Rapid determination of the wide dynamic range of SARS-CoV-2 Spike T cell responses in whole blood of vaccinated and naturally infected

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Declaration of interest

A.T. Tan, N. Le Bert and A. Bertoletti reported a patent for a method to monitor SARS-CoV-2-specific T cells in biological samples pending. W.N. Chia reported a patent for a sublicense agreement with GenScript for the surrogate virus neutralization test pending (Duke-NUS). L. Wang reported a patent application on sVNT pending. A. Bertoletti reported personal fees from Oxford Immunotech and Qiagen outside the submitted work. The other authors have declared that no conflict of interest exists.

Abstract (words 249)

Background: Antibodies and T cells cooperate to control virus infections. The definition of the correlates of protection necessary to manage the COVID-19 pandemic, require both immune parameters but the complexity of traditional tests limits virus-specific T cell measurements.

Methods: We test the sensitivity and performance of a simple and rapid SARS-CoV-2 Spike-specific T cell test based on stimulation of whole blood with peptides covering the SARS-CoV-2 Spike protein followed by cytokine (IFN-γ, IL-2) measurement in different cohorts including BNT162b2 vaccinated (n=112; 201 samples), convalescent asymptomatic (n=62; 62 samples) and symptomatic (n=68; 115 samples) COVID-19 patients and SARS-CoV-1 convalescent individuals (n=12; 12 samples).

Results: The sensitivity of the rapid cytokine whole blood test equates traditional methods of T cell analysis (ELISPOT, Activation Induced Markers). Utilizing this test we observed that Spike-specific T cells in vaccinated preferentially target the S2 region of Spike and that their mean magnitude is similar between them and SARS-CoV-2 convalescents at 3 months after vaccine or virus priming respectively. However, a wide heterogeneity of Spike-specific T cell magnitude characterizes the individual responses irrespective of the time of analysis. No correlation between neutralizing antibody levels and Spike-specific T cell magnitude were found.

Conclusions: Rapid measurement of cytokine production in whole blood after peptide activation revealed a wide dynamic range of Spike-specific T cell response after vaccination that cannot be predicted from neutralizing antibody quantities. Both Spike-specific humoral and cellular immunity should be tested after vaccination to define the correlates of protection necessary to evaluate current vaccine strategies.

Introduction

SARS coronavirus-2 (SARS-CoV-2), the etiological agent of coronavirus disease 2019 (COVID-19), has spread worldwide resulting in a global health and economic crisis that mass vaccinations are trying to resolve. The host's capacity to be protected from viral infection or from the development of severe diseases requires a coordinated activation of different components of the immune system that ultimately lead to the production of neutralizing and antigen binding antibodies and antiviral T cells. Evidence that antibodies and T cells are required for protection have been generated in monkeys challenged with SARS-CoV-2 (1). Similarly, antibodies and T cells are present in the majority of SARS-CoV-2 infected individuals who control infection without severe symptoms (2-6) and a robust CD8 T cells response is associated with mild disease in oncological patients with humoral defects (7).

Recently developed SARS-CoV-2 vaccines that protect more than 90% of the vaccinated individuals from severe COVID-19 can induce Spike-specific antibodies and T cells (8, 9, 10). However, it is not fully clear which level of antibodies and/or T cells is necessary to exert such protection and whether differences do exist in antibody and T cell levels in vaccinated subjects. Efforts to define the protective threshold of antibodies through mathematical modelling (11) have shed some light on this issue but such work on T cell responses have so far been absent. While experimental data has shown that high levels of neutralizing antibodies can be sufficient to protect from experimental infection, lower levels require the presence of T cells (1). The level of neutralizing antibody titers are however extremely heterogeneous after natural infection(12) and while most of the new SARS-CoV-2 vaccines induce high neutralizing antibody levels (13), their persistence over time needs to be evaluated. Instead, virus-specific T cells appear to persist for a long time after viral clearance (i.e. 17 years after SARS-CoV-1 infection) and detection of SARS-CoV-2-specific T cells in COVID-19 patients with waning antibody titers have been reported by different groups (5, 4, 14, 15). Furthermore, the protective role of Spike-specific T cells in vaccinated individuals has also been highlighted by recent analysis of the early profile of Spike-specific immunity (16).

We think that the correlates of protection induced by vaccinations should therefore be derived from large prospective studies where both antibody and T cell levels are measured. However, while tests for antibodies are routinely performed, the technical complexity of SARS-CoV-2 T cell measurements have so far limited, with some exceptions (17), this analysis to few individuals characterized in few specialized laboratories. This is due to the fact that T cells specific for a defined pathogen are a minuscule fraction of the total T cells (often less than 1-3%) present in blood and can be distinguished

mainly by complex functional assays that preserve the viability of the T cells during the assay. In addition, methods that are technically simple and do not require complex laboratory equipment, like ELISPOT, need to be performed in cells that have been purified from whole blood. This introduces into the assay the lengthy and technically demanding processes of peripheral blood mononuclear cell (PBMC) separation. Other assays that can directly measure the frequency and function of virus-specific T cells through expression of activation markers or cytokine production necessitate more complex equipment (i.e. Flow cytometer) and highly specialized personnel which might not be available in every routine diagnostic laboratory.

A possible rapid and simple alternative to these methods is the direct addition of stimulatory antigens or peptides to whole blood that results in the secretion of cytokines (usually IFN-γ) in plasma that is subsequently quantified. This assay is routinely applied for the diagnosis of active Tuberculosis (18) and it has also been shown to measure the presence of SARS-CoV-2-specific T cells in asymptomatic (5) and symptomatic SARS-CoV-2 infected patients (19, 20). However, to our knowledge, its accuracy and validation have not been properly analyzed over time in individuals who have been vaccinated against SARS-CoV-2, and only responses immediately after vaccination have been tested (16). Therefore, herein we utilized a range of cellular methods to measure SARS-CoV-2 T cell responses in individuals vaccinated with the prefusion stabilized, full-length SARS-CoV-2 S protein (BNT162b2) or naturally infected with SARS-CoV-2. We demonstrated that detection and relative quantification of Spike-specific T cells in vaccinated individuals can be easily and rapidly achieved through simple addition of Spike peptide pools to whole blood. Utilization of different peptide pools to stimulate whole blood provides the flexibility to derive rapid information about the kinetics and magnitude of Spike-specific T cell responses induced by vaccination and compare it with the one present in convalescent individuals.

Results

Rapid quantification of SARS-CoV-2 Spike-specific T cells by direct peptide stimulation of whole peripheral blood

We characterized the initial kinetics of Spike-specific T cells induced by two doses of mRNA vaccine (BNT162b2) over a 51 day period with different methods of antigen-specific T cell analysis in fresh blood and in cryopreserved PBMC. Whole blood of 6 healthy individuals was collected before (Day 0) and 7, 10 and 20 days after the prime and 7, 10, 20 and 30 days after the boost dose. Whole fresh blood (2-6 hours from collection) was either directly stimulated with peptides for a cytokine release assay (CRA) or processed to isolated PBMCs by Ficoll density gradient centrifugation (Figure 1A). PBMCs were either used fresh in an IFN-γ ELISPOT assay (Figure 1A) or cryopreserved for further analysis. Fresh blood and fresh PBMCs were stimulated with the SpG peptide pool containing 55 15-mer peptides (Supplementary Table 1) covering Spike-specific T cell epitopes that are immunogenic in 95% of SARS-CoV-2 infected individuals(5). Two negative controls consisting of the vehicle control with identical DMSO concentration present in the SpG peptide pool and a peptide pool covering SARS-CoV-2 nucleoprotein (NP; 41 peptides covering the C-terminal half of nucleoprotein; Supplementary Table 1) were used. Levels of IFN-γ and IL-2 were measured in the whole blood after 14-18 hours of incubation while the numbers of spots were also enumerated in the ELISPOT assay after overnight incubation.

Figure 1B shows that the two different assays detected a predominant Spike-specific response in all the individuals and defined a matching profile of Spike-specific T cell response after the prime and boost vaccination doses. The number of IFN-γ spots detected after boost vaccination matches that observed in the phase 1/2 trial of individuals vaccinated with BNT162b2 (13) and with a similar preparation consisting of the trimerized secreted version of the Spike receptor-binding domain (BNT162b1) (10), including a trial conducted in Chinese individuals vaccinated with BNT162b1 (21). While stimulation with the NP-specific peptide pool remained largely negative, the IFN-γ quantities in blood and the number of IFN-γ spots showed identical peak responses that occurred 7-10 days after the first dose in individuals V4 and V5, and 7-10 days after the second dose in individuals V1, V3 and V6. There were however some minor discrepancies. CRA did not detect the boost of Spike-specific T cells induced by the second vaccine dose in subjects V4 and V5, perhaps in relation to the transient lymphopenia induced by mRNA vaccination (13). IL-2 cytokine measurement (Figure 1C) depicted an equivalent pattern of Spike-specific T cell response with that obtained through IFN-γ release. However, IL-2 levels exceed the level of IFN-γ in all the individuals 21 days after the first and second dose of vaccination. Overall a very strong correlation between IL-2 and IFN-γ secretion with the

number of IFN-γ spots was evident (Figure 1D), which allowed a precise estimation of the quantity of IFN-γ producing cells related to the quantity of cytokines detected in whole blood (Figure 1E).

Assessing the Spike-specific T cell response directly from fresh whole blood yields comparable results to classical T cell assays

Since T cell analysis is often performed in a single centralized laboratory using cryopreserved samples collected at different sites, we also analyzed the Spike-specific T cell response after vaccination using ELISPOT and activation induced cellular markers (AIM) assay performed using cryopreserved samples stimulated with SpG peptide pool and compared the results obtained with that from ELISPOT and CRA performed using the corresponding fresh whole blood. As already shown (22), the quantity of Spike-specific spots detected by ELISPOT in cryopreserved PBMCs was reduced in comparison to the ones detected in freshly isolated PBMCs (Supplementary Figure 1A), but the dynamics of the Spike-specific response remained consistent with fresh PBMCs (Supplementary Figure 1A) as also evidenced from the high correlation between the ELISPOT results from the differently processed samples (Figure 2A; Supplementary Figure 1B). The AIM assay in our hands was less precise in detecting the dynamic expansion and contraction pattern of the Spike-specific T cell response (Supplementary Figure 1C and D) likely due to the negative impact of cryopreserving PBMCs. Nevertheless, the AIM ability to differentiate between CD4 and CD8 T cell response represent an asset that should not be discounted.

We then assessed whether whole blood CRA results could reflect the results obtained using other assays (Figure 2A and B). We correlated the results obtained in all the different assays of Spike-specific T cells in cryopreserved and fresh PBMC samples with the results of the whole blood CRA. Cytokines in whole blood remained well correlated with ELISPOT assays that used cryopreserved or fresh PBMC samples, while its correlation with AIM assay results were generally weaker (Figure 2A and B). These results indicate that the CRA which utilizes freshly collected whole blood is a robust method that can reliably quantify Spike-specific T cell responses with results that are comparable to well-established assays used to analyze T cell responses.

Determining fine specificity of Spike-specific T cell responses through whole blood CRA

We next tested whether the whole blood CRA can be used to rapidly define T cell immunogenic regions of the whole Spike protein in vaccinated individuals. 15-mer peptides with an overlap of 10 AA covering the whole 1273 AA long Spike protein were organized in distinct pools of ~40 peptides that cover the following 7 Spike regions: Pool 1 (1-180), Pool 2 (171-345), Pool 3 (336-510), Pool 4 (501-

705), Pool 5 (696-895), Pool 6 (886-1085), Pool 7 (1076-1273). A schematic representation of the localization of peptide pools 1 to 7 in relation to the S1 (N-terminal), RBD and S2 (C-terminal) regions of Spike is displayed in Figure 3A. These different Spike peptide pools were used in the whole blood CRA and ELISPOT with freshly isolated PBMCs. The results of these different assays performed at the indicated time points is first shown in two representative vaccine recipients (Figure 3B) while the results obtained in all the 6 different individuals are represented as a heat map displayed in Figure 3C. The three different measurements (IFN-γ and IL-2 CRA, and IFN-γ ELISPOT) provided very similar information in relation to the T cell response induced by BNT162b2 in healthy individuals. Even though some differences can be noted, like in individual V3, in whom the dominant IFN-γ response (CRA and ELISPOT) was induced by Pool 3 while Pool 7 induced the dominant IL-2 response, overall all the assays were largely equivalent. Consistent across the three different measurements, Spike-specific T cells preferentially targeted the S2 chain of Spike (covered by Pools 5, 6, and 7 spanning Spike 700-1273 AA) with responses in all the 6 individuals tested at different time points. Whole blood CRA and ELISPOT also showed that the region 501-705 AA contained in Pool 4 is the least immunogenic with only 1 out of the 6 vaccine recipients displaying a response at different time points (Figure 3B and C). Interestingly, analysis of individuals who recovered from SARS-CoV-2 infection (23) and in mRNA vaccine recipients (24) showed a similar reduced frequency of response in the Spike region 500-700 AA for CD4 T cells assayed through AIM detection. Taken together, these data show that direct analysis of cytokines secreted in whole blood pulsed with different peptides constitute a reliable method to gauge the presence and magnitude of functional T cells specific for epitopes covered by the utilized peptides.

