Molecularly distinct memory CD4+ T cells are induced by SARS-CoV-2 infection and mRNA vaccination

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ABSTRACT

Adaptive immune responses are induced by vaccination and infection, yet little is known about how CD4+ T cell memory differs between these two contexts. Notable differences in humoral and cellular immune responses to primary mRNA vaccination were observed and associated with prior COVID-19 history, including in the establishment and recall of Spike-specific CD4+ T cells. It was unclear whether CD4+ T cell memory established by infection or mRNA vaccination as the first exposure to Spike was qualitatively similar. To assess whether the mechanism of initial memory T cell priming affected subsequent responses to Spike protein, 14 people who were receiving a third mRNA vaccination, referenced here as the booster, were stratified based on whether the first exposure to Spike protein was by viral infection or immunization (infection-primed or vaccine-primed). Using multimodal scRNA-seq of activation-induced marker (AIM)-reactive Spike-specific CD4+ T cells, we identified 220 differentially expressed genes between infection- and vaccine-primed patients at the post-booster time point. Infection-primed participants had greater expression of genes related to cytotoxicity and interferon signaling. Gene set enrichment analysis (GSEA) revealed enrichment for Interferon Alpha, Interferon Gamma, and Inflammatory response gene sets in Spike-specific CD4+ T cells from infection-primed individuals, whereas Spike-specific CD4+ T cells from vaccine-primed individuals had strong enrichment for proliferative pathways by GSEA. Finally, SARS-CoV-2 breakthrough infection in vaccine-primed participants resulted in subtle changes in the transcriptional landscape of Spike-specific memory CD4+ T cells relative to pre-breakthrough samples but did not recapitulate the transcriptional profile of infection-primed Spike-specific CD4+ T cells. Together, these data suggest that CD4+ T cell memory is durably imprinted by the inflammatory context of SARS-CoV-2 infection, which has implications for personalization of vaccination based on prior infection history.

One Sentence Summary: SARS-CoV-2 infection and mRNA vaccination prime transcriptionally distinct CD4+ T cell memory landscapes which are sustained with subsequent doses of vaccine.

INTRODUCTION

T cell memory is crucial for long-lived protection against virus and is a well-established correlate of immune protection (*1–5*)**.** In particular, the multifaceted defense established by memory CD4+ T cells occurs via coordination of a suite of innate immune responses, direct help to B cells and CD8+ T cells, and interaction with infected cells (*6, 7*). The quality of memory CD4+ T cell responses has been assessed by interrogation of cellular frequency, cytokine production, provision of help, and T cell receptor (TCR) affinity for antigen (*8–11*). However, the factors that contribute to the heterogeneity of the T cell response are not fully-understood.

The goal of vaccination is to induce long-lasting protection. With onset of the COVID-19 pandemic, SARS-CoV-2 caused widespread infection but also resulted in the rapid deployment of novel vaccines. As a result, many individuals developed T cell memory to Spike protein either through infection or SARS-CoV-2 vaccination. SARS-CoV-2 infection and mRNA vaccination induced robust Spike-specific CD4+ T cell responses skewed towards a Th1 and Tfh profile (*12–17*). Notably, CD4+ T cell responses targeted epitopes that have been relatively conserved across variants to date (*18–20*), which may be increasingly important for mitigating severe disease given emergence of variants demonstrating antibody escape (*21, 22*). In short, mRNA vaccination and SARS-CoV-2 infection each induced memory CD4+ T cell responses that are likely to shape the outcome of future exposure to virus.

Despite induction of a detectable memory CD4+ T cell response by vaccine and virus, little is known about whether, or even if, infection- and vaccine-derived memory CD4+ T cells differ. However, the immune context in which Spike epitopes are presented is not identical. Factors during initial priming such as inflammatory signals, site and persistence of antigen exposure, cell-to-cell interactions, and cytokine milieu imprint resulting memory pools and influence T cell responses upon antigen re-exposure (*23, 24*). Notably, viral infection triggers a widespread high state of inflammation that is largely absent during immunization (*25*). Indeed, COVID-19 patients had increased serum levels of inflammatory cytokines across mild to severe disease severity when compared to healthy controls (*26–28*). In contrast, serum levels of inflammatory cytokines were low following vaccination (*29*) and only mild systemic reactogenicity lasting on average 2-3 days were reported (*30–32*). The difference in concomitant inflammation may have deleterious consequences for T cell memory, however. Indeed, reduced TCR diversity, decreased frequency of antigen-specific memory T cells, and impaired cytokine production have been reported in the setting of excess inflammation (*33–35*). How infection-associated inflammation affects Spike-specific memory CD4+ T cells has not been studied, but examination of the direct effects of inflammation on memory T cell quality will improve our understanding of infection-derived protective immunity for SARS-CoV-2 and broadly inform new strategies for optimal vaccine design.

