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CORONAVIRUS

BNT162b2 vaccination induces durable SARS-CoV-2 specific T cells with a stem cell memory phenotype

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Vaccination against SARS-CoV-2 is effective in preventing hospitalization from severe COVID-19. However, multiple reports of break-through infections and of waning antibody titers have raised concerns on the durability of the vaccine, and current vaccination strategies now propose administration of a third dose. Here, we monitored T cell responses to the Spike protein of SARS-CoV-2 in 71 healthy donors vaccinated with two doses of the Pfizer–BioNTech mRNA vaccine (BNT162b2) for up to 6 months after vaccination. We found that vaccination induced the development of a sustained anti-viral CD4+ and CD8+ T cell response. These cells appeared before the development of high antibody titers, displayed markers of immunological maturity and stem cell memory, survived the physiological contraction of the immune response and persisted for at least 6 months. Collectively, these data show that vaccination with BNT162b2 elicits an immunologically competent and long-lived SARS-CoV-2-specific T cell population.

INTRODUCTION

Approximately half of the world population has developed some immunity to SARS-CoV-2, either through natural infection or through vaccination (1) but it is unclear if this immunity will last longterm. SARS-CoV-2 infection elicits a robust immune response (2–10), as do mRNA vaccines (11–18), inducing both B and T cell responses. Effective humoral immunity involves the generation of high affinity neutralizing antibodies, which are widely used as indicators of protective immunity (19–21) and represent the final output of a coordinated cellular response. The emergence of activated T cells precedes recovery from COVID-19 (7, 22–24), providing evidence of T cell involvement in the immune response to SARS-CoV-2. Initial in vitro studies characterized patient-derived antigen-specific CD4+ and CD8+ lymphocytes reactive with overlapping peptide pools from the SARS-CoV-2 Spike protein (3, 5, 23, 25), showing the establishment of an effective anti-viral response after natural infection. Spike-specific T cells persist in COVID-19 convalescents (2, 26–28), arise following vaccination with mRNA-based SARS-CoV-2 vaccines, and include specialized subsets which can both sustain cytotoxic functions and participate to the maturation of antibody-producing B cells in lymph node germinal centers (11, 15–17, 29, 30). Notably, subjects with undetectable or impaired humoral responses can recover from COVID-19 (31–33), underlining the importance of cellular immunity in clearing SARS-

CoV-2 infection.

Mass-vaccination against COVID-19 is effective and protects against severe COVID-19 in real-world settings (34, 35). Decreases of antibody levels and increases of breakthrough infections point to waning immunity of vaccination over time (36–39). However, despite the decline in antibody levels, protection from severe disease and hospitalization remains high (40, 41), suggesting that persisting cellular immunity drives the immune response and prevents viral dissemination when antibodies disappear. Studies on immune memory to other coronaviruses have shown that cellular immunity can be detected for up to 17 years after initial infection in the absence of antibodies (9, 40–42), with cellular immunity persists for up to 15 months after SARS-CoV-2 infection (43). The study of the cellular immune components induced by vaccination and the assessment of their persistence is fundamental; in this respect, a recent study has shown that robust adaptive immune responses can be detected for up to 6 months following mRNA vaccination (15).

Here, we performed a longitudinal study looking at the T cell responses and Anti-Receptor-Binding Domain (RBD) antibodies in 71 health-care workers and scientists vaccinated with the BNT162b2 vaccine following the European Medicines Agency (EMA)-approved two-dose vaccination schedule, up to 6 months after the first dose. We investigated expression of phenotypic markers associated with the

differentiation, polarization, and cytotoxic functions of Spike-specific T cells, and monitored the evolution of the adaptive immune response induced by mRNA vaccination. Vaccination induced the generation of immunologically competent Spike-specific T cells, including potentially long-lived memory stem cells, pillars of durable immunity. This information provides further information on the durability of the vaccine, which may inform vaccination strategies going forward.

RESULTS

Induction and persistence of anti-RBD antibodies

The antibody response to vaccination with BNT162b2 was measured in serum samples obtained at baseline (T0), on the day of the boost (T1), 14 days later (T2), and 6 months after the first dose (T3). All individuals in our cohort were devoid of anti-RBD antibodies at baseline, and significant levels appeared in 100% of individuals only after the second dose (Fig. S1A), (T1: median 28; T2: median 1786; T3: median 517). Age and sex are key variables in immunity induced by vaccination, whose effectiveness decreases with age and is usually lower in males (44). In our cohort antibody levels induced by BNT162b2 were comparable between males and females (Fig. S1B), similar to other studies (45). Also, we found that antibody titers negatively correlated with age at all time points, confirming previous results (45–47) (Fig. S1C). Thus, the data show that BNT162b2 induces the production of anti-RBD antibodies which decrease over time but are maintained at high levels for at least 6 months.