Total Spike-specific T cell response is accurately represented by the T cells specific for the SpG peptide pool

While whole blood CRA using the 7 overlapping peptide pools of the Spike protein could give us information on the immunogenicity of the different regions of Spike and the total Spike-specific T cell response, the feasibility of assessing the response in larger numbers of individuals requires a more streamlined approach. As such, we analyzed the relation between the total Spike-specific T cell response and the response against our selected SpG peptide pool. A schematic representation of the localization of the peptides contained in the SpG peptide pool is displayed in Figure 4A. By correlating the results obtained from 3 different assays (IFN-γ and IL-2 CRA, and IFN-γ ELISPOT) where both the total Spike protein and SpG peptide pool -specific T cell response was determined in the same sample through stimulation with the corresponding peptide pools, we observed a strong positive linear relationship indicating that the T cell response against the SpG peptide pool is highly representative

of the total Spike T cell response (Figure 4B). In fact, the SpG peptide pool-specific T cell response constitutes ~60-80% of the total T cell response against the entire Spike protein (Figure 4C). Hence, we proceeded to analyze the Spike-specific T cell response in a larger cohort of BNT162b2 vaccinated individuals and in individuals who have recovered from SARS-CoV-2 and SARS-CoV-1 infection using the whole blood CRA with SpG peptide pool as a stimulant.

T cell response to Spike after vaccination or after natural infection with SARS-CoV-2 or SARS-CoV-1

A total of 112 BNT162b2 vaccinated individuals (201 samples), 62 and 68 individuals who recovered from symptomatic (115 samples) and asymptomatic (62 samples) SARS-CoV-2 infection respectively, and 12 individuals who recovered from SARS-CoV-1 infection 18 years ago (12 samples) were studied longitudinally using the whole blood CRA with SpG peptide pool and measuring both IFN-γ and IL-2.

Firstly, we analyzed samples collected ≥3 months post boost vaccination (3 months) or SARS-CoV-2 infection clearance (3-12 months) to understand if the whole blood CRA remains reliable in quantifying the Spike-specific T cell responses at later time points beyond that tracked in Figure 1 (Supplementary Figure 3). Linear regression analysis of IFN-γ and IL-2 secretion from whole blood CRA and the corresponding frequency of IFN-γ spots in ELISPOT showed a good correlation across all subject groups at time points 3 months and beyond (Supplementary Figure 3). This indicates that whole blood CRA can quantify Spike-specific T cell responses accurately in vaccinated and infected individuals within 3 and 12 months after boost vaccination or infection resolution respectively.

Analyzing all the time points studied, we observed that vaccinated individuals exhibited a pronounced Spike-specific T cell response at the earliest analyzed timepoint (~14 days) after the boost vaccination dose which gradually declined and started to stabilize above the positivity threshold at 2-3 months (34/35 IFN-γ positive at 3 months), consistent with an initial T cell clonal expansion induced by vaccination and subsequent normalization (Figure 5A; Supplementary Figure 4). Similar kinetics were observed in natural SARS-CoV-2 infection where Spike-specific T cell responses were high around 1 month after infection clearance which gradually declined and stabilized above the positivity threshold (Asymp.: 23/27 positive; Symp.: 46/55 IFN-γ positive at 9-12 months) regardless of the symptomatic presentation during infection (Figure 5A; Supplementary Figure 4). Even in individuals who recovered from SARS-CoV-1 infection 17 years ago, T cells specific for SARS-CoV-2 Spike protein also remained detectable (8/12 IFN-γ positive) similar to the nucleoprotein-specific T cells described previously (25), despite the low AA conservation of the SpG peptides between the two viruses (Figure 5A). Clearly,

whether T cells induced by vaccines will be maintained as the ones induced by natural infection beyond the 3 months period will have to be analyzed.

We also assessed whether differences in the magnitude of the response were present between the different groups. We compared the Spike-specific T cell response detected at similar time points 2-3 months post vaccine boost or viral clearance. Unlike reports of higher quantities of neutralizing antibodies in vaccinees (13), individuals with symptomatic SARS-CoV-2 infection and vaccinees mount an equivalent magnitude of Spike-specific T cell response (both IFN-γ and IL-2 secretion) while higher levels of IFN-γ secretion were only detected in individuals who had an asymptomatic SARS-CoV-2 infection (Figure 5B). The latter observation is in line with previous analysis of asymptomatic and symptomatic SARS-CoV-2 infected individuals within 1 month of viral clearance where the former showed increased cytokine production with comparable frequencies of virus-specific T cells (5). Subtle qualitative differences in the Spike-specific T cell response were also observed in the different groups. Upon vaccination, Spike peptide induced IFN-γ and IL-2 secretion was comparable, but gradually diverged with time leading to higher levels of detectable IL-2 2-3 months post boost vaccination (Figure 5C). Symptomatic individuals also produced significantly more IL-2 than IFN-γ >6 months after viral clearance while this difference was less pronounced in asymptomatic individuals even at the latest time points tested (9-12 months post viral clearance) which did not reach statistical significance (Figure 5C). In individuals with previous SARS-CoV-1 infection, whole blood CRA also detected higher IL-2 secreting Spike-specific T cell responses 18 years after resolution of infection (Figure 5A). Hence, quantifying IL-2 secretion provides better sensitivity over IFN-γ in the detection of individuals with a long-term Spike-specific memory T cell response.

Whole blood CRA detects the wide dynamic range and heterogeneous function of Spike-specific T cell responses in vaccinated individuals

In addition to evaluating the kinetics, quantitative and qualitative differences in the T cell response, the whole blood CRA also detected a wide range of Spike-specific T cell response in vaccinated individuals. Figure 6A shows the paired longitudinal samples of 27 vaccinees at ~14 and 90 days post boost vaccination. Secreted IFN-γ and IL-2 amounts in whole blood CRA differed between the two time points and among individuals. Interestingly, the quantity of cytokines detected two weeks after boost dose vaccination does not always predict the level of Spike-specific T cell response measurable at day 90. Some individuals exhibit >20-fold reduction at day 90 after boost vaccination, while in others, particularly for IL-2, the level was more stable (Figure 6). Indeed some individuals have a more

295 pronounced decline of IFN-γ than IL-2 or vice versa as indicated in Figure 6B where the trajectory of
296 the level of IFN-γ and IL-2 at day 14 and day 90 in each individual was plotted (Figure 6B).
297
298 **Spike-specific T cell responses do not correlate with neutralizing antibody quantities**
299 Lastly, since the quantification of serum neutralizing antibodies is the mainstay of SARS-CoV-2
300 humoral immunity assessment, we determined if there is a predictive relationship between the
301 quantity of neutralizing antibodies and Spike-specific T cell responses. We performed linear
302 regression analysis of the T cell response in vaccinated and convalescent asymptomatic and
303 symptomatic COVID-19 patients quantified through whole blood CRA (both IFN-γ and IL-2) with the
304 serum neutralizing antibody quantities assessed through RBD-hACE2 binding inhibition assay (Figure
305 7). No substantial correlations were observed in any of the analyzed groups. As also demonstrated
306 previously in convalescent {PhD:2021bm} and in vaccinated individuals (26), serum neutralizing
307 antibody quantities cannot predict the corresponding Spike-specific T cell responses in an individual
308 and this further stresses how T cell response information from the whole blood CRA complements
309 existing antibody assessments.
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311
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Discussion

The complexity of virus-specific T cell characterization relegates their analysis to studies performed in selected laboratories accustomed with the complex methods of T cell analysis. The rapidity, simplicity and accuracy of the cytokine release assay (CRA) in whole blood can allow a routine measurement of SARS-CoV-2 T cells in large populations and thus help in understanding the role of antiviral T cells during the current COVID-19 pandemic. It is important to note that cytokine release in the stimulated whole blood does not detect the mere presence of T cells but also their functionality. This is an important feature of the assay that differentiates the CRA (and other assays like ELISPOT and AIM) from the recently developed test (T-Detect COVID, Adaptive Biotechnologies), which uses next generation sequencing of T cell receptors (TCR) to determine the presence/absence of cellular immunity to SARS-CoV-2 (27). We think that the ability to measure the wide dynamic range of functional Spike-specific T cells and not only their presence will be an important asset that will more precisely evaluate the protective ability of T cells after infection or vaccination.

In this work, by sequentially testing vaccinated and SARS-CoV-2 convalescent individuals, we show that IL-2 and IFN-γ quantification in whole blood measured Spike-specific T cell response with an accuracy equivalent to ELISPOT assays performed in freshly purified PBMC. Minor discrepancies between the magnitudes of T cells were detected only at early time points when CRA was able to detect a signal in the absence of ELISPOT results. Furthermore, analysis at 2 and 3 months after vaccination showed, on average, a better sensitivity of IL-2 over IFN-γ in the detection of subjects with Spike-specific T cell responses. The superior ability of IL-2 to detect long-term memory Spike-specific T cells was also supported by the analysis of SARS-CoV-2 convalescent individuals 12 months after infection and also 17 years after infection in the case of SARS-CoV-1 infected individuals. Interestingly, this difference in sensitivity is less pronounced in convalescent COVID-19 patients with asymptomatic infection. Though speculative at the moment, this lack of difference between IFN-γ and IL-2 secretion could reflect a better functionality of Spike-specific T cell response and hence plausibly contribute to the benign disease trajectory in these individuals.

The ability of CRA to measure the dynamic range of Spike-specific T cell responses allowed us to study a wide group of vaccinated and COVID-19 convalescent individuals. We observed that despite the homogeneous cohort of vaccinated adults (21-60 years of age, healthy and SARS-CoV-2 naive individuals), the Spike-specific T cell response was quantitatively different immediately after both the prime and vaccination doses (~14 days after vaccination) and after 3 months, similar to what was detected in a more heterogeneous population of COVID-19 convalescent individuals with mild or

asymptomatic disease. In the vaccinated cohort, we first observed that half of the vaccinated individuals showed a peak of their Spike-specific T cell response ~14 days after the prime dose while half reached their peak response after the boost dose. These data were derived from a very limited sample size since we studied only 6 individuals at multiple time points after vaccination. However, the inability of the second vaccine dose to boost Spike-specific T (and antibody) responses in some individuals has been observed so far only after vaccination of SARS-CoV-2 convalescents and not, like in our study, in SARS-CoV-2 naïve. A possibility is that the pre-existing Spike-specific T cells primed by other coronaviruses could have influenced this different kinetic.

The analysis of a much larger cohort of vaccinated individuals (n=112) studied with less frequent sampling up to 3 months upon completion of vaccination further confirmed the heterogeneity of the magnitude of the induced Spike-specific cellular response. The quantity and the kinetics of decline of the Spike-specific cellular responses diverged among the vaccinated individuals with CRA IFN-γ and IL-2 concentrations that spans from 10-500pg/ml and 40-500pg/ml respectively. Interestingly, some individuals have a constant cytokine secretion level over the observation period while in others, the cytokines quantity drops precipitously. In addition, the longitudinal quantification of two cytokines further increased the heterogeneity of the vaccine-induced immunity in different individuals as IFN-γ and IL-2 quantities did not decrease in parallel in all the individuals. Some vaccinated individuals displayed a stable IL-2 production associated with a profound decrease of IFN-γ, while others showed exactly the opposite. Whether these differences can be attributed to the presence of different populations of effector/memory T cells or different ratios of CD4 and CD8 T cells and whether such differences have an impact on protection will need to be analyzed in a large clinical study. In addition, it will be important to continue monitoring the Spike-specific T cell response beyond the 3 months observation period to understand whether the Spike-specific T cell response induced by vaccines will behave like the one observed after natural infection. Importantly, the rapid cytokine assay was able to detect Spike-specific T cells in ~84% of SARS-CoV-2 individuals 1 year after infection and also in 8 out of 12 SARS-CoV-1 patients 18 years after infection.

Another important observation that further supports the concept of marked heterogeneity of the immune response induced by the BNT162b2 vaccination was the lack of correlation between the magnitude of humoral and cellular immunity. The substantial independence of different components of the immune system after the initial induction phase has been demonstrated in COVID-19 convalescents (28, 29) and can be explained by recent data showing that neutralizing antibodies can be produced without follicular T cell help (30). The CRA also reveals a similar profile both in COVID-

19 convalescent and vaccinated individuals where an independence of the neutralizing antibody titers and the magnitude of cytokines secreted was detected. COVID-19 convalescents were studied over 1 year after infection and displayed different levels of neutralizing antibodies, while vaccinated individuals mostly displayed a consistently high neutralizing antibody titers with little variations among the tested individuals 3 months after vaccination. In other studies, antibody persistence was still observed up to 6 months post mRNA vaccination at the time of writing(31). We do not know whether antibodies induced by vaccination will show a similar rate of decline as observed after natural infection beyond the 6 months follow up till date, which can make the analysis of cellular immunity even more important. Our data at the moment show that three months after vaccination, the levels of neutralizing antibodies cannot be used as a surrogate of Spike-specific cellular immunity induced by vaccination.

This quantitative heterogeneity was however not mirrored by the regions of Spike protein targeted by T cells induced by BNT162b2 vaccination. We observed a substantial similarity of immunodominance among the different vaccinated individuals, with large part of the T cell response directed towards the Spike 2 chain and with an almost complete lack of T cell determinants within the N terminal region of S1 (region 501-705AA). A reduced presence of T cell epitopes in this region was already observed in SARS-CoV-2 convalescents (23). It will be interesting to test whether this documented profile of Spike-T cell specificity will also occur in individuals vaccinated with different products where subtle differences of codon usage, signal peptide and amino acid modifications (pre-fusion conformation stabilization and furin cleavage site mutations) were introduced(32) (33-35).