Due to the difference in inflammatory context between infection and vaccination at the time of memory CD4+ T cell priming, we hypothesized that qualitative differences in Spike-specific memory CD4+ T cells may have been established. To test this hypothesis, we explored the transcriptional profiles of Spike-specific CD4+ T cell responses pre- and post-third vaccine dose, referenced as the “booster” dose. We evaluated Spike-specific memory CD4+ T cells from fourteen individuals whose first exposure to Spike protein was either infection (infection-primed) or immunization (vaccine-primed) as well as five vaccine-primed participants who had breakthrough infection using multimodal scRNA-seq following overnight Spike peptide pool stimulation in the activation-induced markers (AIM) assay (*12, 36–39*). We found that Spike-specific CD4+ T cells from infection-primed individuals exhibited an increased cytotoxic signature compared to vaccine-primed individuals. Although both vaccine-primed and infection-primed participants had received their primary mRNA vaccination series, we uncovered greater expression of interferon-stimulated genes (ISGs) and interferon-related pathways in Spike-

specific CD4+ T cells from infection-primed adults. In contrast, proliferative pathways were enriched in Spike-specific CD4+ T cells from vaccine-primed participants. To test whether Spike-specific memory CD4+ T cells from vaccine-primed individuals could be affected by subsequent inflammation, we evaluated the same individuals who later had breakthrough infection. We found that breakthrough infection modestly altered the transcriptional profile of vaccine-primed participants but did not introduce the ISG-associated gene signatures seen in Spike-specific CD4+ T cells from infection-primed individuals. These data suggest a durable, inflammatory imprint on memory CD4+ T cells due to viral infection and highlight the importance of understanding the inflammatory context of initial antigen exposure for memory CD4+ T cell development.

RESULTS

Spike-specific CD4+ T cells form a distinct cluster in the Activation-Induced Marker assay

SARS-CoV-2 infection and mRNA vaccination induced Spike-specific memory CD4+ T cell responses, as assessed by ELISpot and Activation-Induced Marker (AIM) assay (*13–17*). However, we and others found subtle differences in the cellular responses based on the route of initial priming (*12*, *13*, *40*), and we observed muted CD4+ T cell responses to primary mRNA vaccination of infection-primed participants (*12*). Given that the magnitude of interferon-stimulated gene (ISG) activity differed during the acute response to vaccination or infection (*25*, *28*, *29*), we sought to understand the extent to which concomitant inflammation affected the generation of Spike-specific memory CD4+ T cells. To understand whether the initial priming affected subsequent responses, we chose to study Spike-specific memory CD4+ T cells during quiescence and following re-exposure to Spike antigens via mRNA vaccination.

To do this, we collected peripheral blood for 14 individuals around 8 months after second vaccination, referred to pre-booster time point, and one month post-booster to longitudinally assess immune memory (**Fig. 1A**). Seven individuals had confirmed diagnosis of SARS-CoV-2 infection in the spring of 2020 (labeled infection-primed), received two doses of mRNA vaccination ~9 months later, and received their third vaccination around 20 months after onset of COVID-19 (**Table S1**). Of these seven, six had mild or minimally symptomatic COVID-19 and one had severe disease (**Table S2**). In addition, we evaluated seven individuals who had not had COVID-19, as confirmed by N antibody ELISA and thus whose first exposure to Spike protein was mRNA vaccination (labeled vaccine-primed). Of these seven participants, five had later breakthrough SARS-CoV-2 infection at a median of around 5 months after their third vaccine dose during the Omicron wave; for these individuals, peripheral blood was collected one month post onset of symptoms (**Table S3**). Participants' ages ranged from 28 - 54, with a median age of 45 for infection-primed individuals and 38 for vaccine-primed individuals (**Table S1**).

To identify Spike-specific memory CD4+ T cells in a manner that did not require HLA-matched reagents, we used activation-induced markers (AIM) assay (*12*, *36–39*, *41*). Briefly, PBMCs were stimulated overnight with overlapping Spike peptide pools to evaluate surface expression. By flow cytometry, AIM reactive CD4+ T cells were identified based on co-expression of CD69 and CD200 (*12*, *36*). Stimulated cells demonstrated higher frequencies of CD69+CD200+ CD4+ T cells than paired unstimulated controls (P = 0.028; n=5; Wilcoxon matched-pairs signed rank test), thus demonstrating detection of antigen-specific cells (**Fig. 1B**).

We wanted to evaluate for differences at the transcriptional level in Spike-specific memory CD4+ T. To do this, we performed droplet-based multimodal single-cell RNA sequencing (*42*) following AIM assay and magnetic bead enrichment for CD69 and CD137, two

highly expressed surface markers among AIM reactive cells (*12*, *39*) (**Fig. 1A**). In total, we recovered 110,764 T cells. We evaluated the AIM assay in high-dimensional clustering at single cell resolution. Dimension reduction was performed on gene expression to generate a uniform manifold approximation and projection (UMAP), and using graph-based clustering (*43*), we identified 7 major clusters, two of which were nearly absent in unstimulated controls (**Fig. 1C, S1A-B**). Differential gene expression analysis revealed enriched expression of genes associated with activation such as *IFNG*, *IL2*, *LTA* in these two clusters (**Fig. 1C, S1A**). We denoted these two clusters as AIM Reactive CD4+ and AIM Reactive CD8+ T cells. In prior studies (*12*), we identified fewer Spike-specific CD8+ T cells than Spike-specific CD4+ T cells using AIM. We therefore focused on Spike-specific CD4+ T cells for the remaining analyses.