Induction and durability of the Spike-specific T cell response

To investigate the cellular immune response induced by vaccination, we exposed freshly obtained peripheral blood mononuclear cells (PBMC) to peptide pools spanning the entire sequence of the Spike (S) protein. Blood collection was performed at the same timepoints as outlined above. To fully capture the antigen-specific T cell response and to maximize sensitivity, two separate assays for the detection of the expression of surface Activation-Induced Markers (AIM) and for Intracellular Cytokine Staining (ICS) were set up (Fig. S2 for gating strategy). All assays were performed on freshly isolated PBMCs in order to obtain accurate absolute cell counts.

AIM+ CD4+ T cells were defined by up-regulation of CD40L and CD69 (48), while CD137 and CD69 expression identified the AIM+ CD8+ subset (5) (Fig. 1A). After paired background subtraction from parallel unstimulated cultures, and having set a threshold for positivity at 0.008% for CD4+ T cells and 0.079% for CD8 T cells (see Methods section for further details), we found that 97% of donors had detectable numbers of AIM+ CD4+ cells at baseline (T0, Fig. 1B, 1C and Fig. S3A). 21 days after the first dose of vaccine these cells were expanded 6-fold compared to baseline (T1), and further still at 14 days after the boost (T2). Six months after the first

dose (T3) AIM+ CD4+ cell numbers were still 5-fold higher compared to baseline and were detected in all donors. AIM+ CD8+ T lymphocytes were present at baseline in only 18% of the donors (Fig. 1B, 1C and Fig. S3A). Following vaccination, 87% showed Spike-specific CD8+ T cells which had expanded 11-fold from baseline; following further expansion after the second dose, these cells remained detectable after 6 months in 88% of donors (Fig. 1B, 1C and Fig. S3A). The total magnitude of the T cell response (including both CD4+ and CD8+ cells) increased significantly following priming (T1) and re-challenge (T2), but decreased 6 months after the first dose (T3) (Fig. 1D and S3B). The Stimulation Index (S.I., the ratio of AIM+ T cells in stimulated over unstimulated samples) was used to determine T cell activation (49), and the cut-off for positive responses was arbitrarily set at 3. The S.I. of AIM+ CD4+ cells increased dramatically after the first dose, remained at high levels after boosting, and decreased after 6 months, remaining more than 5-fold higher compared to baseline (Fig. 1E). The SI of AIM+ CD8+ T cells peaked 14 days after the boost, and declined by 1/3 at the latest time-point (Fig. 1E). Notably, over 70% of individuals in our cohort showed AIM+ CD4+ cells with S.I. >3 already at baseline, and this proportion increased to 100% 6 months after receiving the first dose (Fig. 1F). On the other hand, only 7% of individuals showed AIM+ CD8+ with SI>3 cells at baseline, with this fraction increasing to 60% and 75% after priming and boosting, and then decreasing to 63% at the latest time points (Fig. 1F). The frequency of S-specific CD4+ (and not CD8+) T cells induced by vaccination inversely correlated with age only at T1, although a tendency toward reduced numbers of activated T cells with increasing age was observed at all time points (Fig. S3C), again with no significant differences between males and females (Fig. S3D).

In univariate regression, the number of AIM+ cells correlated with antibody levels only after priming and not at subsequent time points (Fig. S3E), suggesting that humoral and cellular immune responses follow different kinetics. Multivariate regression modelling indicated that the number of AIM+ CD4+ cells 21 days after priming was the best predictor of anti-RBD levels after 6 months, as expected from a T cell-dependent B cell response (Fig. 1G).

Two donors in the cohort presented exceptionally high frequencies of CD4+ and CD8+ AIM+ cells at baseline (Fig. S4A). Vaccination boosted the frequency of AIM+ cells, but antibody responses remained within the 25th and 75th percentile of the distribution of the whole cohort at all time points (Fig. S4B). Thus, vaccination induces detectable and robust antigen-specific T cells that develop before high antibody titers, with most T cell expansion occurring after the first dose and persisting for up to 6 months.