In conclusion, we show here that the rapid measurement of cytokine production in whole blood after peptide specific activation is a quick and simple assay that can reliably detect the wide dynamic range of functionally heterogeneous Spike-specific T cell response induced after vaccination or infection in different individuals. Even though T cells cannot prevent infection in the absence of antibodies, their pivotal role in the protection from disease severity has been shown in natural infection of normal (2) (3), oncological patients (7) and in vaccinated individuals (16). As such, since the quantity of Spike-specific T cells cannot be predicted by the simple measurement of antibodies, this higher throughput and simple assay represents a feasible approach to implement in routine testing to complement existing antibody measurements and thus help to define the correlates of protection necessary for the design of current vaccine strategies.

Materials and Methods

Subject details

Individuals who have recovered from SARS-CoV-2 infection (Asymptomatic: n=62; Symptomatic: n=68), vaccinated with BNT162b2 (n=112) or had SARS-CoV-1 infection 17 years ago (n=12) were enrolled in this work as part of the PROTECT study (National Healthcare Group Domain Specific Review Board, NHG DSRB ref. 2012/00917), Healthcare Worker Vaccination study (SingHealth Centralised Institutional Review Board, CIRB ref. 2021/2014), Novel Pathogens study (CIRB ref. 2018/3045) and SARS Recall study (NHG DSRB ref. 2020/00091). All participants provided written informed consent. Vaccinated individuals were between 21-60 years of age, healthy and SARS-CoV-2 infection naïve. Whole blood and serum samples were collected at the indicated intervals for serological and T cell response analysis.

Peptides

15-mer peptides that are overlapping by 10 amino acids (AA) spanning the entire SARS-CoV-2 Spike protein (GISAID EPI_ISL_410713) were synthesized (Genscript) and pooled into 7 pools of approximately 40 peptides in each pool (Supplementary Table 1). 55 Spike peptides covering the immunogenic regions of the SARS-CoV-2 Spike protein that represents 40.5% of the whole Spike protein forms the SpG peptide pool as described previously (5).

Cytokine release assay (CRA) from whole peripheral blood stimulated with SARS-CoV-2 Spike peptide pools

320 µl of whole blood drawn on the same day were mixed with 80 µl RPMI and stimulated with the indicated SARS-CoV-2 Spike peptide pools (Supplementary Table 1) at 2 µg/ml or with DMSO as a control. After 16 hours of culture, the culture supernatant (plasma) was collected and stored at −80ºC. Cytokine concentrations in the plasma were quantified using an Ella machine with microfluidic multiplex cartridges measuring IFN-γ and IL-2 following the manufacturer's instructions (ProteinSimple). The level of cytokines present in the plasma of DMSO controls was subtracted from the corresponding peptide pool stimulated samples. The positivity threshold was set at 10x times the lower limit of quantification of each cytokine (IFN-γ = 1.7pg/ml; IL-2 = 5.4pg/ml) after DMSO background subtraction.

Peripheral blood mononuclear cell isolation

Peripheral blood of all individuals was collected in heparin containing tubes and peripheral blood mononuclear cells from all collected blood samples were isolated by Ficoll-Paque density gradient centrifugation.

SARS-CoV-2 Spike-specific T cell quantification

The frequency of SARS-CoV-2 Spike-specific T cells was quantified as described previously (5).Briefly, freshly isolated or cryopreserved PBMC (as indicated) were stimulated with SpG peptide pool in an IFN-γ ELISPOT assay. ELISPOT plates (Millipore) were coated with human IFN-γ antibody overnight at 4°C. 400,000 PBMC were seeded per well and stimulated for 18h with the SpG peptide pool at 2 µg/ml. The plates were then incubated with human biotinylated IFN-γ detection antibody, followed by Streptavidin-AP and developed using the KPL BCIP/NBT Phosphatase Substrate. To quantify positive peptide-specific responses, 2x mean spots of the unstimulated wells were subtracted from the peptide-stimulated wells, and the results expressed as spot forming cells (SFC)/10^6 PBMC. Results were excluded if negative control wells had >30 SFC/10^6 PBMC or if positive control wells (PMA/Ionomycin) were negative.

RBD-hACE2 binding inhibition assay

Antibodies inhibiting virus binding to host cell was measured using a commercial RBD-human angiotensin-converting enzyme 2 (hACE2) binding inhibition assay called cPASS™ (GenScript). As per manufacturer's instructions, serum was diluted 1:10 in the kit sample buffer and mixed 1:1 with HRP-conjugated RBD and incubated for 30 mins at 37°C. RBD-antibody mixtures were then transferred and incubated for 15 mins at 37°C in enzyme-linked immunosorbent assay (ELISA) plates coated with recombinant hACE2 receptor. Following incubation, plates were washed with the kit wash solution, incubated with TMB substrate for 15 mins and reaction stopped with stop solution. Absorbance was measured at OD450 nm. Percent inhibition of RBD-hACE2 binding was computed using the following equation: % inhibition=(1-[(OD of serum+RBD)/(OD of negative control+RBD)])×100. As described by the cPASS™ kit, a cutoff of 20% and above was used to determine positive RBD-hACE2 inhibition.

Activation induced cell marker assay

Cryopreserved PBMCs were thawed and stimulated for 24 hr at 37^{o}C with the SpG peptide pool (2µg/ml) in AIM-V media supplemented with 2% AB serum. Cells were then stained with the Fixable Near-IR Live/Dead fixable cell stain kit (Invitrogen) followed with surface markers as previously described (16): anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-CD134 (OX40) and anti-CD137 (4-1BB).

All samples were acquired on a BD-LSR II Analyzer (BD) and analyzed with FlowJo software (BD). Gating strategy is shown in Supplementary Figure 2.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v9. Where applicable, the statistical tests used and the definition of centre were indicated in the figure legends. Statistical significance was defined as having a P-value of less than 0.05. In all instances, "n" refers to the number of patients analysed.

Author contributions

A.T. Tan, N. Le Bert and A. Bertoletti designed the experiments; W.N. Chia, R. Alwiss, E.E. Ooi and L-F. Wang performed and analyzed the antibody experiments; J.M.E. Lim, K. Kunasegaran, A. Chia, M.D.C. Qui and N. Tan performed all other experiments and analyzed the data; A.T. Tan, N. Le Bert and A. Bertoletti analyzed and interpreted all the data; A.T. Tan, N. Le Bert and A. Bertoletti prepared the figures and wrote the paper; D. Ying, M.I. Cheng, B. Young, L.Y. Hsu, J.GH. Low and D.C. Lye recruited all the COVID-19 patients, SARS-recall patients and the vaccinees and provided all clinical samples and data; A. Bertoletti designed and coordinated the study.

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Figure Legends

Figure 1. Detection of SARS-CoV-2 Spike-specific T cells by peptide stimulation of whole peripheral blood from vaccinated individuals. A) Comparative schematic representation of the workflow for the direct peptide stimulation of whole peripheral blood and the subsequent detection of cytokine secretion, and a standard IFN-γ ELISPOT assay. **B)** 6 healthy individuals were vaccinated with 2 doses of BNT162b2 according to the recommended schedule (21 days apart) and whole blood samples were longitudinally analyzed at 7, 10, 20 and 30 days after each dose. The collected whole blood was either directly stimulated for 16hrs with peptide pools specific for the Spike (line) or NP protein (shaded area), or immediately processed with Ficoll-density gradient centrifugation to isolate PBMCs. A standard IFN-γ ELISPOT assay using the SpG- or NP-specific peptide pools was then setup using the freshly isolated PBMCs. The quantity of secreted IFN-γ in stimulated whole blood (red line) was compared to the frequency of peptide reactive PBMCs quantified by IFN-γ ELISPOT (black line). **C)** The quantity of secreted IL-2 (blue line) in whole blood stimulated with SpG peptide pool are compared to the amount of IFN-γ detected. **D)** Linear regression analysis of the concentration of IFN-γ and IL-2 in SpG-specific peptide pool stimulated whole blood and the corresponding frequency of Spike-specific PBMCs (n=6; 48 samples). Dotted lines denote the 95% confidence interval. **E)** Table shows the estimated IFN-γ SFU/10^6 PBMCs derived from the concentrations of IFN-γ and IL-2 in SpG peptide pool stimulated whole blood based on the linear regression analysis in D.

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Figure 3. Immunodominance of Spike-specific T cells in vaccinated individuals. A) Schematic representation of the 7 Spike-specific peptide pools containing 15-mer overlapping peptides spanning the entire Spike protein. Pools 1-4 contain peptides from the signal peptide and the S1 chain while pools 5-6 encompass the S2 chain together with the transmembrane and cytoplasmic domains. **B)** Plots show the longitudinal evaluation of Spike-specific T cell responses (pools 1-7) through the quantification of IFN-γ (left) or IL-2 (middle) in peptide stimulated whole blood, or through IFN-γ ELISPOT (right) in 2 representative vaccinees. **C)** Heatmap shows the Spike-specific T cell responses

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631 Linear regression analysis of the T cell response against SpG peptide pool and the total spike protein
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641 SpG peptide pool stimulation. Cross-reactive SARS-CoV-2 Spike-specific T cells were also quantified in
642 the whole blood of individuals who were infected with SARS-CoV-1 18 years ago (n=12; 12 samples).
643 The response of individuals before receiving BNT162b2 vaccination are shown for reference. Pie chart
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645 CoV-2 and SARS-CoV-1. The sampling timespan (highlighted in yellow) is shown and the number of
646 samples analysed at each time point were indicated in parentheses. Dashed lines denote the
647 detection cut-off for the measured cytokines**.** Significant differences in each group were analysed by
648 one-way ANOVA and the adjusted p-value (adjusted for multiple comparison) are shown. ns = not
649 significant P>0.05; * = P≤0.05; ** = P≤0.01; *** = P≤0.001; **** = P≤0.0001. **B)** The quantities of
650 secreted IFN-γ (red) and IL-2 (blue) in SpG peptide pool stimulated whole blood of vaccinees and
651 COVID-19 patients sampled 2-3 months post-boost vaccination dose or viral clearance are shown. Bars
652 denote the median value of each group and the dashed lines denote the detection cut-off for the
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654 dynamics of secreted IFN-γ (red) and IL-2 (blue) in SpG peptide pool stimulated whole blood of
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658 **Figure 6. Heterogeneity of Spike-specific T cell response in vaccinated individuals. A)** SARS-CoV-2
659 Spike-specific T cell response evaluated by SpG peptide pool stimulation of whole blood from

vaccinated individuals (n=27) at 2 weeks (D~14; green circle) and 3 months (D90; red circle) after boost vaccination dose. Secreted IFN-γ and IL-2 concentrations detected are shown. **B)** Bivariate dot plots of secreted IFN-γ and IL-2 concentrations. Arrows connect paired individuals analyzed at D~14 and D90. Dashed lines denote the detection cut-off for the measured cytokines**.**

Figure 7. Spike-specific T cell responses do not correlate with the quantity of neutralizing antibodies in the serum. Linear regression analysis of the concentration of IFN-γ and IL-2 in SpG peptide pool stimulated whole blood and the corresponding SARS-CoV-2 neutralizing capacity of serum from vaccinated individuals (n=91; 112 samples), asymptomatic (n=62; 62 samples) and symptomatic (n=64; 107 samples) COVID-19 patients sampled at all timepoints available. Dotted lines denote the 95% confidence interval.

Supplementary Figure 1. Longitudinal response dynamics of ELISPOT assay performed using fresh and cryopreserved PBMCs. A) Standard IFN-γ ELISPOT assays using SpG peptide pool were performed in parallel using freshly isolated (black) or cryopreserved PBMCs (red) obtained from vaccinated individuals at different time points post vaccination (n=6; 24 samples). **B)** Linear regression analysis of IFN-γ SFU/10^6 PBMCs quantified using freshly isolated or cryopreserved PBMCs shows a strong positive correlation. Dotted lines denote the 95% confidence interval. **C)** The frequency of Spike-specific CD4 and CD8 T cells 10 days after the second dose of BNT162b2 were quantified from cryopreserved PBMC samples through the induction of activation markers (CD69, 4-1BB and/or OX40) after SpG peptide pool stimulation (n=6; 27 samples). **D)** Flow cytometry bivariate dot plots showing AIM+ CD8 and CD4 T cells frequencies after SpG peptide pool stimulation in a representative vaccinee 10 days after the boost dose.

Supplementary Figure 2. Representative gating strategy for AIM+ CD4+ (CD134/OX40+ CD137/4-1BB+; blue) and CD8+ (CD69+ CD137/4-1BB+; purple) T cells in PBMCs with and without peptide stimulation. Frequencies of gated populations relative to parent are affixed into the contour plots.

