell and antibody responses (NL63 is negatively correlated and OC43 is positively correlated), substantiating the findings that immunity may be defined by immunocompetence and previous exposure to circulating coronaviruses.

To further delineate this observation, we performed integrative analysis using baseline parameters only (measured on day 28 after infection), and this led to the second key finding – identification of an early immunological signature that is associated with durable and protective SARS-CoV-2 immunity. Using hierarchical clustering approach and integrated baseline cellular and humoral immune parameters, we observed distinct clustering of high and low responders at this early time point. High anti-N IgG, along with more robust overall T cell responses (including IFN-γ ELISpot and proliferation) at baseline with a low response to seasonal coronaviruses (NL63 and 229e) dominated in the high responder group, whilst low responders had lower anti-N IgG and overall T cell responses and had more pronounced cross-reactive seasonal CoV responses (NL63 and 229e) at baseline. The final major finding was the ability to predict if the individual will generate durable and protective SARS-CoV-2 immunity 6 months post infection based on the early immunological signature one month after infection. With the use of SIMON data mining tool and generation of more than 3,500 predictive machine learning models, we identified upregulation of antibody responses (spike and NP, with pseudoneutralising and ADCD functions) combined with the more robust T cell responses as predictors of individuals who will generate durable and protective immunity 6 months post infection (high responders). The predictive model built by SIMON suggests a link between both arms of the immune response - cellular and humoral immunity – with the durability of the SARS-CoV-2 protective immunity. Thus, this early immunological signature may determine essential differences of the trajectory that each individual will take after SARS-CoV-2 infection. Importantly, the sera of the individuals who will go on to generate durable and protective SARS-CoV-2 immunity (high responders) 6 months post infection, were better able to neutralise both

744 the Victoria strain (the likely infection strain), and also the VOCs (B.1.1.7 - alpha and B.1.351 - beta)
745 one month after infection, and such protective neutralising antibody responses were durable (as
746 measured 6 months post infection). In contrast, those who were low responders 6 months after infection
747 showed a reduction in the capacity to neutralise the Victoria strain, with a severe loss of neutralisation
748 against both VOC - particularly B1.351.
749
750 Overall, our data reveal the highly variable range of immunity after SARS-CoV-2 infection and suggest
751 that immune events primed during early SARS-CoV-2 infection may define the subsequent trajectories
752 leading to the effective maintenance or loss of long-term SARS-CoV-2 protective immunity as measured
753 by neutralising antibodies. Importantly, previous infection may not give ongoing protection against VOC
754 months later, and people with asymptomatic infection had lower responses at all time points across
755 many of the immune parameters we measured. Maintenance of immune memory over time is critically
756 required for the effective neutralisation of VOC that is most likely to confer sterilising immunity, whilst
757 other immune mechanisms including non-neutralising antibodies and T cells may account for the
758 protection against severe disease, including for VOC [57, 58, 59, 60]. This study provides a basis for more
759 targeted vaccination programme of previously infected individuals based on early immunological
760 signature 28 days after infection.
761
762 **Figure Legends**
763
764 **Figure 1: Longitudinal humoral immune responses in individuals with PCR confirmed SARS-**
765 **CoV-2 asymptomatic, mild or severe infection.**
766 Humoral immune responses were assessed in acute and convalescent by binding antibody ELISA for
767 total IgG specific to the **(A)** Nucleopcapsid and **(B)** Spike glycoprotein, quantification of **(C)** IgG
768 memory B cells specific to the spike glycoprotein, and **(D)** pseudoneutralisation antibody titres. Boxplots
769 represent the median with interquartile range, a Wilcoxon rank-sum test was used to compare between
770 study time points. A generalised additive mixed model (GAMM) by restricted maximum likelihood —
771 right-hand plots — was used to fit the immunological measures (log10 transformed) taken at multiple
772 study time points, using Gaussian process smooth term. Disease severity group was included in the
773 GAMM as a linear predictor and a participant identifier was included as a random effect. See Table S1
774 for number of individuals evaluated per assay.
775
776 **Figure 2: Antibody isotype, subclass and function in individuals with PCR confirmed SARS-**
777 **CoV-2 asymptomatic, mild or severe infection.**
778 SARS-CoV-2 spike-specific antibody isotype and subclasses measured post-infection: **(A)** IgM, **(B)** IgA,
779 **(C)** IgG1 and **(D)** IgG3. Antibody function measure post-SARS-CoV-2 infection: **(E)** antibody-dependent
780 NK cell activation (ADNKA), **(F)** antibody-dependent neutrophil phagocytosis (ADNP), **(G)** antibody-
781 dependent monocyte phagocytosis (ADMP) and **(H)** antibody-dependent complement deposition
782 (ADCD). **(I)** Polar plot of various antibody isotype, subclass and function data, minimum-maximum
783 normalised. Boxplots represent the median with interquartile range, a Wilcoxon rank-sum test was
784 used to compare between study time points. A generalised additive mixed model (GAMM) by restricted