Cytokine production by S-specific T cells

Cytokine production was assessed by intracellular

cytokine staining (ICS) following stimulation overnight with the peptide pools in the presence of monensin and brefeldin (Fig. 2A). Measurement of cytokine production showed that at baseline 45% and 46% of the tested donors had IFN γ + CD4+ and CD8+ cells, respectively (Fig. 2B and 2C). Three weeks after the first dose, 83% of individuals had IFN γ + CD4+ T cells, while CD8+IFN γ + were found in 71% of individuals. Two weeks after the boost, 81% and 68% of individuals had IFN γ + CD4+ and CD8+ T cells, respectively; these cells were maintained for 6 months. 53% of donors at baseline showed IL-2+ CD4+ T cells; this fraction increased to 84% after the first dose then reached 94% after the boost, and remained detectable after 6 months. Polyfunctional IFN γ +IL-2+ CD4+ T cells were induced in 99% of the individuals only following the booster dose, and persisted for at least 6 months after the first dose (Fig. 2D). CD8+ T cells co-expressing IFN γ + and lysosomal associated membrane glycoprotein (CD107a) increased significantly after the boost (Fig. 2D). Induction of cytokine-secreting S-specific T cells was equivalent in both sexes at all time points (Fig. S5A). The number of TNF α -secreting CD4+ and CD8+ cells was highest at the peak of the secondary response, 14 days after the second dose (Fig. S5B); at the same time point polyfunctional TNF α +IFN γ + cells decreased significantly, and after 6 months they were at pre-vaccination levels. Further analysis of CD107a, TNF α and Granzyme B co-expression among CD4+ and CD8+ T cells producing IFN γ + revealed changing patterns between time points ($p < 0.001$ for all comparisons except for IFN γ + CD4+ T cells between T1 and T2, Fig. 2E), with the fraction of polyfunctional CD4+ cells increasing at each time point, and a predominance of CD8+ cells with 2 functions at the latest time point, indicating dynamic changes of functional programs in antigen-specific T cells following boost and contraction.

At the peak of the response (T2), cytokine secretion was also measured with a MACSplex assay in the supernatants of cultures stimulated with the peptide pools for the AIM assays. Production of high levels of IFN γ and IL-2 was found, whereas IL-17 and IL-4 were barely detectable (< 5 pg/ml), confirming the Th1 differentiation profile of S-specific cells (Fig. S5C). Thus, vaccination induces the emergence of a robust CD4+ and CD8+ cytokine response by T cells after priming, while full effector functions marked by polyfunctionality are acquired following the boost, and then maintained for at least 6 months.

Differentiation features of Spike-specific T cells

We then characterized the antigen-specific T cells induced by vaccination through the definition of their differentiation status using CCR7 and CD45RA markers to define naïve (N), central memory (CM), effector memory (EM) and terminally differentiated effector (EMRA) populations (Fig. S2 and Fig. 3A and B). The study of the frequencies of these subsets

within AIM+ cells in the peripheral blood showed that at all time points effector memory (EM) cells dominate the CD4+ T cell subset, while CD8+ T cells also present a large proportion of terminally differentiated cells (Fig. 3A and B). Six months after the first dose, CD4+ S-specific cells which had survived the physiological contraction of the immune response were mostly central- and effector memory cells, while in the CD8+ subset these cells also included a significant fraction of terminally differentiated effectors (Fig. 3A and B).

Among the desirable outcomes of vaccination lies the generation of a pool of memory stem cells, which can rapidly and efficiently differentiate in highly effective and polyfunctional lymphocytes in case of re-encounter with their nominal antigen (50). Stemness includes long-term persistence, a key aspect in this age of pandemics and uncertainties on the durability of the novel vaccines. Thus, we searched for these cells within the antigen-responsive CD4+ and CD8+ subsets. After the first dose, CD4+AIM+CD45RA+CD27+CCR7^{high}CD95+ cells, representing CD4+T_{SCM} (T Stem Cell Memory) cells (51), were detectable in 88% of individuals (see Fig. S6 for gating strategy); 2 weeks after the second dose, this fraction was still 88%; after 6 months, 91% of individuals showed these cells (Fig. 3C). CD8+ T_{SCM} followed similar kinetics, with 94%, 87%, and 96% of individuals showing these cells at T1, T2, and T3 respectively.

To investigate the possible impact of T_{SCM} on future immune responses, we used a General Linear Model with stepwise selection aiming to optimize Akaike Information Criterion (AIC) with Leave-One-Out cross-validation. We tested if the number of early (i.e., T1) CD4+ T_{SCM} induced by vaccination significantly predicted immunological outcomes at future time points. We found that the number of T_{SCM} induced after priming was a significant predictor of the number of both CD4+ and CD8+ activated T cells at the latest time point (Fig. 3D). To exclude the possibility that outliers were present in the CD4+ T_{SCM} distribution we performed multiple Grubbs and Rosner tests and no outliers were found (Table S1 and S2). These findings show that vaccination with BNT162b2 induces a T cell population with features of longevity, which remain numerically stable in the peripheral blood for at least 6 months and predict future T cell responses.