Supplementary Figure 3. Whole blood CRA quantifies Spike-specific memory T cell responses ≥3 months post-boost vaccination dose or viral clearance. Linear regression analysis of the concentration of IFN-γ and IL-2 in SpG peptide pool stimulated whole blood and the corresponding frequency of Spike-specific T cells quantified by IFN-γ ELISPOT in vaccinated individuals (grey; n=30; 30 samples), asymptomatic (green; n=51; 51 samples) and symptomatic (red; n=38; 62 samples)

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702 Significant differences in each group were analysed by one-way ANOVA and the adjusted p-value
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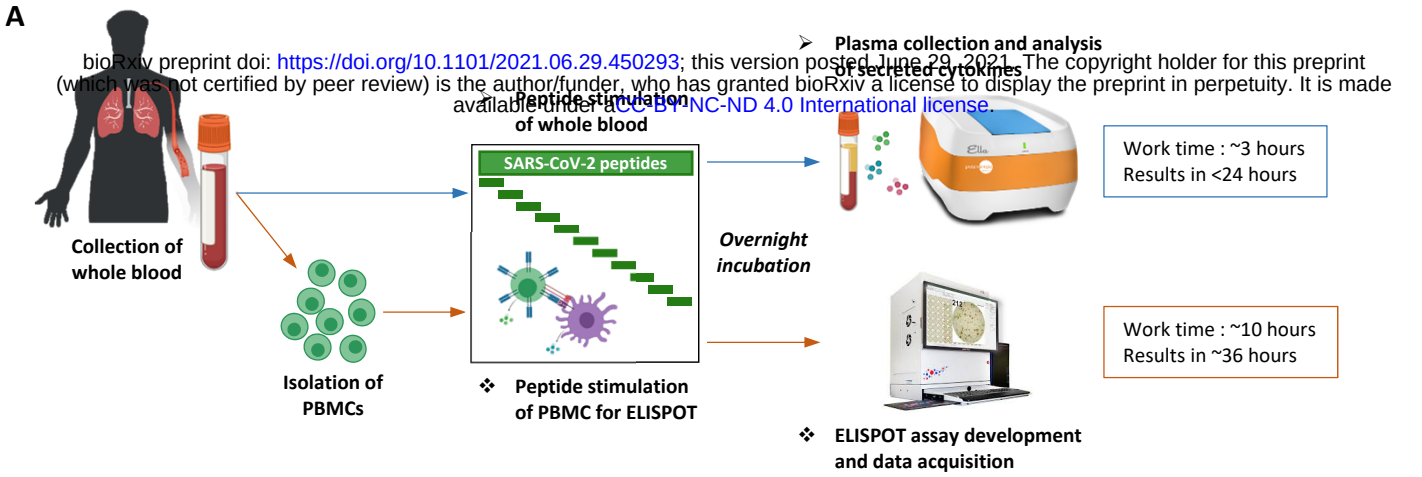
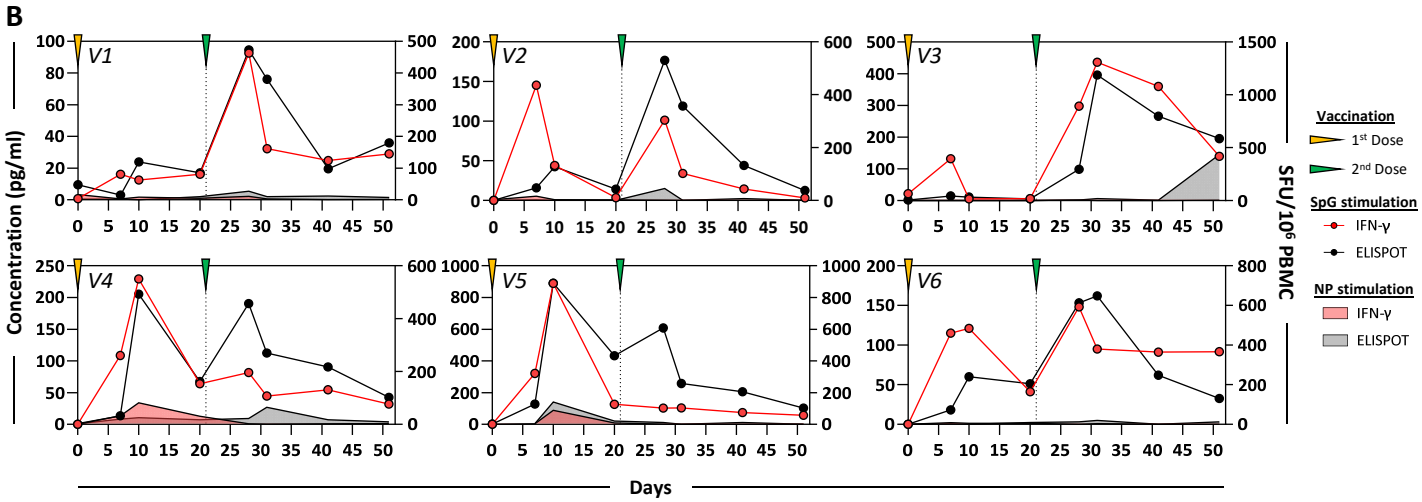
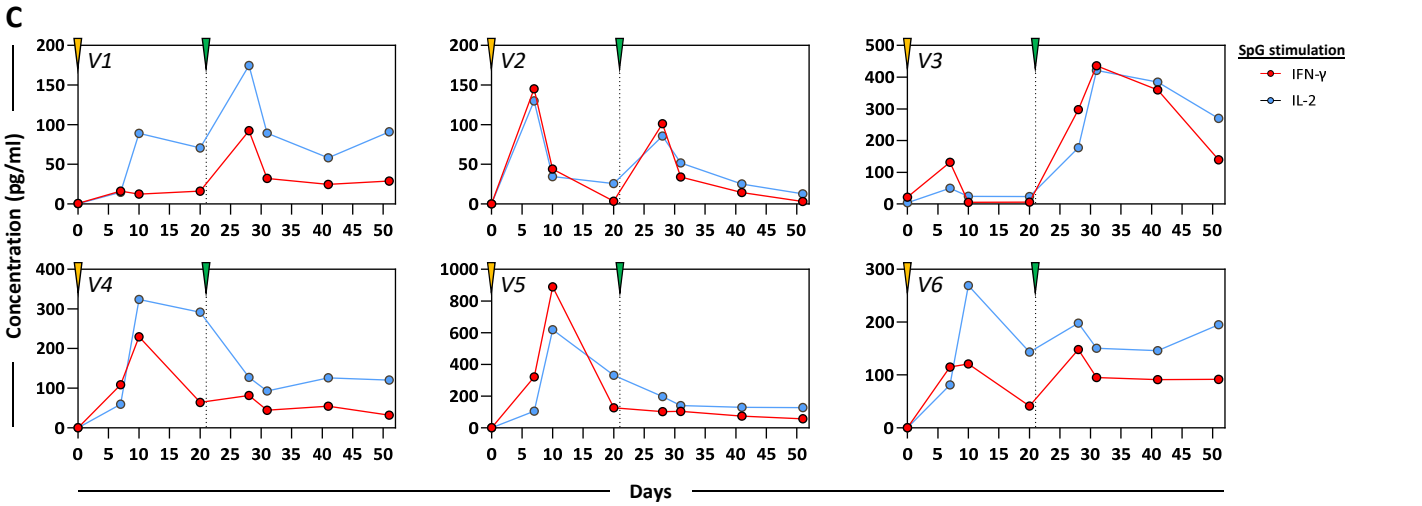
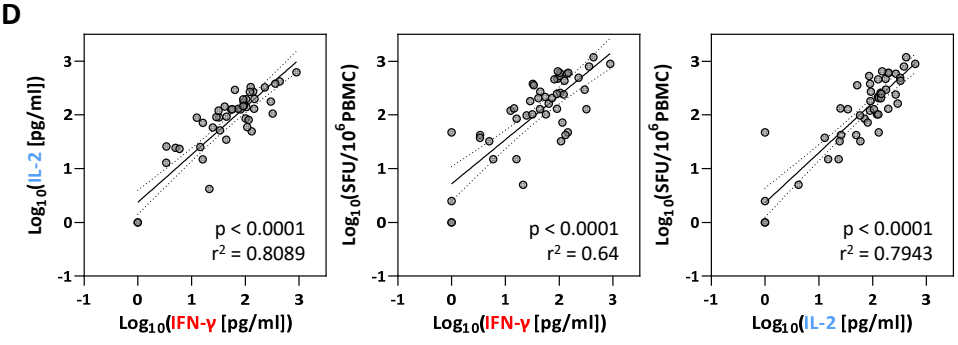
705
706 **Supplementary Table 1.** Details of the peptides found in the SpG pool, and the overlapping peptide
707 pools covering the entire SARS-CoV-2 Spike protein and the C-terminal half of the nucleoprotein.









E

IFN-γ / IL-2 concentration (pg/ml)	SFU/10⁶ PBMC estimated from IFN-γ concentration	SFU/10⁶ PBMC estimated from IL-2 concentration
1	5.2	2.3
10	34.6	19.8
25	73.6	46.4
50	130.1	88.3
75	181.7	128.7
100	230.2	168.2
200	407.1	320.3
300	568.3	466.9
400	720.0	609.9
500	865.1	750.5
750	1207.7	1093.9
1000	1530.2	1429.1

Figure 1

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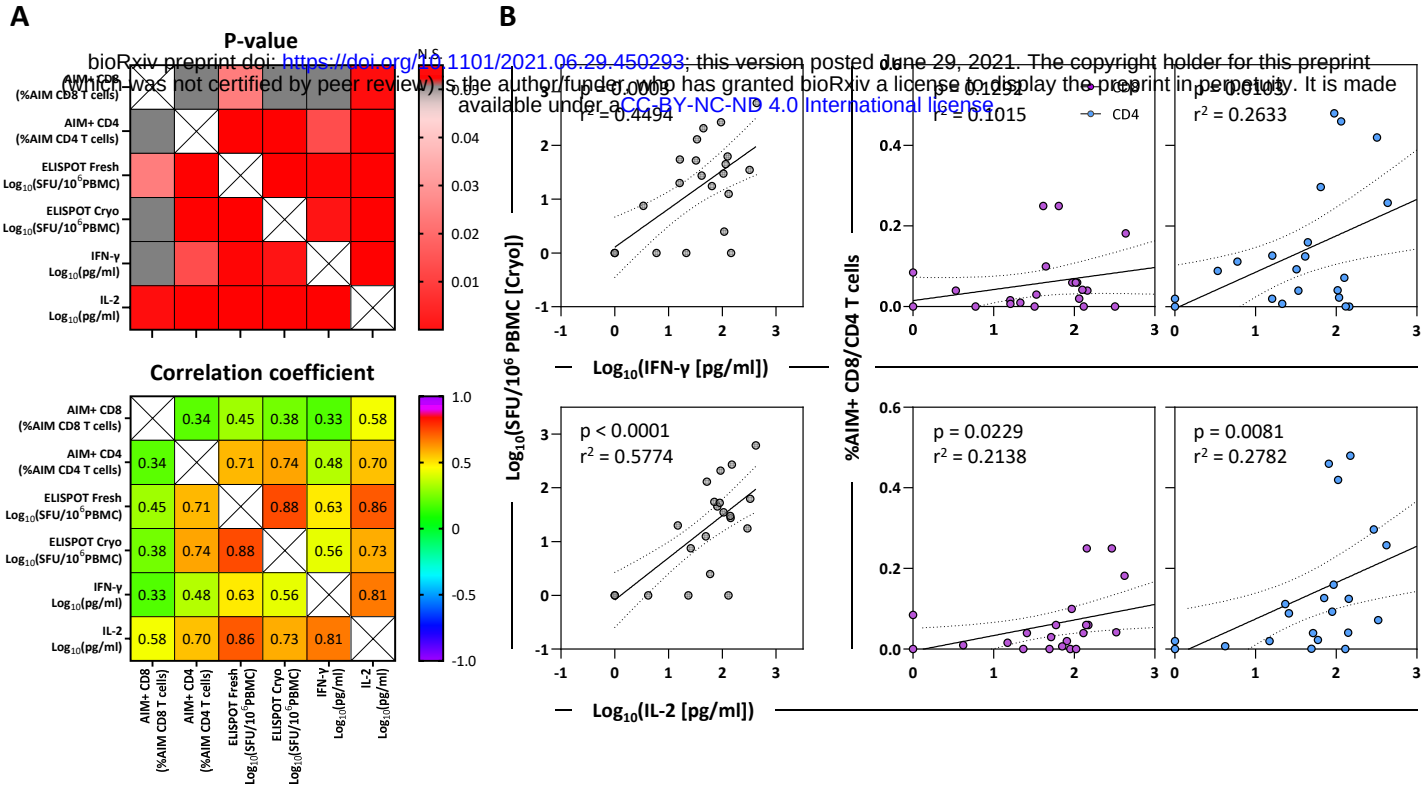