maximum likelihood — right-hand plots — was used to fit the immunological measures (log10 transformed) taken at multiple study time points, using Gaussian process smooth term. Disease severity group was included in the GAMM as a linear predictor and a participant identifier was included as a random effect. See Table S1 for number of individuals evaluated per assay.

Figure 3: Longitudinal specific-IgG and memory B cell responses to spike protein from non-SARS-CoV-2 coronaviruses.

(A) Meso Scale Discovery (MSD) multiplexed immunoassay (MIA) platform measurements of antibody levels to spike protein from non-SARS-CoV-2 coronaviruses. **(B)** Memory B cells responses to spike protein from non-SARS-CoV-2 coronaviruses. See Table S1 for number of individuals evaluated per assay.

Figure 4 Magnitude of SARS-CoV-2 specific Effector T cell Response.

(A) *Ex vivo* IFN-γ ELISpot showing the effector T cell responses to summed SARS-CoV-2 peptide pools spanning spike, accessory and structural proteins (summed total of SARS-CoV-2 proteins tested, S1, S2, NSP3B, M, NP, ORF 3, ORF8 and the CEFT positive control peptides for T cell responses) in 78 individuals 28, 90 and 180 days after mild or asymptomatic SARS-CoV-2 infection (onset of symptoms for mild cases, PCR positive test for asymptomatic participants). **(B)** Heatmap displaying unsupervised hierarchical clustering of the ELISpot data in (A) and disease severity (mild or asymptomatic) for the original SARS-CoV-2 diagnosis. Sfu / million PBMCs = spot forming units per million peripheral blood mononuclear cells, with background subtracted. D28, d90 and d180 = days after SARS-CoV-2 diagnosis. Grey regions on heatmap represent missing data due to insufficient cells. Plots show median with error bars indicating +/- IQR. Friedman test with Dunn's multiple comparisons test was performed.

Figure 5. Proliferative responses to SARS-CoV-2 peptide pools at 1- and 6-months post infection

Proliferative responses against **(A)** SARS-CoV-2 proteins S1, S2, M, NP, ORF3 and ORF8 presented in CD4+ (Left hand panel) and CD8+ (Right hand panel) T cells measured at 28 and 180 days pso for volunteers with mild disease or days post PCR positivity for asymptomatic disease (asymptomatic n = 8, mild disease n = 49). Kruskal Wallis T test, all P values are all stated on plots. **(B)** shows unsupervised hierarchical clustering showing visual representation of SARS-CoV-2 specific responses at day 28 and 180 in both CD4+ and CD8+ T cell compartments and **(C)** comparative analysis of SARS-CoV-2 specific CD4+ and CD8+ T cell responses at day 28 (top panel) and day 180 (bottom panel) in both asymptomatic and mild groups (analysed as one group). Kruskal Wallis T test, all P values are all stated on plots.

Figure 6. Integrative analysis of clinical and longitudinal immunological data reveals distinct immunophenotypic groups of SARS-CoV-2 infected individuals. (A) Clinical study overview. **(B)** t-SNE map of integrated clinical and immunological data color-coded based on timepoint or disease severity. **(C)** Clustered t-SNE analysis. **(D)** Heatmap of clinical and immune parameters across three identified clusters. **(E)** PCA plot representing integrated immunological data, grouped based on the

disease severity. Percentage indicates the variance explained by the principal component (PC). **(F)** Variable correlation plot. Positively correlated variables are grouped together, while negatively correlated variables are positioned on opposite quadrants. The distance between variables and the origin measures the quality of the variables on the factor map, while the colour indicated the quality of representations as cos2. **(G)** Quality of variable representations (color-coded, cos2) and contributions of variables to principal components 1 and 2 (size of the circle). **(H)** Top 10 variables and their contribution to PC 1 and 2. **(I)** Correlations of immunological parameters with time component across samples. Spearman's correlation coefficient (colour coded) and only significant values shown (after adjusted FDR <0.05). Black boxes indicate clusters (hierarchical clustering).