Effector features of Spike-Specific CD4+ and CD8+ cells

We next explored the phenotypic changes occurring in AIM+ T cells after boosting and 6 months thereafter by unbiased computational analysis. We used FlowSOM clustering to identify four CD4+ T cell clusters segregated by the expression pattern of CD137, CD39, ICOS, PD-1, HLA-DR, CD25, CXCR5, CD95, CCR7, CD45RA, CD38, and CD127 (Table S3) and superimposed those clusters in a UMAP plot generated

by embedding the same set of markers (Fig. 4A). AIM+ CD4+ T cells were distributed differently in the UMAP plot at the three different time points (Fig. 4B), highlighting an overall phenotypic shift. Among these cell clusters, cluster 3 and 4 were unchanged in frequency, while clusters 1 and 2 showed significant changes over time (Fig. 4C). Cluster 2 increased in frequency at each time point and was characterized by high expression of activation markers (CD25, CD38, CD39, HLA-DR, CD137) and of the typical markers of T follicular helper (Tfh) cells (i.e., expression of ICOS, PD-1, and CXCR5, Fig. 4A and C). Cluster 1 was composed of cells which showed the lowest expression of CD25, CD38, CD39, HLA-DR, CD137, corresponding to a non-activated profile, and decreased with time (Fig. 4A and C).

Data analysis through manual gating and measurement of single marker expression on the antigen-specific T cells revealed the emergence and persistence in time, albeit at lower levels after 6 months, of CD4+ subsets displaying PD-1, ICOS, and CXCR5 (Fig. S7), typical Tfh markers. At the 6-month time point expression of activation markers such as CD25, CD39, CD38, and HLA-DR were greatly reduced. Linear regression modelling indicated that the fraction of PD1+ICOS+ within CD4+ AIM+ T cells was a significant predictor of antibody levels at the 6 months' time point (Fig.S8).

S-specific CD8+ lymphocytes were remodeled by vaccine doses, as shown by the distinct distribution with time along the UMAP axes (Fig. 4E). Two clusters, comprising highly (Cluster 4) and slightly less (Cluster 3) activated AIM+ CD8+ T cells, showed transient expansion after the booster dose (Fig. 4D and F). This was in contrast to Cluster 1 (naïve-like cells) which remained stable after a decrease in frequency at T2, and to Cluster 2 (CCR7+ CD127+ CD45RA- central memory CD8+ T cells) which was not significantly reduced at T2 but increased in size at 6 months. Manual analysis of AIM+ CD8+ T cells confirmed the transient increase at T2 of CD38+, HLA-DR+ and CD25+ cell frequencies, whereas CD39+ and PD-1+ cells steadily decreased or increased, respectively, at T2 and T3 compared to T1 (Fig. S7). Thus, antigen-specific T cells acquire phenotypic features of activation and functional capability after the booster, and most of these attributes are less evident and partially replaced, in the long run, by characteristics distinctive of more quiescent memory cells.

DISCUSSION

Multiple different vaccines are being administered globally to prevent the infection and spread of SARS-CoV-2. Here, we studied the adaptive immune response induced by vaccination with BNT162b2 to characterize Spike-specific T cell responses, and to understand if vaccine-induced T cells displayed features of longevity. We found Spike-specific CD4+ and CD8+ memory T cells which peaked two weeks after the boost and remained detectable in the peripheral blood for up

to 6 months. These findings are in line with those of Goel *et al.* (15), who have recently described the establishment and durability of memory T cells following mRNA vaccination, and with previous studies investigating cellular immunity following SARS-CoV2 infection (2, 26, 28). Also, we found antigen-specific population of T_{SCM} which persisted overtime.

In our cohort, nearly all individuals harbored Spike-specific T cells at baseline, likely due to the presence of a pool of memory clones cross-reactive with other coronaviruses. Preexisting T cell cross-reactivity to endemic coronaviruses, such as common cold coronaviruses, have been described in various proportions of SARS-CoV-2-naïve individuals (5, 9, 23, 24, 49, 53–55). Bacher *et al.* (55), using freshly isolated cells, detected Spike-specific T cells in all tested donors, similar to our findings. The presence of SARS-CoV-2 cross-reactive T cells associates with an enhanced cellular and humoral response to vaccination (49). The individuals in our cohort with exceptionally high numbers of AIM+ CD4+ cells at baseline did not respond to vaccination with an equally exceptional antibody response. This could be due to the fact that cross-reactive T cells mainly target the S-II region of the Spike protein (49), which does not contain the RBD-binding region; thus, measurement of only anti-RBD antibodies may not inform on the whole spectrum of the humoral response induced by vaccination.