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Figure 2

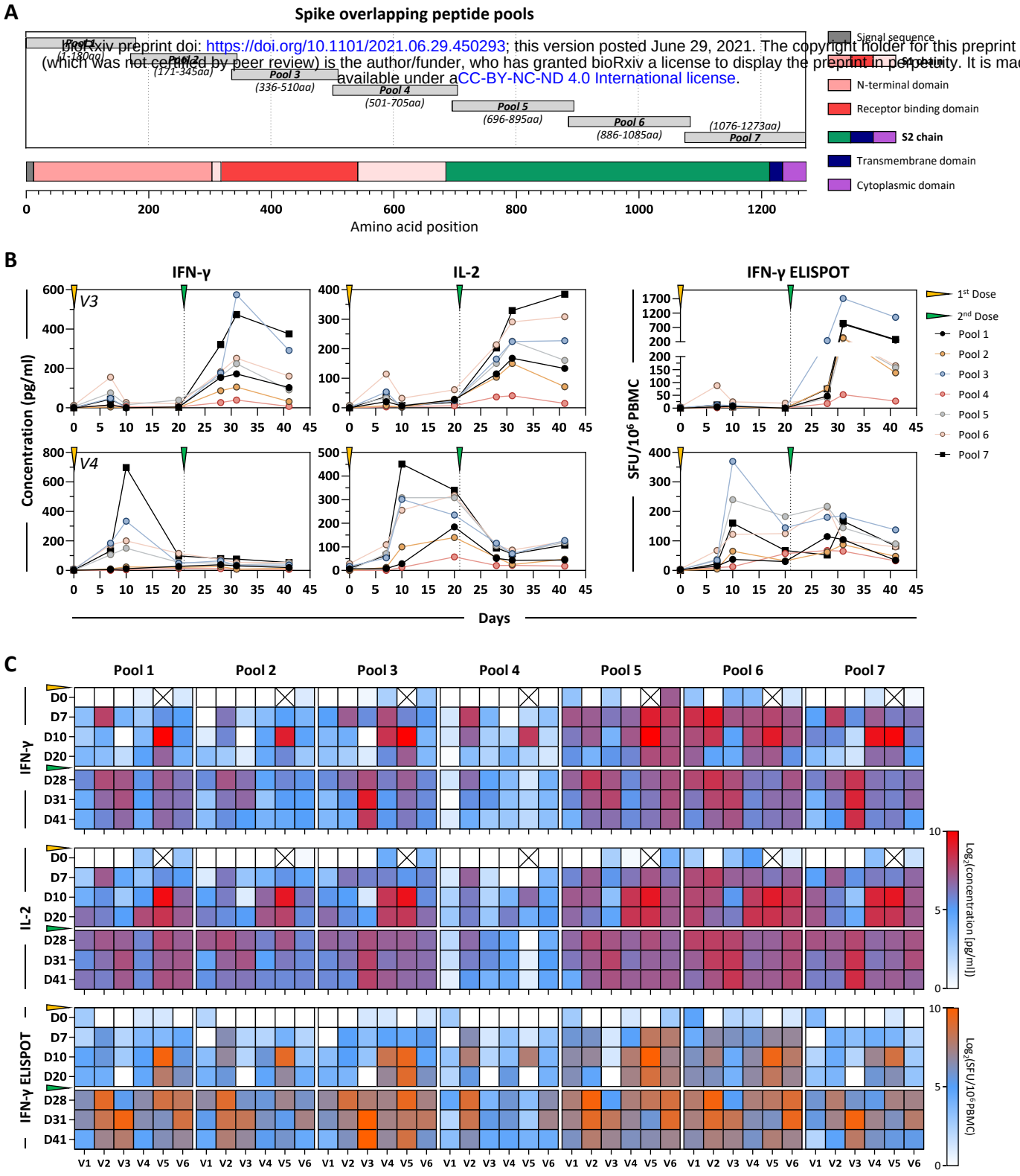

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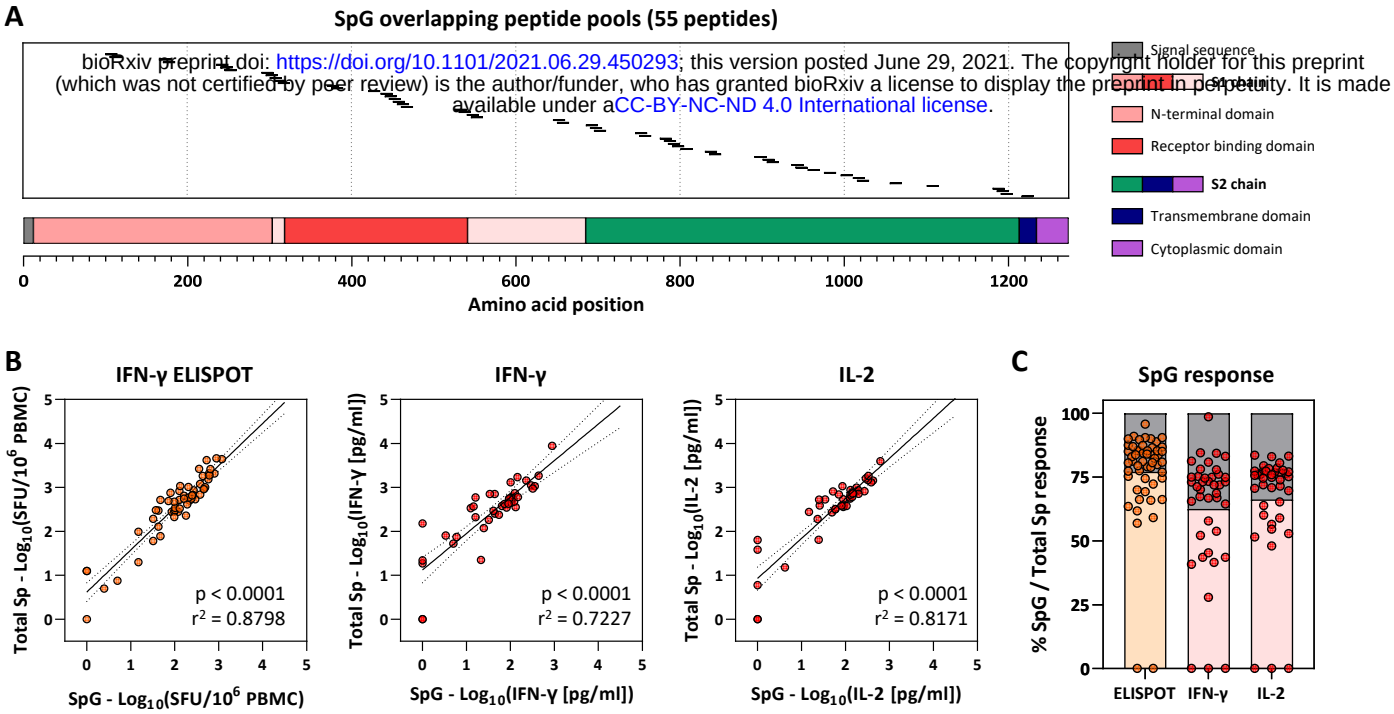
Figure 3



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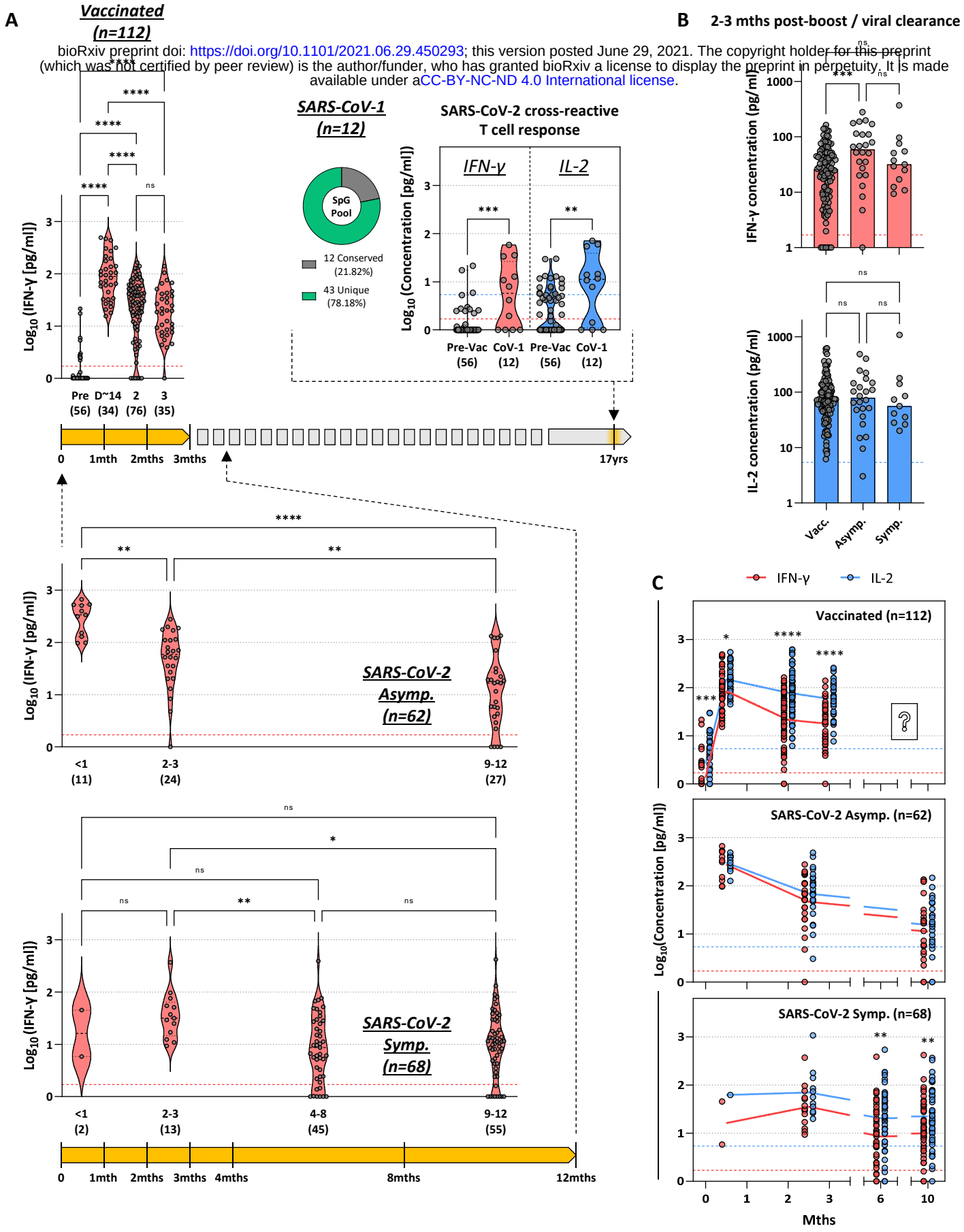

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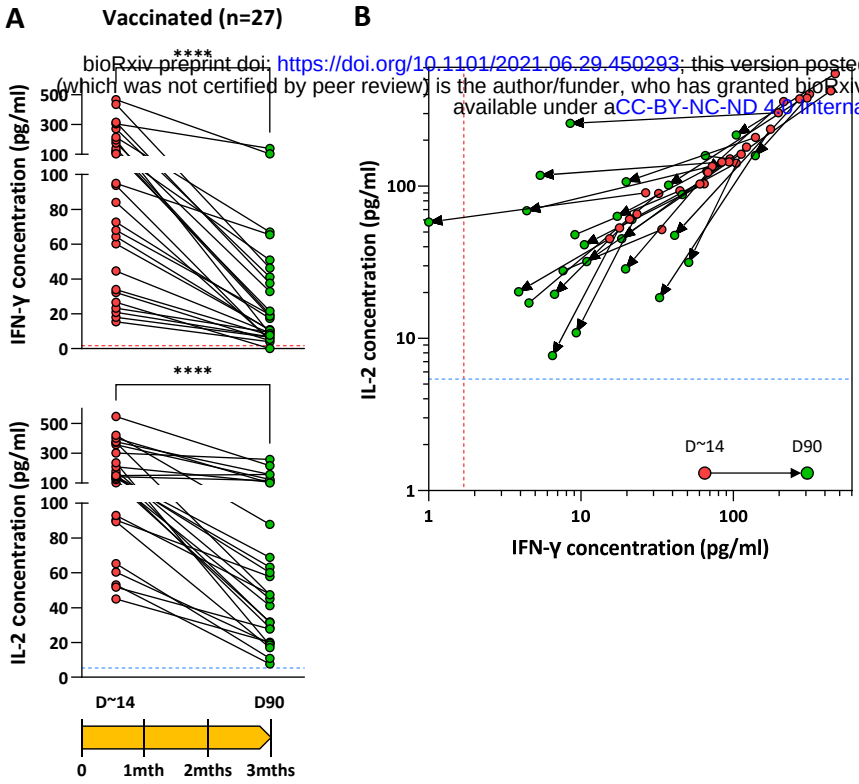