Figure 7. Early signature of durable SARS-CoV2 protective immunity. (A) Hierarchical clustering heatmap of immune parameters on day 28 pso, grouping by responder status 6 months pso and disease severity. Results obtained using complete linkage agglomeration method, dendrogram ordered tightest cluster first. **(B)** Integrative immunological dataset containing 3,626 datapoints (49 features and 74 donors) was used for SIMON analysis to predict if the individual will generate high or low anti-N antibody responses 6 months pso. In total, 184 ML algorithms were tested and 2,556 model built. ROC plot of the best performing model built with the svmPoly algorithm. Train AUROC (black line) is determined using 10-fold cross-validation and test AUROC evaluated on the independent test set (25% of the initial dataset). **(C)** Top variables that contribute to the model and are increased in high relative to low responders. **(D)** Frequency of selected variables on day 28pso (bars show mean with SEM). Mann-Whitney test ($p<0.05$). **(E)** Neutralisation assay against wild-type SARS-CoV2 (Victoria), and two novel variants (B1.1.7 and B1.351) between high and low responders on two timepoints (one and 6 months pso). Plots show mean with SEM. Kruskal-Wallis, with Dunn's multiple comparison test ($p<0.05$) was performed.

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Figures

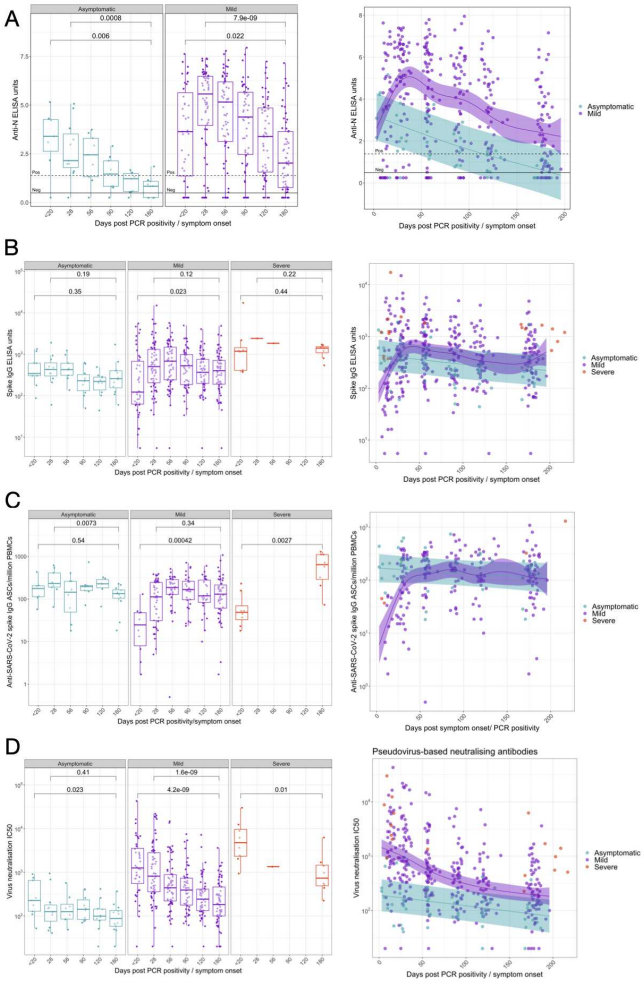

Figure 1

Figure 1

Figure 1: Longitudinal humoral immune responses in individuals with PCR confirmed SARS-CoV-2 asymptomatic, mild or severe infection. Humoral immune responses were assessed in acute and convalescent by binding antibody ELISA for total IgG specific to the (A) Nucleopcapsid and (B) Spike

glycoprotein, quantification of (C) IgG memory B cells specific to the spike glycoprotein, and (D) pseudoneutralisation antibody titres. Boxplots represent the median with interquartile range, a Wilcoxon rank-sum test was used to compare between study time points. A generalised additive mixed model (GAMM) by restricted maximum likelihood — right-hand plots — was used to fit the immunological measures (log10 transformed) taken at multiple study time points, using Gaussian process smooth term. Disease severity group was included in the GAMM as a linear predictor and a participant identifier was included as a random effect.