An optimal antibody response is the consequence of a competent underlying T cell response, which relies on the presence of Tfh cells that interact with B lymphocytes in germinal centers to promote high affinity antibody production and the generation of long-lived memory B cells (56). High antibody titers induced by influenza vaccination positively correlate with the frequency of T cells expressing follicular helper molecules, including CXCR5 and ICOS (57, 58). Induction of Tfh cells also occurs in response to the Spike protein (59, 60), and our finding of Spike-specific cells expressing CXCR5, PD1, and ICOS suggests that mRNA vaccination is effective in generating CD4+ cells able to interact with B cells, in line with other studies (13, 15, 17, 61). Accordingly, the simultaneous measurement of serum levels of anti-RBD antibodies showed the effective induction of the humoral arm of the adaptive response in all individuals in our cohort. Although antibody titers did decline with time, they were maintained at high levels for the entire period of our observation. Our data also converges with a recent study showing that the magnitude of antigen-specific CD4 responses at early time points predicted antibody levels after 6 months, confirming the central role of CD4+ T cells in instructing the humoral response (15).

CD4+ T cells also sustain immune responses through the production of cytokines, and we find that mRNA vaccination elicits a vigorous Th1-skewed response, with production of IFN γ , IL2, and TNF α by Spike-specific cells, and undetectable

levels of IL4 and IL17. After 6 months, T cell responses were maintained, with the persistence of a numerically consistent pool of polyfunctional memory antigen-specific T cells (62). A correlation between both magnitude and polyfunctionality of T cell responses and resistance to infection or favorable disease evolution has been described in successful vaccine settings (63–69), and polyfunctional T cells correlate to protection against influenza (70). Polyfunctional cells were also detected in the CD8+ Spike-specific T cell subset, which produced IFN γ , TNF α , and expressed granzyme B and the surrogate marker of degranulation CD107a, in agreement with previous studies (12, 15, 17, 71, 72). CD8+ T cells, however, were less expanded compared to the CD4+ subset, and showed lower S.I., probably due the fact that the peptide pool used in this study, consisting of 15-mers, is not optimal for presentation through MHC-I and thus for CD8+ T cell recognition (73), so these responses might have been underestimated.

We found that mRNA vaccination also induced CD4+ and CD8+ T_{SCM}, which were stable throughout the 6-month period of this study. T_{SCM} cells provide a memory reservoir with multipotent capacity, and have been shown to persist for decades (74–77). The detection of T_{SCM} in vaccinated individuals is thus suggestive of the establishment of long-lived immunity. The number of T_{SCM} induced after priming predicted future T cell responses, and the stability of this memory population may point in the direction of durable immunity against SARS-CoV2.

Finally, immune responses to vaccines have been shown to be higher in females (44, 78). Moreover, the male sex is associated with severe COVID-19 and death (79). Thus, we searched for a sex-bias in the immunological parameters investigated. Importantly, we did not find differences in vaccine-induced immune responses between males and females.

This study has the inevitable limits of human studies, consisting mainly of the wide genetic and immunological variability of the study population, and with blood being the only available source of material. Thus, we only studied circulating lymphocytes, which may be different from those who reside at mucosal barriers and which confer immediate protection against infection. Also, we did not investigate Spike-specific B lymphocytes, and dosage of antibody titers was the only read-out of successful induction of humoral immunity. Tfh and T_{SCM} cells were identified only by the phenotypic characterization of surface markers, and functional studies, such as measurement of IL21 production to identify Tfh cells, were not carried out to match the phenotype. Further time points will be necessary to measure effective durability of anti-SARS-CoV-2 immune responses. Moreover, although donors were equally distributed for age and sex, our sample was limited in size.

On the whole, the results of this study can be visualized

as the dynamic and integrated emergence of a Spike-specific adaptive immune response, characterized by a T cell response which precedes the development of high levels of anti-RBD antibodies (Fig. 4G). T cells induced after the first encounter with the Spike protein are mostly effector cells, which secrete intermediate levels of cytokines, express the highest levels of Granzyme B, are not polyfunctional, and only some have Tfh characteristics; cells with features of stemness and longevity also appear. After the boost, the peak of the response shows a fully activated, cytotoxically empowered, multifunctional T cell population, including cells of the Tfh phenotype; at this time point, the highest levels of anti-RBD antibodies are detected in the serum. What remains after 6 months is a population of T cells with features of polyfunctionality and markers of Tfh cells; at this time point, Spike-specific T cells which have survived the immunological contraction are highly specific and theoretically prone to give rise to effective and rapid antiviral responses, both by possibly sustaining the production of antibodies and by exerting cytotoxicity toward already infected cells; concomitantly, the pool of T_{SCM} is stably maintained. This is in line with clinical real-world data showing that vaccine effectiveness in preventing severe COVID-19 is maintained above 90% at least for 6 months (80–82). Protection from infection, however, does significantly decrease with time and likely correlates to the waning antibody titers (21, 38), which provide an immediate shield against infection.