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Figure 6

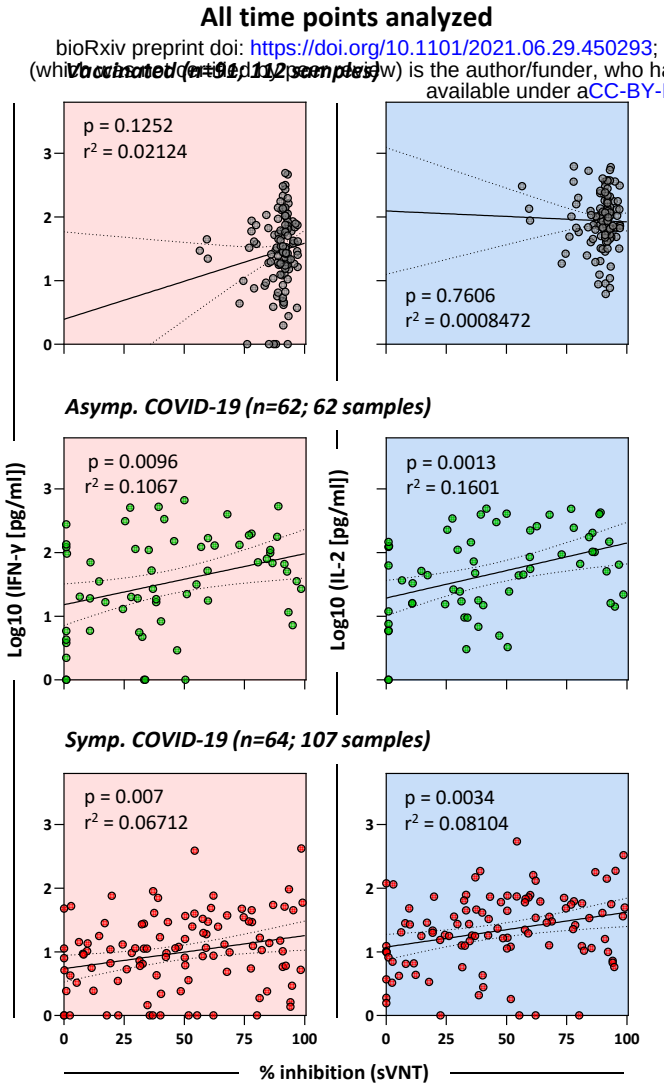
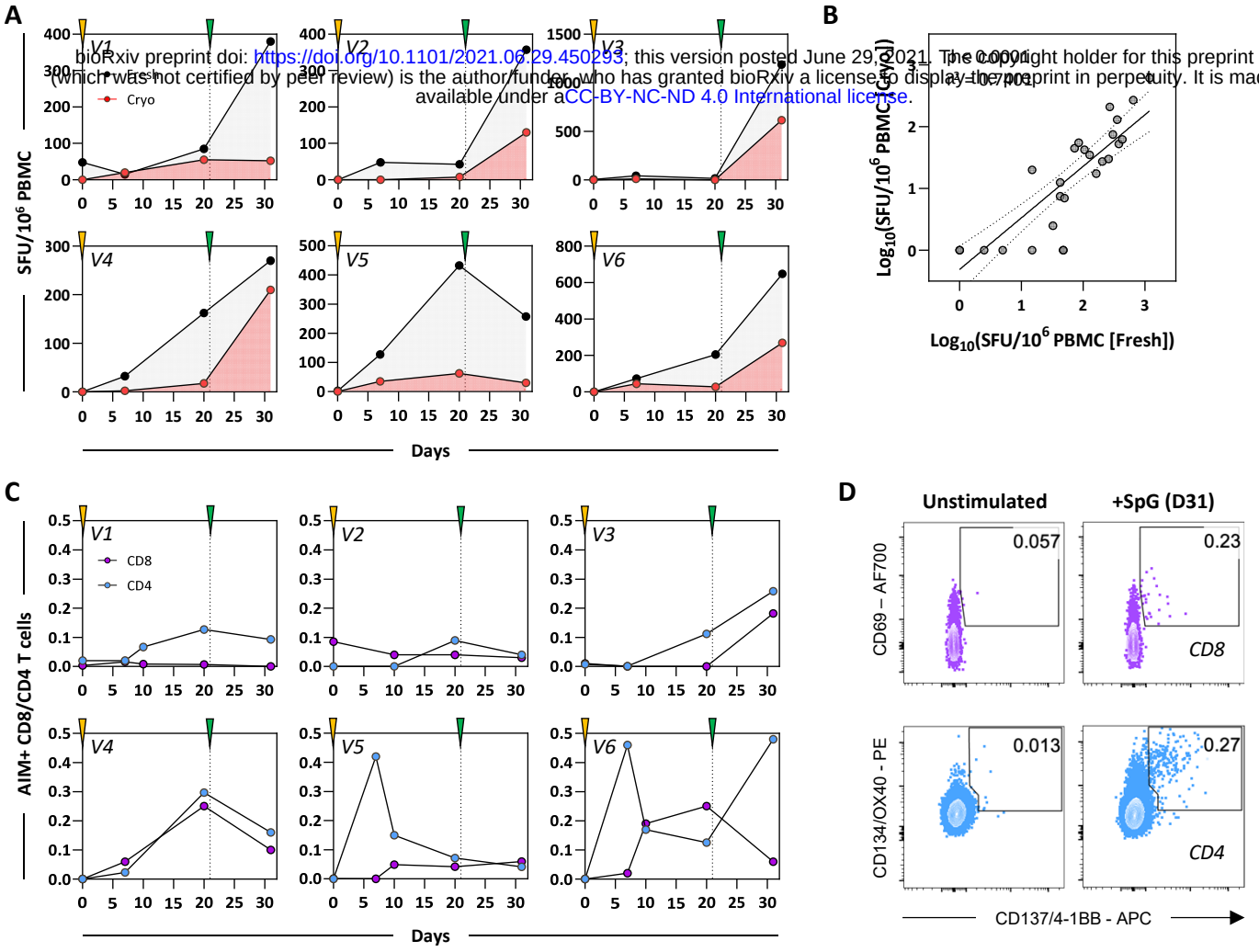
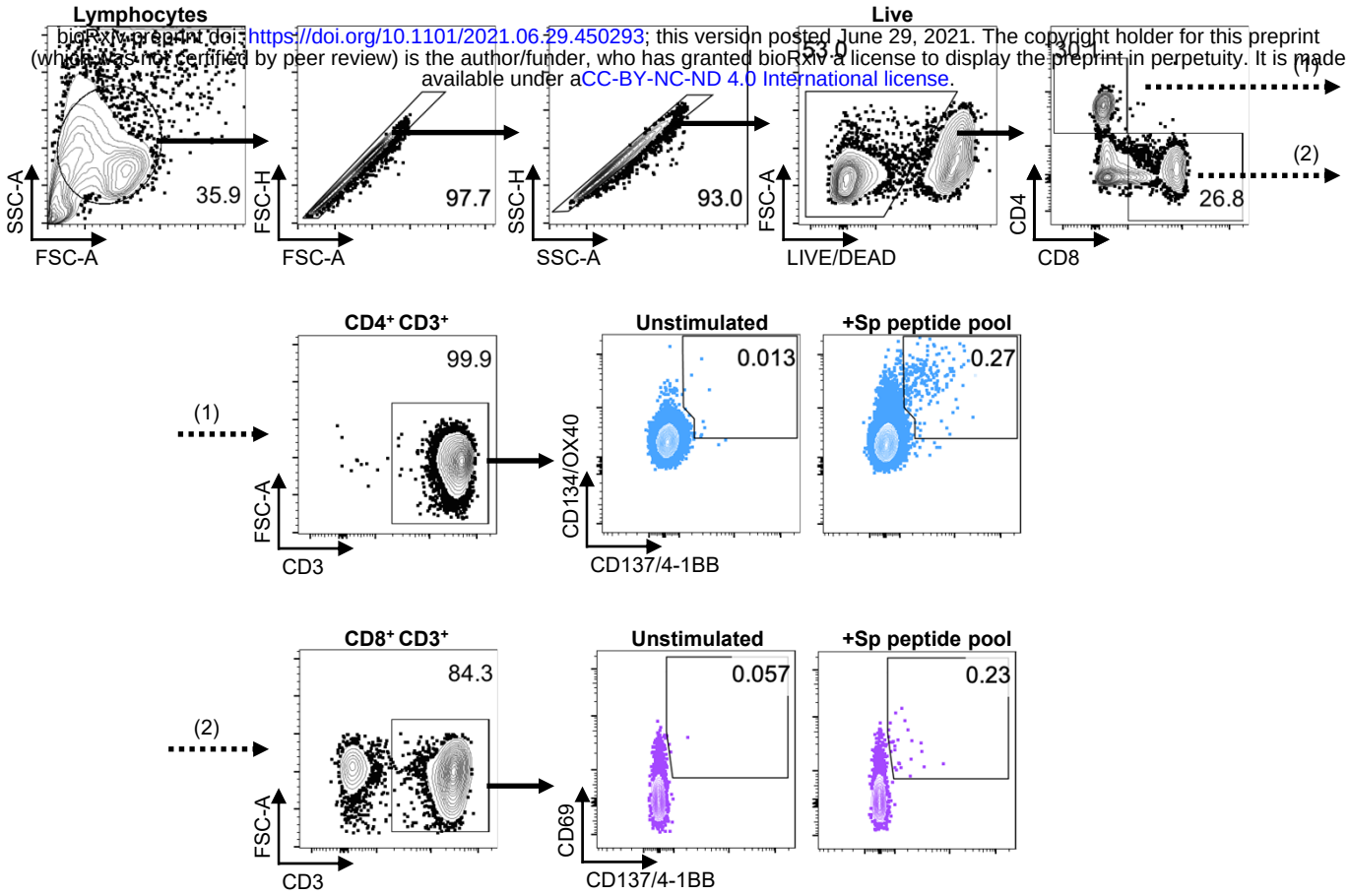


Figure 7. Spike-specific T cell responses do not correlate with the quantity of neutralizing antibodies in the serum. Linear regression analysis of the concentration of IFN-γ and IL-2 in SpG peptide pool stimulated whole blood and the corresponding SARS-CoV-2 neutralising capacity of serum from vaccinated individuals (n=91; 112 samples), asymptomatic (n=62; 62 samples) and symptomatic (n=64; 107 samples) COVID-19 patients sampled at all timepoints available. Dotted lines denote the 95% confidence interval.



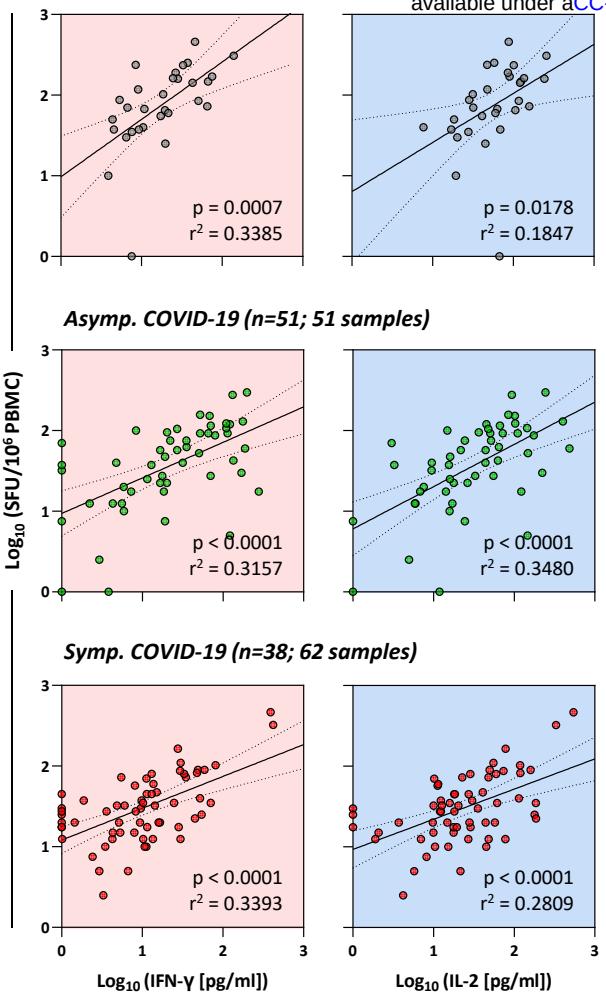
Supplementary Figure 1. Longitudinal response dynamics of ELISPOT assay performed using fresh and cryopreserved PBMCs. A) Standard IFN-γ ELISPOT assays using SpG peptide pool were performed in parallel using freshly isolated (black) or cryopreserved PBMCs (red) obtained from vaccinated individuals at different time points post vaccination (n=6; 24 samples). **B)** Linear regression analysis of IFN-γ SFU/10^6 PBMCs quantified using freshly isolated or cryopreserved PBMCs shows a strong positive correlation. Dotted lines denote the 95% confidence interval. **C)** The frequency of Spike-specific CD4 and CD8 T cells 10 days after the second dose of BNT162b2 were quantified from cryopreserved PBMC samples through the induction of activation markers (CD69, 4-1BB and/or OX40) after SpG peptide pool stimulation (n=6; 27 samples). **D)** Flow cytometry bivariate dot plots showing AIM+ CD8 and CD4 T cells frequencies after SpG peptide pool stimulation in a representative vaccinee 10 days after the boost dose.