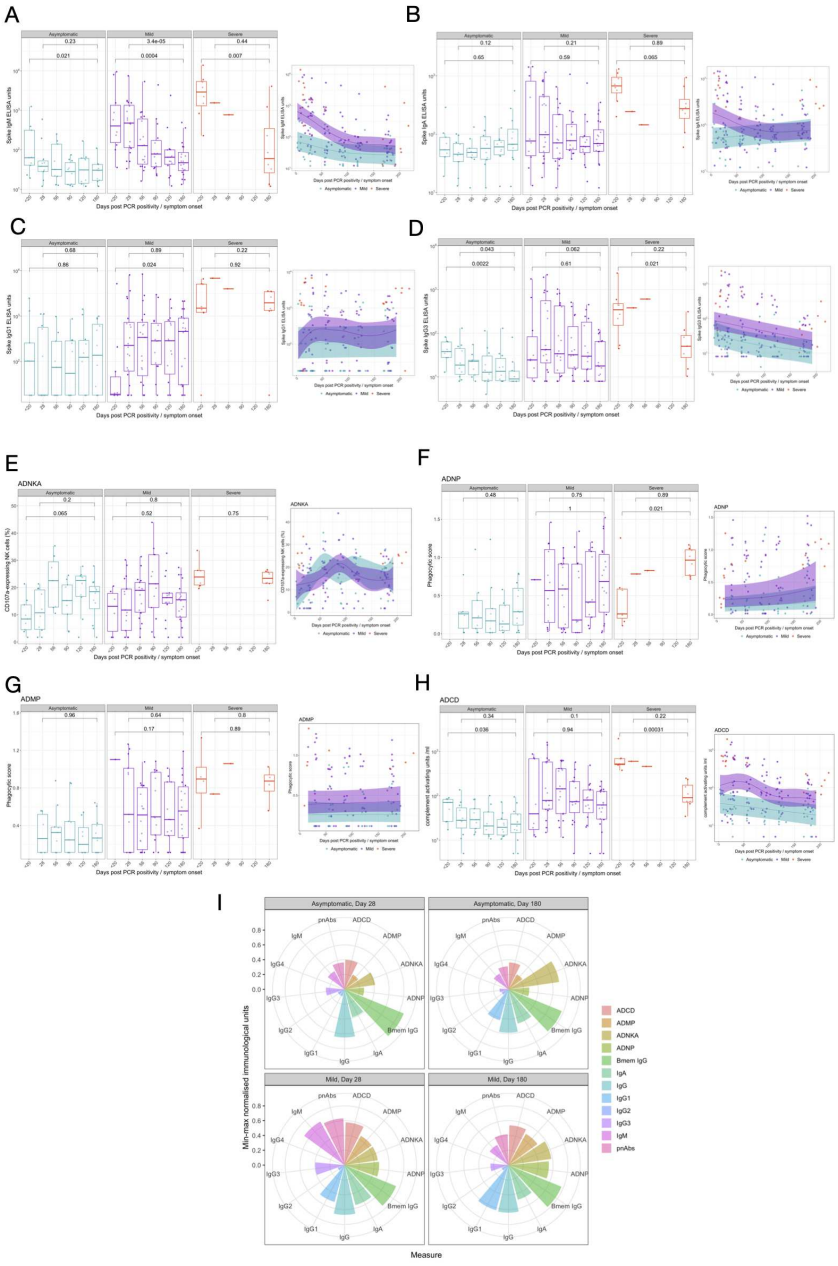

Figure 2

Figure 2

Figure 2: Antibody isotype, subclass and function in individuals with PCR confirmed SARS-CoV-2 asymptomatic, mild or severe infection. SARS-CoV-2 spike-specific antibody isotype and subclasses measured post-infection: (A) IgM, (B) IgA, (C) IgG1 and (D) IgG3. Antibody function measure post-SARS-CoV-2 infection: (E) antibody-dependent NK cell activation (ADNKA), (F) antibody-dependent neutrophil phagocytosis (ADNP), (G) antibody-dependent monocyte phagocytosis (ADMP) and (H) antibody-dependent complement deposition (ADCD). (I) Polar plot of various antibody isotype, subclass and function data, minimum-maximum normalised. Boxplots represent the median with interquartile range, a Wilcoxon rank-sum test was used to compare between study time points. A generalised additive mixed model (GAMM) by restricted maximum likelihood — right-hand plots — was used to fit the immunological measures (log10 transformed) taken at multiple study time points, using Gaussian process smooth term. Disease severity group was included in the GAMM as a linear predictor and a participant identifier was included as a random effect.