Cellular responses to mRNA vaccination thus include richly heterogeneous and dynamic memory T cell populations showing features associated with protective immunity. Previous studies performed at early time points after vaccination have described the induction of memory Th1 and Tfh CD4+ T cells along with cytotoxic CD8+ T cells (13, 16, 17, 83, 84). Observations at more distant time points (6 months) have shown stability of SARS-CoV-2 memory T cells, with a faster decline of the CD8+ subset (15). Another study found equivalent levels of CD4+ and CD8+ cells 6 months after vaccination, as observed following natural infection (13). Here, we have shown that a sizable population of T_{SCM} is promptly induced by vaccination, but whether these memory progenitors will provide added protection upon viral exposure remains to be established.

MATERIALS AND METHODS

Study Design This work started to define cellular immune responses against the SARS-CoV-2 Spike-protein induced by mRNA vaccine administration. To this aim, we enrolled 71 individuals from health-care workers from the sanitary personnel and scientists operating at the Santa Lucia Foundation scheduled for vaccination with Pfizer-BioNTech BNT162b2 between January 12th and February 2nd 2021 (Table S4). All donors signed informed consent forms approved by the Ethical Committee of the Santa Lucia Foundation.

Venous blood was obtained immediately before the first dose (T0), 21 days thereafter, at the time of boosting (T1), two weeks after the second dose (T2), and 6 months after the first dose (T3). The use of freshly obtained blood cells permitted the detection of fragile markers, avoided the bias introduced by freezing/thawing procedures, and provided the possibility to precisely calculate absolute cell counts, a measure routinely used to guide clinical decisions in infectious diseases, such as HIV infection (52).

Evaluation of anti-SARS-CoV-2 antibodies The measurement of anti-RBD antibodies was performed by electrochemiluminescence sandwich immunoassay (ECLIA) through Roche Elecsys Anti-SARS-CoV-2 S (Roche diagnostics, Switzerland). Antibody levels were measured on a Cobas 601 modular analyzer (Roche diagnostics, Switzerland), using a cut-off of 0.8 U/ml. Elecsys Anti-SARS-CoV-2 S U/mL measurements are equivalent to WHO International Standard Binding Arbitrary Units per mL (BAU/mL).

AIM assay

In vitro stimulation of freshly obtained PBMC was performed as described (85). In brief, 200 μ l of cell suspensions (10×10^6 cells/ml) were seeded in U-bottom 96-well plates and stimulated for 18 hours with PepTivator SARS-CoV-2 protein S, S1 and S+ peptide pools (1 mg/ml each, Miltenyi Biotec) or with an equal volume of water. Purified α CD40 (0.5 μ g/ μ l, Miltenyi Biotec) was added at culture start. Supernatants and cells from these culture wells were collected for cytokine measurement and flow cytometry, respectively. The threshold for positivity for background-subtracted values was set using the median-75th percentile of values obtained in negative control cultures (86).

Intracellular Cytokine Staining PBMC were incubated with peptide pools or water for one hour and then BV421-conjugated antiCD107a (1/200 dilution, BD Biosciences), monensin and brefeldin A (5 μ M and 10 μ g/ml, respectively, both from Sigma-Aldrich) were added to the cultures. After additional 17 hours, cells were harvested and directly stained for flow cytometry.

Flow cytometry staining and acquisition Post-culture cells were pelleted in V-bottom 96-well plates and resuspended in 30 μ l of antibodies at pre-optimized concentrations and diluted in Brilliant Stain Buffer (BD Biosciences), then incubated for 15' at RT. Cell pellets were fixed either in FoxP3 fixation/permeabilization Buffer (ThermoFisher) for 20' at RT (AIM Assay) or in Formaldehyde 4% in PBS (ICS Assay). ICS was performed by incubating cells in 30 μ l of antibodies against cytokines and Granzyme B diluted in a Saponin (Sigma-Aldrich) 0.5% W/V solution. The complete list of antibodies used for surface and intracellular staining is shown in Table S5 and S6, respectively. Samples were acquired on a fully equipped Cytoflex LX within 6 hours from staining and fixation. Quality Control beads (Beckman