Supp Figure 1



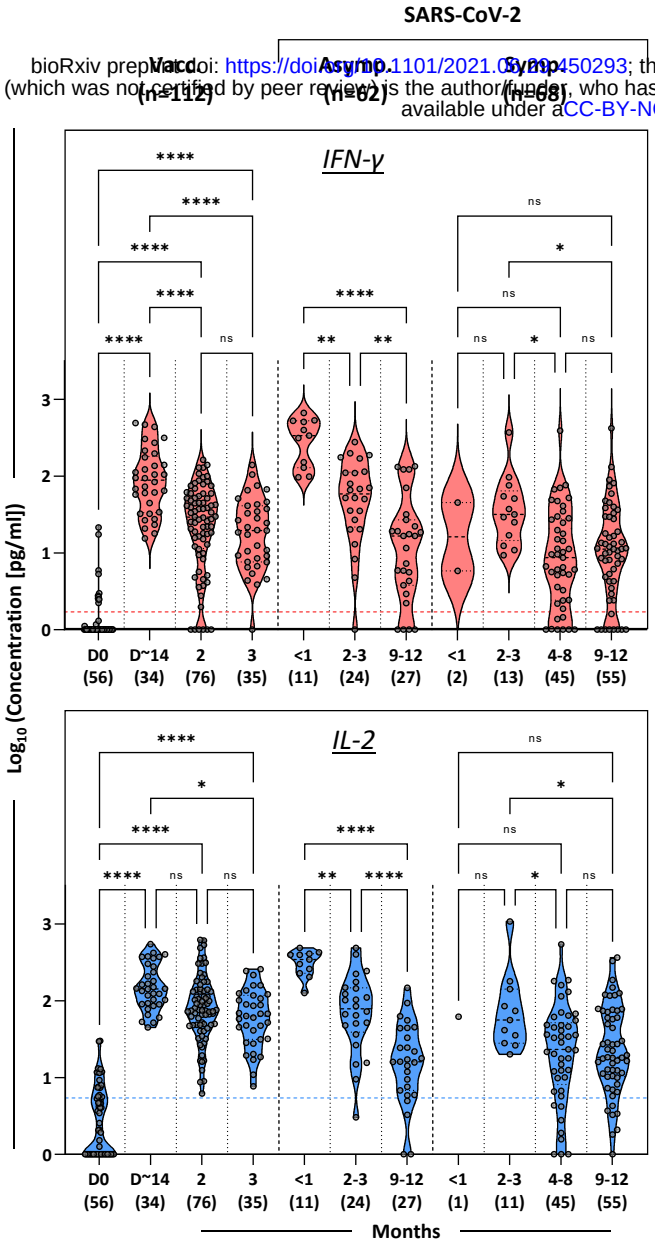
Supplementary Figure 2. Representative gating strategy for AIM+ CD4+ (CD134/OX40+ CD137/4-1BB+; blue) and CD8+ (CD69+ CD137/4-1BB+; purple) T cells in PBMCs with and without peptide stimulation. Frequencies of gated populations relative to parent are affixed into the contour plots.

Supp Figure 2



Supplementary Figure 3. Whole blood CRA quantifies Spike-specific memory T cell responses ≥3 months post-boost vaccination dose or viral clearance. Linear regression analysis of the concentration of IFN-γ and IL-2 in SpG peptide pool stimulated whole blood and the corresponding frequency of Spike-specific T cells quantified by IFN-γ ELISPOT in vaccinated individuals (grey; n=30; 30 samples), asymptomatic (green; n=51; 51 samples) and symptomatic (red; n=38; 62 samples) COVID-19 patients sampled ≥3 months post-boost vaccination dose or viral clearance. Dotted lines denote the 95% confidence interval.

Supp Figure 3



Supplementary Figure 4. SARS-CoV-2 spike specific T cells in the whole blood of vaccinated and infected individuals. A) SARS-CoV-2 Spike-specific T cell response in vaccinated individuals (n=112; 201 samples), asymptomatic (n=62; 62 samples) and symptomatic (n=68; 115 samples) COVID-19 patients were longitudinally quantified by measuring IFN-γ (red) and IL-2 (blue) secretion in whole blood after SpG peptide pool stimulation. Dashed lines denote the detection cut-off for the measured cytokines**.** The number of samples analysed at each time point were indicated in parentheses. Significant differences in each group were analysed by one-way ANOVA and the adjusted p-value (adjusted for multiple comparison) are shown. ns = not significant P>0.05; * = P≤0.05; ** = P≤0.01; *** = P≤0.001; **** = P≤0.0001.