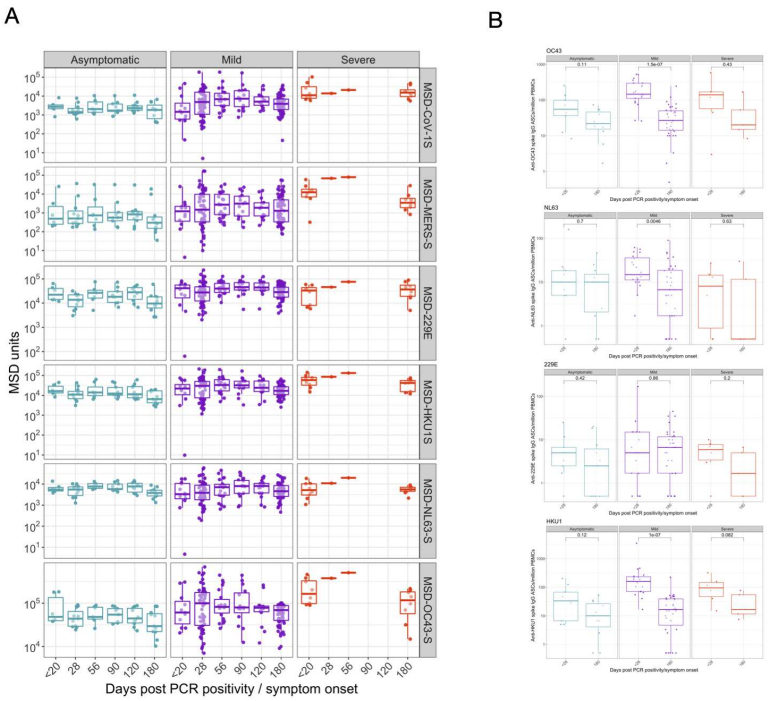

Figure 3

Figure 3

(A) Meso Scale Discovery (MSD) multiplexed immunoassay (MIA) platform measurements of antibody levels to spike protein from non-SARS-CoV-2 coronaviruses. (B) Memory B cells responses to spike protein from non-SARS-CoV-2 coronaviruses.