Coulter) were used daily to check and standardize instrument performances. Absolute counts were obtained by a volumetric, lyse-no-wash, flow cytometry approach. Briefly, whole blood was stained (Table S7). After 15 min of incubation at RT, FACS lysing solution (BD Biosciences) was added. After complete RBC lysis samples run on a Cytoflex with a volume-calibrated peristaltic pump. The number of cells/ml automatically derived by the instrument was then multiplied by 10000 to determine the absolute CD4 and CD8 T cells on a ml basis. Absolute counts of AIM+, cytokine+, and related subsets was then performed multiplying their proportions by the absolute count of parent lineages. Data was analyzed with FlowJo v. 10.7. UMAP embedding and FlowSOM clustering of AIM+ cells were performed with FlowJo (UMAP ver3.1 and FlowSOM ver2.6 plugins). A total of 2500 CD4 and 990 CD8 AIM+ T cells, equally sampled from 10 donors/three time points, were used to run each algorithm.

Cytokine measurements IL-4, IL-17, TNF α and IFN γ were measured in frozen culture supernatants from the AIM assays described above by a bead-based sandwich ELISA (MACSplex Cytokine Kit, Miltenyi Biotec) according to manufacturer instructions.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9, Spice 6.1 and R programming language using the following libraries: caret 6.0, FactoMineR 2.4 and factoextra 1.0.7. Details of the statistical tests applied for each experiment are illustrated in the respective figure. Time points were compared by Kruskal Wallis or Friedman test followed by post hoc Dunn's or two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. Statistical significance was inferred if p values were below 0.05. In all figures, p values are indicated by: *p < 0.05; **p < 0.01; *** p < 0.001; **** p < 0.0001. Outlier detection was performed with multiple Grubbs' test and Rosner's test using the outliers and EnvStats packages in R, respectively.

SUPPLEMENTARY MATERIALS

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Figs. S1 to S8

Tables S1 to S8

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Fig. 1. Spike-specific T cell responses induced by vaccination with BNT162b2. A) Representative flow cytometry plots gated on CD4+ or CD8+ T cells showing up-regulation of activation markers (CD69 and CD40L for CD4+ cells and CD69 and CD137 for CD8 cells) following overnight stimulation with a pool of overlapping peptides covering the wt Spike protein at baseline (T0), 21 days after the first dose (T1), 14 days after the second dose (T2), and 6 months after initial vaccination (T3). Numbers in gates represent percentages of positive cells. B) Longitudinal analysis of Spike-specific CD4+ and CD8+ absolute cell counts, following background subtraction in paired unstimulated samples. Time points were compared by non parametric Kruskal Wallis and Dunn's multiple comparison test; lines represent median with interquartile range. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; no symbol, not significant. C) Fraction of individuals showing spike-specific AIM+ CD4+ and CD8+ cells above threshold at each time point. D) Magnitude of T cell responses. AIM+ CD4+ and CD8+ absolute cell counts were aggregated for each donor and displayed in the bar charts at the different time points, ordered by height. E) Stimulation indices (ratio of AIM+ cells stimulated and paired unstimulated samples) of CD4+ and CD8+ cells in the different time points; time points were compared by non parametric Kruskal-Wallis followed by Dunn's *post hoc* tests; bars represent median with interquartile range. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; no symbol, not significant. Dotted line indicates S.I. =3. F) Fraction of individuals showing S.I. >3 in CD4+ and CD8+ cells at the different time points. G) A linear regression model was fitted to test for T1 significant predictors of T3 anti-RBD antibody serum levels. Variables were log10-scaled when their distribution wasn't normal. Model's R^2 was 0.18. ** $p < 0.01$. The number of AIM+ CD4+ cells/ml was deemed as significant ** $p < 0.01$. Continuous and dotted lines represent linear regression and 95% confidence intervals, respectively.

Fig. 2. Spike-specific T cell responses characterized by cytokine production and expression of cytotoxicity markers. A) Representative flow cytometry plots gated on CD4+ or CD8+ T cells showing production of IFN γ and IL2 by CD4+ cells (top) and of both IFN γ production and up-regulation of CD107a (LAMP-1) by CD8+ cells (bottom) following peptide pool stimulation. Numbers in gates indicate percentages of positive cells. B) Longitudinal analysis of absolute cell counts of spike-specific IFN γ -producing CD4+ and CD8+ cells. Time points were compared by non parametric Kruskal-Wallis Dunn's multiple comparison tests; lines represent median with interquartile range. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; no symbol, not significant. C) Fraction of individuals showing spike-specific IFN γ + CD4+ and CD8+ cells at each time point. D) Longitudinal analysis of Spike-specific CD4+ cells producing IL2 or both IL2 and IFN γ (left and center panels) and of CD8+ cells expressing both CD107a and IFN γ (left panel). p values were determined as in Fig. 2B. E) Coexpression of functional markers (CD107a, Granzyme B and TNF α) in IFN γ -producing S-specific CD4+ and CD8+ T cells. The percentage of T cells positive for the specified number of functions is indicated by the pie slices for each timepoint, with functions=1 indicating the fraction of cells that produce only IFN γ (gating parameter). A partial permutation test (10000 iterations, Monte Carlo simulation) was applied on distributions into combinatorial gates. *** $p < 0.001$; **** $p < 0.0001$.