Supp Figure 4

SpG peptide pool				Sp-1 peptide pool				Sp-2 peptide pool				Sp-3 peptide pool				Sp-4 peptide pool				Sp-5 peptide pool				Sp-6 peptide pool				Sp-7 peptide pool				NP peptide pool			
No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa	No	Peptide	Sequence	aa
1	SP_21	IRGWIFGTTLDSKTQ	101-115	1	SP_1	MFVFLVLLPLVSSQC	1-15	1	SP_35	VSQPFLMDLEGKQGN	171-185	1	SP_68	CPFGEVFNATRFASV	336-350	1	SP_101	NGVGYQPYRVVVLSF	501-515	1	SP_140	TMSLGAENSVAYSNN	696-710	1	SP_178	WTFGACAALQIPFAM	886-900	1	SP_216	TTAPAICHDGKAHFP	1076-1090	1	NP_42	SPARMAGNGCDAALA	206-220
2	SP_22	FGTTLDSKTQSLLIV	106-120	2	SP_2	VLLPLVSSQCVNLTT	6-20	2	SP_36	LMDLEGKQGNFKNLR	176-190	2	SP_69	VFNATRFASVYAWNR	341-355	2	SP_102	QPYRVVVLSFELLHA	506-520	2	SP_141	AENSVAYSNNSIAIP	701-715	2	SP_179	GAALQIPFAMQMAYR	891-905	2	SP_217	ICHDGKAHFPRECVF	1081-1095	2	NP_43	ACNGCDAALALLLLD	211-225
3	SP_34	CTFEYVSQPFLMDLE	166-180	3	SP_3	VSSQCVNLTTRTQLP	11-25	3	SP_37	GKQGNFKNLREFVFK	181-195	3	SP_70	RFASVYAWNRKRISN	346-360	3	SP_103	VVLSFELLHAPATVC	511-525	3	SP_142	AYSNNSIAIPTNFTI	706-720	3	SP_180	IPFAMQMAYRFNGIG	896-910	3	SP_218	KAHFPRECVFVSNGT	1086-1100	3	NP_44	DAALALLLLDRLNQL	216-230
4	SP_35	VSQPFLMDLEGKQGN	171-185	4	SP_4	VNLTTRTQLPPAYTN	16-30	4	SP_38	FKNLREFVFKNIDGY	186-200	4	SP_71	YAWNRKRISNCVADY	351-365	4	SP_104	ELLHAPATVCGPKKS	516-530	4	SP_143	SIAIPTNFTISVTTE	711-725	4	SP_181	QMAYRFNGIGVTQNV	901-915	4	SP_219	RECVFVSNGTHWFVT	1091-1105	4	NP_45	LLLLDRLNQLESKMS	221-235
5	SP_48	TRFQTLLALHRSYLT	236-250	5	SP_5	RTQLPPAYTNSFTRG	21-35	5	SP_39	EFVFKNIDGYFKIYS	191-205	5	SP_72	KRISNCVADYSVLYN	356-370	5	SP_105	PATVCGPKKSTNLVK	521-535	5	SP_144	TNFTISVTTEILPVS	716-730	5	SP_182	FNGIGVTQNVLYENQ	906-920	5	SP_220	VSNGTHWFVTQRNFY	1096-1110	5	NP_46	RLNQLESKMSCKGQQ	226-240
6	SP_49	LLALHRSYLTPGDSS	241-255	6	SP_6	PAYTNSFTRGVYYPD	26-40	6	SP_40	NIDGYFKIYSKHTPI	196-210	6	SP_73	CVADYSVLYNSASFS	361-375	6	SP_106	GPKKSTNLVKNKCVN	526-540	6	SP_145	SVTTEILPVSMTKTS	721-735	6	SP_183	VTQNVLYENQKLIAN	911-925	6	SP_221	HWFVTQRNFYEPQII	1101-1115	6	NP_47	ESKMSGKGQQQQGQT	231-245
7	SP_50	RSYLTPGDSSSGWTA	246-260	7	SP_7	SFTRGVYYPDKVFRS	31-45	7	SP_41	FKIYSKHTPINLVRD	201-215	7	SP_74	SVLYNSASFSTFKCY	366-380	7	SP_107	TNLVKNKCVNFNFNG	531-545	7	SP_146	ILPVSMTKTSVDCTM	726-740	7	SP_184	LYENQKLIANQFNSA	916-930	7	SP_222	QRNFYEPQIITTDNT	1106-1120	7	NP_48	GKGQQQQGQTVTKKS	236-250
8	SP_59	CALDPLSETKCTLKS	291-305	8	SP_8	VYYPDKVFRSSVLHS	36-50	8	SP_42	KHTPINLVRDLPQGF	206-220	8	SP_75	SASFSTFKCYGVSPT	371-385	8	SP_108	NKCVNFNFNGLTGTG	536-550	8	SP_147	MTKTSVDCTMYICGD	731-745	8	SP_185	KLIANQFNSAIGKIQ	921-935	8	SP_223	EPQIITTDNTFVSGN	1111-1125	8	NP_49	QQGQTVTKKSAAEAS	241-255
9	SP_60	LSETKCTLKSFTVEK	296-310	9	SP_9	KVFRSSVLHSTQDLF	41-55	9	SP_43	NLVRDLPQGFSALEP	211-225	9	SP_76	TFKCYGVSPTKLNDL	376-390	9	SP_109	FNFNGLTGTGVLTES	541-555	9	SP_148	VDCTMYICGDSTECS	736-750	9	SP_186	QFNSAIGKIQDSLSS	926-940	9	SP_224	TTDNTFVSGNCDVVI	1116-1130	9	NP_50	VTKKSAAEASKKPRQ	246-260
10	SP_61	CTLKSFTVEKGIYQT	301-315	10	SP_10	SVLHSTQDLFLPFFS	46-60	10	SP_44	LPQGFSALEPLVDLP	216-230	10	SP_77	GVSPTKLNDLCFTNV	381-395	10	SP_110	LTGTGVLTESNKKFL	546-560	10	SP_149	YICGDSTECSNLLLQ	741-755	10	SP_187	IGKIQDSLSSTASAL	931-945	10	SP_225	FVSGNCDVVIGIVNN	1121-1135	10	NP_51	AAEASKKPRQKRTAT	251-265
11	SP_62	FTVEKGIYQTSNFRV	306-320	11	SP_11	TQDLFLPFFSNVTWF	51-65	11	SP_45	SALEPLVDLPIGINI	221-235	11	SP_78	KLNDLCFTNVYADSF	386-400	11	SP_111	VLTESNKKFLPFQQF	551-565	11	SP_150	STECSNLLLQYGSFC	746-760	11	SP_188	DSLSSTASALGKLQD	936-950	11	SP_226	CDVVIGIVNNTVYDP	1126-1140	11	NP_52	KKPRQKRTATKAYNV	256-270
12	SP_63	GIYQTSNFRVQPTES	311-325	12	SP_12	LPFFSNVTWFHAIHV	56-70	12	SP_46	LVDLPIGININTRFQT	226-240	12	SP_79	CFTNVYADSFVIRGD	391-405	12	SP_112	NKKFLPFQQFGRDIA	556-570	12	SP_151	NLLLQYGSFCTQLNR	751-765	12	SP_189	TASALGKLQDVVNQN	941-955	12	SP_227	GIVNNTVYDPLQPEL	1131-1145	12	NP_53	KRTATKAYNVTQAFG	261-275
13	SP_75	SASFSTFKCYGVSPT	371-385	13	SP_13	NVTWFHAIHVSGTNG	61-75	13	SP_47	IGININTRFQTLLALH	231-245	13	SP_80	YADSFVIRGDEVRQI	396-410	13	SP_113	PFQQFGRDIADTTDA	561-575	13	SP_152	YGSFCTQLNRALTGI	756-770	13	SP_190	GKLQDVVNQNAQALN	946-960	13	SP_228	TVYDPLQPELDSFKE	1136-1150	13	NP_54	KAYNVTQAFGRRGPE	266-280
14	SP_76	TFKCYGVSPTKLNDL	376-390	14	SP_14	HAIHVSGTNGTKRFD	66-80	14	SP_48	TRFQTLLALHRSYLT	236-250	14	SP_81	VIRGDEVRQIAPGQT	401-415	14	SP_114	GRDIADTTDAVRDPQ	566-580	14	SP_153	TQLNRALTGIAVEQD	761-775	14	SP_191	VVNQNAQALNTLVKQ	951-965	14	SP_229	LQPELDSFKEELDKY	1141-1155	14	NP_55	TQAFGRRGPEQTQGN	271-285
15	SP_85	YNYKLPDDFTGCVIA	421-435	15	SP_15	SGTNGTKRFDNPVLP	71-85	15	SP_49	LLALHRSYLTPGDSS	241-255	15	SP_82	EVRQIAPGQTGKIAD	406-420	15	SP_115	DTTDAVRDPQTLEIL	571-585	15	SP_154	ALTGIAVEQDKNTQE	766-780	15	SP_192	AQALNTLVKQLSSNF	956-970	15	SP_230	DSFKEELDKYFKNHT	1146-1160	15	NP_56	RRGPEQTQGNFCDQE	276-290
16	SP_88	WNSNNLDSKVGGNYN	436-450	16	SP_16	TKRFDNPVLPFNDGV	76-90	16	SP_50	RSYLTPGDSSSGWTA	246-260	16	SP_83	APGQTGKIADYNYKL	411-425	16	SP_116	VRDPQTLEILDITPC	576-590	16	SP_155	AVEQDKNTQEVFAQV	771-785	16	SP_193	TLVKQLSSNFGAISS	961-975	16	SP_231	ELDKYFKNHTSPDVD	1151-1165	16	NP_57	QTQGNFCDQELIRQG	281-295
17	SP_89	LDSKVGGNYNYLYRL	441-455	17	SP_17	NPVLPFNDGVYFAST	81-95	17	SP_51	PGDSSSGWTACAAAY	251-265	17	SP_84	GKIADYNYKLPDDFT	416-430	17	SP_117	TLEILDITPCSFGGV	581-595	17	SP_156	KNTQEVFAQVKQIYK	776-790	17	SP_194	LSSNFGAISSVLNDI	966-980	17	SP_232	FKNHTSPDVDLGDIS	1156-1170	17	NP_58	FCDQELIRQGTDYKH	286-300
18	SP_90	GGNYNYLYRLFRKSN	446-460	18	SP_18	FNDGVYFASTEKSNI	86-100	18	SP_52	SGWTACAAAYYVGYL	256-270	18	SP_85	YNYKLPDDFTGCVIA	421-435	18	SP_118	DITPCSFGGVSVITP	586-600	18	SP_157	VFAQVKQIYKTPPIK	781-795	18	SP_195	GAISSVLNDILSRLD	971-985	18	SP_233	SPDVDLGDISGINAS	1161-1175	18	NP_59	LIRQGTDYKHWPQIA	291-305
19	SP_91	YLYRLFRKSNLKPFE	451-465	19	SP_19	YFASTEKSNIIRGWI	91-105	19	SP_53	GAAAYYVGYLQPRTF	261-275	19	SP_86	PDDFTGCVIAWNSNN	426-440	19	SP_119	SFGGVSVITPGTNTS	591-605	19	SP_158	KQIYKTPPIKDFGGF	786-800	19	SP_196	VLNDILSRLDKVEAE	976-990	19	SP_234	LGDISGINASVVNIQ	1166-1180	19	NP_60	TDYKHWPQIAQFAPS	296-310
20	SP_92	FRKSNLKPFERDIST	456-470	20	SP_20	EKSNIIRGWIFGTTL	96-110	20	SP_54	YVGYLQPRTFLLKYN	266-280	20	SP_87	GCVIAWNSNNLDSKV	431-445	20	SP_120	SVITPGTNTSNQVAV	596-610	20	SP_159	TPPIKDFGGFNFSQI	791-805	20	SP_197	LSRLDKVEAEVQIDR	981-995	20	SP_235	GINASVVNIQKEIDR	1171-1185	20	NP_61	WPQIAQFAPSASAFF	301-315
21	SP_93	LKPFERDISTEIYQA	461-475	21	SP_21	IRGWIFGTTLDSKTQ	101-115	21	SP_55	QPRTFLLKYNENGTI	271-285	21	SP_88	WNSNNLDSKVGGNYN	436-450	21	SP_121	GTNTSNQVAVLYQDV	601-615	21	SP_160	DFGGFNFSQILPDPS	796-810	21	SP_198	KVEAEVQIDRLITGR	986-1000	21	SP_236	VVNIQKEIDRLNEVA	1176-1190	21	NP_62	QFAPSASAFFGMSRI	306-320
22	SP_106	GPKKSTNLVKNKCVN	526-540	22	SP_22	FGTTLDSKTQSLLIV	106-120	22	SP_56	LLKYNENGTITDAVD	276-290	22	SP_89	LDSKVGGNYNYLYRL	441-455	22	SP_122	NQVAVLYQDVNCTEV	606-620	22	SP_161	NFSQILPDPSKPSKR	801-815	22	SP_199	VQIDRLITGRLQSLQ	991-1005	22	SP_237	KEIDRLNEVAKNLNE	1181-1195	22	NP_63	ASAFFGMSRIGMEVT	311-325
23	SP_107	TNLVKNKCVNFNFNG	531-545	23	SP_23	DSKTQSLLIVNNATN	111-125	23	SP_57	ENGTITDAVDCALDP	281-295	23	SP_90	GGNYNYLYRLFRKSN	446-460	23	SP_123	LYQDVNCTEVPVAIH	611-625	23	SP_162	LPDPSKPSKRSFIED	806-820	23	SP_200	LITGRLQSLQTYVTQ	996-1010	23	SP_238	LNEVAKNLNESLIDL	1186-1200	23	NP_64	GMSRIGMEVTPSGTW	316-330
24	SP_109	FNFNGLTGTGVLTES	541-555	24	SP_24	SLLIVNNATNVVIKV	116-130	24	SP_58	TDAVDCALDPLSETK	286-300	24	SP_91	YLYRLFRKSNLKPFE	451-465	24	SP_124	NCTEVPVAIHADQLT	616-630	24	SP_163	KPSKRSFIEDLLFNK	811-825	24	SP_201	LQSLQTYVTQQLIRA	1001-1015	24	SP_239	KNLNESLIDLQELGK	1191-1205	24	NP_65	GMEVTPSGTWLTYTG	321-335
25	SP_110	LTGTGVLTESNKKFL	546-560	25	SP_25	NNATNVVIKVCEFQF	121-135	25	SP_59	CALDPLSETKCTLKS	291-305	25	SP_92	FRKSNLKPFERDIST	456-470	25	SP_125	PVAIHADQLTPTWRV	621-635	25	SP_164	SFIEDLLFNKVTLAD	816-830	25	SP_202	TYVTQQLIRAAEIRA	1006-1020	25	SP_240	SLIDLQELGKYEQYI	1196-1210	25	NP_66	PSGTWLTYTGAIKLD	326-340
26	SP_130	RAGCLIGAEHVNNSY	646-660	26	SP_26	VVIKVCEFQFCNDPF	126-140	26	SP_60	LSETKCTLKSFTVEK	296-310	26	SP_93	LKPFERDISTEIYQA	461-475	26	SP_126	ADQLTPTWRVYSTGS	626-640	26	SP_165	LLFNKVTLADAGFIK	821-835	26	SP_203	QLIRAAEIRASANLA	1011-1025	26	SP_241	QELGKYEQYIKWPWY	1201-1215	26	NP_67	LTYTGAIKLDDKDPN	331-345
27	SP_131	IGAEHVNNSYECDIP	651-665	27	SP_27	CEFQFCNDPFLGVYY	131-145	27	SP_61	CTLKSFTVEKGIYQT	301-315	27	SP_94	RDISTEIYQAGSTPC	466-480	27	SP_127	PTWRVYSTGSNVFQT	631-645	27	SP_166	VTLADAGFIKQYGDC	826-840	27	SP_204	AEIRASANLAATKMS	1016-1030	27	SP_242	YEQYIKWPWYIWLGF	1206-1220	27	NP_68	AIKLDDKDPNFKDQV	336-350
28	SP_138	SVASQSIIAYTMSLG	686-700	28	SP_28	CNDPFLGVYYHKNNK	136-150	28	SP_62	FTVEKGIYQTSNFRV	306-320	28	SP_95	EIYQAGSTPCNGVEG	471-485	28	SP_128	YSTGSNVFQTRAGCL	636-650	28	SP_167	AGFIKQYGDCLGDIA	831-845	28	SP_205	SANLAATKMSECVLC	1021-1035	28	SP_243	KWPWYIWLGFIACLI	1211-1225	28	NP_69	DKDPNFKDQVILLNK	341-355
29	SP_139	SIIAYTMSLGAENSV	691-705	29	SP_29	LGVYYHKNNKSWMES	141-155	29	SP_63	GIYQTSNFRVQPTES	311-325	29	SP_96	GSTPCNGVEGFNCYF	476-490	29	SP_129	NVFQTRAGCLIGAEH	641-655	29	SP_168	QYGDCLGDIAARDLI	836-850	29	SP_206	ATKMSECVLGQSKRV	1026-1040	29	SP_244	IWLGFIAGLIAIVMV	1216-1230	29	NP_70	FKDQVILLNKHIDAY	346-360
30	SP_140	TMSLGAENSVAYSNN	696-710	30	SP_30	HKNNKSWMESEFRVY	146-160	30	SP_64	SNFRVQPTESIVRFP	316-330	30	SP_97	NGVEGFNCYFPLQSY	481-495	30	SP_130	RAGCLIGAEHVNNSY	646-660	30	SP_169	LGDIAARDLICAQKF	841-855	30	SP_207	ECVLGQSKRVDFCGK	1031-1045	30	SP_245	IAGLIAIVMVTIMLC	1221-1235	30	NP_71	ILLNKHIDAYKTFPP	351-365
31	SP_150	STECSNLLLQYGSFC	746-760	31	SP_31	SWMESEFRVYSSANN	151-165	31	SP_65	QPTESIVRFPNITNL	321-335	31	SP_98	FNCYFPLQSYGFQPT	486-500	31	SP_131	IGAEHVNNSYECDIP	651-665	31	SP_170	ARDLICAQKFNGLTV	846-860	31	SP_208	QSKRVDFCGKGYHLM	1036-1050	31	SP_246	AIVMVTIMLCCMTSC	1226-1240	31	NP_72	HIDAYKTFPPTEPKK	356-370
32	SP_151	NLLLQYGSFCTQLNR	751-765	32	SP_32	EFRVYSSANNCTFEY	156-170	32	SP_66	IVRFPNITNLCPFGE	326-340	32	SP_99	PLQSYGFQPTNGVGY	491-505	32	SP_132	VNNSYECDIPIGAGI	656-670	32	SP_171	CAQKFNGLTVLPPLL	851-865	32	SP_209	DFCGKGYHLMSFPQS	1041-1055	32	SP_247	TIMLCCMTSCCSCLK	1231-1245	32	NP_73	KTFPPTEPKKDKKKK	361-375
33	SP_156	KNTQEVFAQVKQIYK	776-790	33	SP_33	SSANNCTFEYVSQPF	161-175	33	SP_67	NITNLCPFGEVFNAT	331-345	33	SP_100	GFQPTNGVGYQPYRV	496-510	33	SP_133	ECDIPIGAGICASYQ	661-675	33	SP_172	NGLTVLPPLLTDEMI	856-870	33	SP_210	GYHLMSFPQSAPHGV	1046-1060	33	SP_248	CMTSCCSCLKGCCSC	1236-1250	33	NP_74	TEPKKDKKKKADETQ	366-380
34	SP_157	VFAQVKQIYKTPPIK	781-795	34	SP_34	CTFEYVSQPFLMDLE	166-180									34	SP_134	IGAGICASYQTQTNS	666-680	34	SP_173	LPPLLTDEMIAQYTS	861-875	34	SP_211	SFPQSAPHGVVFLHV	1051-1065	34	SP_249	CSCLKGCCSCCSCCK	1241-1255	34	NP_75	DKKKKADETQALPQR	371-385
35	SP_158	KQIYKTPPIKDFGGF	786-800													35	SP_135	CASYQTQTNSPRRAR	671-685	35	SP_174	TDEMIAQYTSALLAG	866-880	35	SP_212	APHGVVFLHVTYVPA	1056-1070	35	SP_250	GCCSCCSCCKKFDED	1246-1260	35	NP_76	ADETQALPQRQKKQQ	376-390
36	SP_159	TPPIKDFGGFNFSQI	791-805													36	SP_136	TQTNSPRRARSVASQ	676-690	36	SP_175	AQYTSALLAGTITSG	871-885	36	SP_213	VFLHVTYVPAQEKNF	1061-1075	36	SP_251	GSCCKKFDEDDSEPV	1251-1265	36	NP_77	ALPQRQKKQQTVTLL	381-395
37	SP_161	NFSQILPDPSKPSKR	801-815													37	SP_137	PRRARSVASQSIIAY	681-695	37	SP_176	ALLAGTITSGWTFGA	876-890	37	SP_214	TYVPAQEKNFTTAPA	1066-1080	37	SP_252	FDEDDSEPVLKGVKL	1256-1270	37	NP_78	QKKQQTVTLLPAADL	386-400
38	SP_167	AGFIKQYGDCLGDIA	831-845													38	SP_138	SVASQSIIAYTMSLG	686-700	38	SP_177	TITSGWTFGAGAALQ	881-895	38	SP_215	QEKNFTTAPAICHDG	1071-1085	38	SP_253	SEPVLKGVKLHYT	1261-1273	38	NP_79	TVTLLPAADLDDFSK	391-405
39	SP_168	QYGDCLGDIAARDLI	836-850													39	SP_139	SIIAYTMSLGAENSV	691-705																	39	NP_80	PAADLDDFSKQLQQS	396-410
40	SP_179	GAALQIPFAMQMAYR	891-905																													40	NP_81	DDFSKQLQQSMSSAD	401-415
41	SP_181	QMAYRFNGIGVTQNV	901-915																													41	NP_82	QLQQSMSSADSTQA	406-419
42	SP_182	FNGIGVTQNVLYENQ	906-920																																
43	SP_188	DSLSSTASALGKLQD	936-950																																
44	SP_189	TASALGKLQDVVNQN	941-955																																
45	SP_192	AQALNTLVKQLSSNF	956-970																																
46	SP_196	VLNDILSRLDKVEAE	976-990																																
47	SP_200	LITGRLQSLQTYVTQ	996-1010																																
48	SP_203	QLIRAAEIRASANLA	1011-1025																																
49	SP_204	AEIRASANLAATKMS	1016-1030																																
50	SP_212	APHGVVFLHVTYVPA	1056-1070																																
51	SP_221	HWFVTQRNFYEPQII	1101-1115																																
52	SP_237	KEIDRLNEVAKNLNE	1181-1195																																
53	SP_238	LNEVAKNLNESLIDL	1186-1200																																
54	SP_239	KNLNESLIDLQELGK	1191-1205																																
55	SP_244	IWLGFIAGLIAIVMV	1216-1230																																