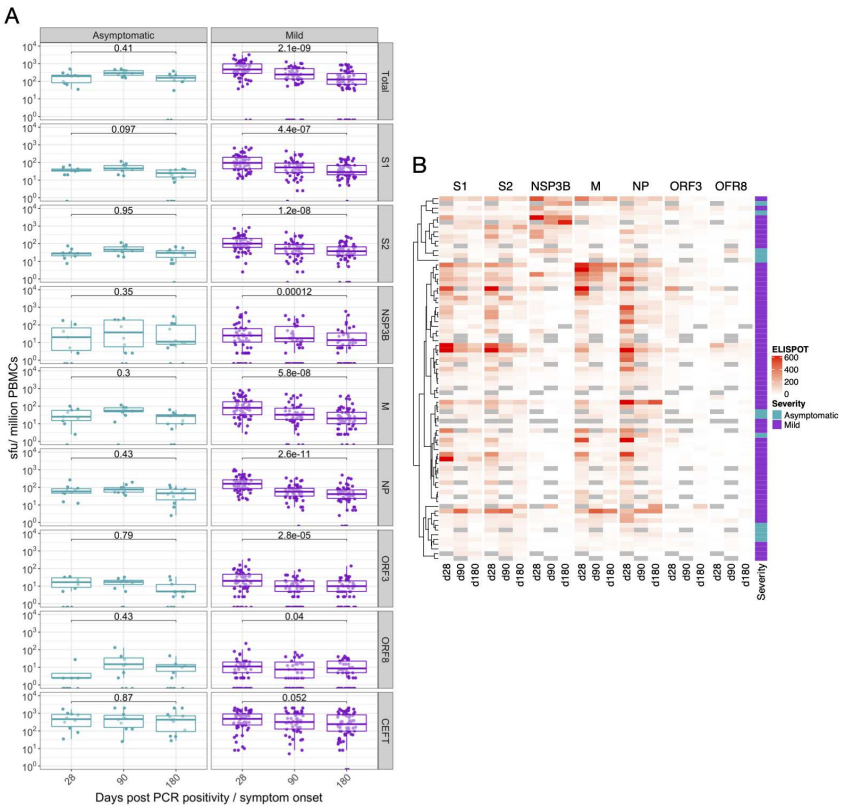

Figure 4

Figure 4

Figure 4 Magnitude of SARS-CoV-2 specific Effector T cell Response. A. Ex vivo IFN-γ ELISpot showing the effector T cell responses to summed SARS-CoV-2 peptide pools spanning spike, accessory and structural proteins (M, NP, NSP3B, ORF 3, ORF8, S1, S2, summed total of SARS-CoV-2 proteins tested and the CEFT positive control peptides for T cell responses) in 78 individuals 28, 90 and 180 days after SARS-CoV-2 (onset of symptoms for mild cases, PCR positive test for asymptomatic participants). Heatmap

displaying unsupervised hierarchical clustering of the ELISpot data in (A) and disease severity (mild or asymptomatic) for the original SARS-CoV-2 diagnosis. Sfu / million PBMCs = spot forming units per million peripheral blood mononuclear cells, with background subtracted. D28, d90 and d180 = days after SARS-CoV-2 diagnosis. Grey regions on heatmap represent missing data due to insufficient cells. Plots show median with error bars indicating +/- IQR. Friedman test with Dunn's multiple comparisons test was performed.

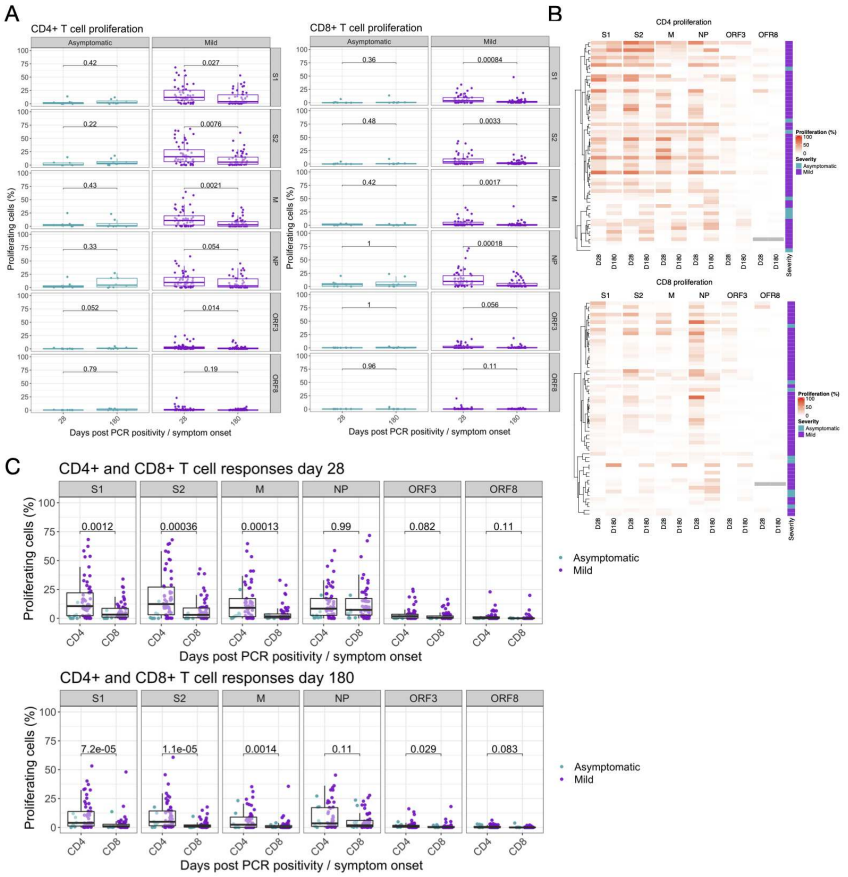

Figure 5

Figure 5

Figure 5. Proliferative responses to SARS-CoV-2 peptide pools at 1- and 6-months post infection Proliferative responses against (A) SARS-CoV-2 proteins S1, S2, M, NP, ORF3 and ORF8 presented in CD4+ (Left hand panel) and CD8+ (Right hand panel) T cells measured at 28 and 180 days pso for volunteers with mild disease or days post PCR positivity for asymptomatic disease (asymptomatic n = 8, mild disease n = 49). Kruskal Wallis T test, all P values are all stated on plots. (B) shows unsupervised hierarchical clustering showing visual representation of SARS-CoV-2 specific responses at day 28 and 180 in both CD4+ and CD8+ T cell compartments and (C) comparative analysis of SARS-CoV-2 specific CD4+ and CD8+ T cell responses at day 28 (top panel) and day 180 (bottom panel) in both asymptomatic and mild groups (analysed as one group). Kruskal Wallis T test, all P values are all stated on plots.