Fig. 3. Differentiation status of Spike-specific CD4+ and CD8+ T cells. A) Frequency of naïve, central memory (CM), effector memory (EM) and CD45RA+ effector memory (EMRA) within AIM+ CD4+ (top panels) and CD8+ (bottom panels), at each time point. Time points were compared by non parametric repeated measures Friedman and Dunn's multiple comparison tests; lines represent median with interquartile range. *p < 0.05; **p < 0.01; *** p < 0.001; ****p < 0.0001; no symbol, not significant. B) Fraction of AIM+ or total CD4+ (top panels) and CD8+ (bottom panels) that belong to the indicated subsets at each time point. C) Absolute cell counts of CCR7+CD45RA+CD27+CD95+ AIM+ CD4+ (left) and CD8+ (right) cells at the different time points. Timepoints were compared by non parametric Kruskal-Wallis test; lines represent median with interquartile range. *p < 0.05; no symbol, not significant. D) The relevance of T1 CD4+ T_{SCM} cells/ml in predicting T3 AIM+ CD4+ (top) and CD8+ (bottom) cells/ml was tested with two Generalized Linear Models with stepwise selection aiming to optimize Akaike Information Criterion (AIC). Model's R² were 0.16 and 0.34 for AIM+ CD4+ and CD8+ cells respectively. The number of CD4+ T_{SCM} in T1 cells/ml was deemed as significant in predicting both CD4+ and CD8+ AIM+ cell numbers in T3 (*p < 0.05). Continuous and dotted lines represent linear regression and 95% confidence intervals, respectively.

Fig. 4. Phenotypic shifts of AIM+ T cells over time. **A)** Top panel. UMAP embedding and FlowSOM clustering based on the expression of the indicated markers on AIM+ CD4+ T cells. The cell clusters identified by FlowSOM were superimposed on the UMAP plots and manually contoured to highlight cluster boundaries. Bottom panel. Heatmaps with two-way hierarchical clustering of scaled and centered median fluorescence intensity values for the indicated markers expressed by the AIM+ CD4+ T cell clusters. **B)** UMAP plots with AIM+ CD4+ T cells selected from each time point. **C)** Bar plots showing relative frequency of cell clusters at indicated time points among AIM+ CD4+ T cells. **D)** Top panel. UMAP embedding and FlowSOM clustering based on the expression of the indicated markers on AIM+ CD8+ T cells. The cell clusters identified by FlowSOM were superimposed on the UMAP plots and manually contoured to highlight cluster boundaries. Bottom panel. Heatmaps with two-way hierarchical clustering of scaled and centered median fluorescence intensity values for the indicated markers expressed by the AIM+ CD8+ T cell clusters. **E)** UMAP plots with AIM+ CD8+ T cells selected from each time point. **F)** Bar plots showing relative frequency of cell clusters at indicated time points among AIM+ CD8+ T cells. Statistical significance was assessed by Friedman test followed by *post hoc* two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **G)** T cell marker measurements from both CD4+ and CD8+ AIM+ cells were normalized across the 3 time points, and the relative positivity for each marker is displayed on the radar plots. Measurements from CD4+ T cells are shown in red, those from CD8+ cells are blue, and cumulative measurements from both subsets are purple. The resulting plots illustrate the main features of T cells responding to a pool of peptides derived from the Spike protein of SARS-CoV-2. Dashed circles indicate neutralizing anti-Spike antibody levels. Histograms represent CD4+ (red) and CD8+ (blue) cell counts along the timeline. Syringes indicate the time point of vaccine administration, and tubes correspond to the day of blood sampling. Cyto+: aggregation of absolute numbers of CD4+ and CD8+ cell producing at least one cytokine among IFN γ , IL2, and TNF α ; IFN γ MFI: mean fluorescence intensity of the IFN γ signal in IFN γ + CD8+ (blue) or CD4+ (red) cells.