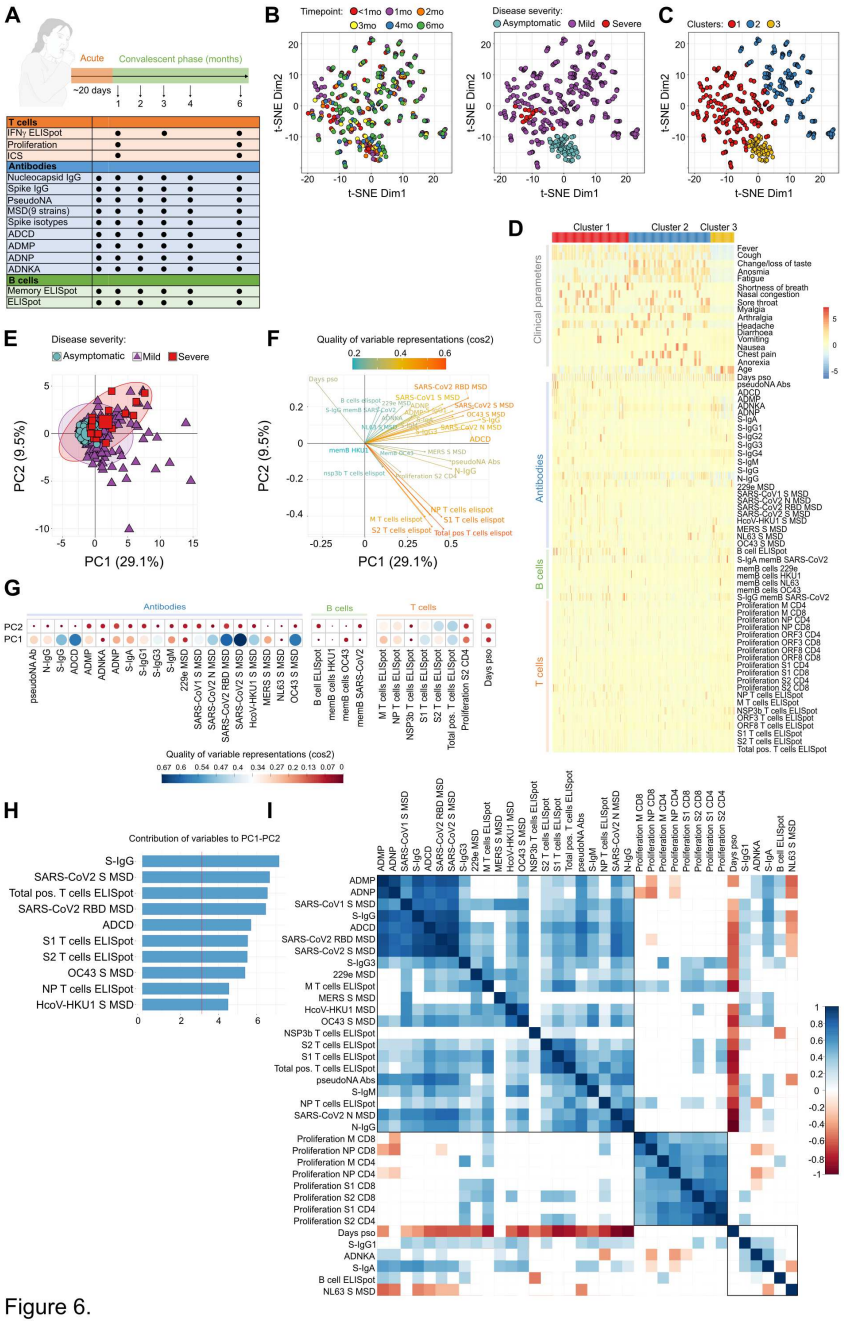

Figure 6.

Figure 6

Figure 6. Integrative analysis of clinical and longitudinal immunological data reveals distinct immunophenotypic groups of SARS-CoV-2 infected individuals. (A) Clinical study overview. (B) t-SNE map of integrated clinical and immunological data color-coded based on timepoint or disease severity. (C) Clustered t-SNE analysis. (D) Heatmap of clinical and immune parameters across three identified clusters. (E) PCA plot representing integrated immunological data, grouped based on the disease severity.

Percentage indicates the variance explained by the principal component (PC). (F) Variable correlation plot. Positively correlated variables are grouped together, while negatively correlated variables are positioned on opposite quadrants. The distance between variables and the origin measures the quality of the variables on the factor map, while the colour indicated the quality of representations as cos2. (G) Quality of variable representations (color-coded, cos2) and contributions of variables to principal components 1 and 2 (size of the circle). (H) Top 10 variables and their contribution to PC 1 and 2. (I) Correlations of immunological parameters with time component across samples. Spearman's correlation coefficient (colour coded) and only significant values shown (after adjusted FDR <0.05). Black boxes indicate clusters (hierarchical clustering).

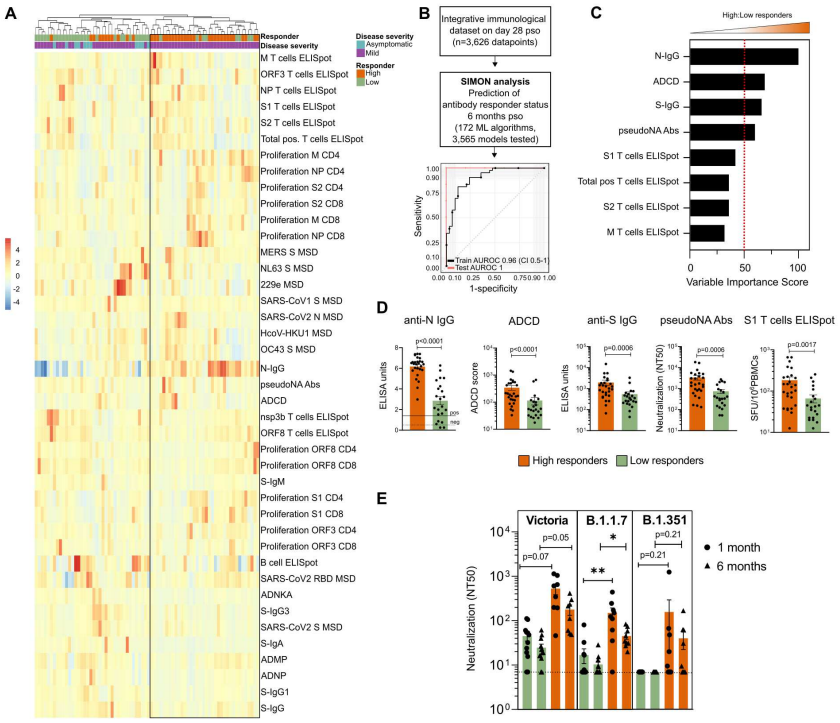

Figure 7.

Figure 7

Figure 7. Early signature of durable SARS-CoV2 protective immunity. (A) Hierarchical clustering heatmap of immune parameters on day 28 pso, grouping by responder status 6 months pso and disease severity. Results obtained using complete linkage agglomeration method, dendrogram ordered tightest cluster first. (B) Integrative immunological dataset containing 3,626 datapoints (49 features and 74 donors) was used for SIMON analysis to predict if the individual will generate high or low anti-N antibody responses 6

months pso. In total, 184 ML algorithms were tested and 2,556 model built. ROC plot of the best performing model built with the svmPoly algorithm. Train AUROC (black line) is determined using 10-fold cross-validation and test AUROC evaluated on the independent test set (25% of the initial dataset). (C) Top variables that contribute to the model and are increased in high relative to low responders. (D) Frequency of selected variables on day 28pso (bars show mean with SEM). Mann-Whitney test ($p<0.05$). (E) Neutralisation assay against wild-type SARS-CoV2 (Victoria), and two novel variants (B1.1.7 and B1.351) between high and low responders on two timepoints (one and 6 months pso). Plots show mean with SEM. Kruskal-Wallis, with Dunn's multiple comparison test ($p<0.05$) was performed.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Suppfig1.tiff
- Suppfig2.tiff
- Suppfig3.tiff
- Suppfig4.tiff
- Suppfig5.tiff
- AppendixSupplementaryFiguresandAdditionalMethods.pdf