To further interrogate transcriptional changes induced by peptide stimulation, we evaluated the CD4+ cluster that appeared following stimulation. We first interrogated genes associated with other activation-induced markers. We found upregulation of genes such as *TNFRSF9* (CD137), consistent with the magnetic bead enrichment performed. Genes such as *TNFRSF4* (OX40) and *TFRC* (CD71) were also upregulated in the AIM Reactive CD4+ T cell cluster, as well as genes such as *LTA* (**Fig. 1D**). We then performed differential expression analysis on the AIM-reactive CD4+ T cell cluster relative to CD4+ T cell cluster, which revealed over 200 positively enriched genes. Furthermore, gene ontology analysis of genes differentially expressed by AIM-reactive CD4+ T cells demonstrated enrichment for terms associated with cellular response to stimuli and TCR signaling in the AIM Reactive CD4+ T cluster compared to the CD4+ T cluster (**Fig. 1E, S1C**). Together, these data demonstrated robust activation of a subset of CD4+ T cells by a Spike peptide pool.

In previous studies, the AIM assay identified antigen-specific cells as validated by MHC Class II tetramer analysis (*36*). To further test whether AIM-reactive CD4+ T cells were Spike-specific, we compared TCR sequences in our AIM-reactive CD4+ T cells to a public database of ~3000 TCRB published in the literature (*44*). For four of ten participants, there was substantial overlap between the AIM-reactive CD4+ CDR3B amino acid sequences and those of the public SARS-CoV-2 TCRB database (*44*) ($P < 0.05$, Fisher's exact test, **Fig. S1D**). Although TCR overlap was not observed in other participants, this may have been due to the relatively limited number of TCRB sequences available for comparison. The TCR overlap further supported the notion that the AIM-reactive CD4+ T cell subset is Spike-specific.

Infection-primed Spike-specific CD4+ T cells have a cytotoxic phenotype

Prior studies in the literature described Th1 and Tfh subsets following SARS-CoV-2 infection and vaccination as well as expression of cytokines including IFNg, TNF, IL-2, and granzyme B (*12–14*, *17*, *40*, *45*). To ask whether these cytokines were detectable using exCITE-seq, we focused on these three as well as the cytotoxic marker *GZMB*. Indeed, overnight stimulation with Spike peptides induced transcripts for these cytokines in the AIM-reactive cluster relative to non-activated CD4+ T cells (**Fig S2A**).

We next wanted to further evaluate the subsets of Spike-specific CD4+ T cells using transcriptional profiling. Subsetting on the AIM Reactive CD4+ T cell cluster, we applied dimensionality reduction with 27 select parameters (*IFNG*, *TNF*, *IL2*, *IL12A*, *CXCR3*, *CCR5*, *STAT4*, *TBX21*, *RUNX3*, *IL4*, *IL5*, *IL13*, *CXCR4*, *GATA3*, *STAT6*, *CCR4*, *IL21*, *IL17A*, *RORC*, *STAT3*, *CCR6*, *IL10*, *IL2RA*, *FOXP3*, *CCR7*, *TGFB1*, *CXCR5*) which revealed 11 unique clusters (**Fig. 2A**). The distribution of cells across the 11 clusters were similar between pre- and post-booster timepoints for both cohorts (**Fig. S2B**). The majority of Spike-specific CD4+ T cells in vaccine- and infection-primed subjects were found in cluster 0 which strongly expressed *TCF7*, *CD27*, and *SELL*, suggesting a central memory state (**Fig 2B**). The next two most abundant clusters, clusters 1 and 2 which had strong expression of *TNF* and *IFNG*, revealed subtle differences across the two cohorts. Following booster vaccination, more infection-primed Spike-specific CD4+ T cells were found in cluster 1 than in other clusters ($P = 0.003$, two-way

ANOVA, **Fig 2B-C, S2C**) which differentially expressed *GZMB*, *CCL3, CCL4* as well as other cytotoxic genes such as *PRF1*, *GZMH*, *NKG7*. These data demonstrated that infection-primed Spike-specific CD4+ T cells have greater expression of cytotoxic genes compared to vaccine-primed Spike-specific CD4+ T cells.

Polyfunctionality of CD4+ T cell responses has been identified as a correlate of protection in other settings (*10*). To assess whether polyfunctionality was similar between the two cohorts, we evaluated transcripts for *IFNG*, *IL2*, *TNF*, and *GZMB* before and after booster vaccination. Overall, the two cohorts were similar in polyfunctionality, and booster immunization did not dramatically change the polyfunctionality of Spike-specific CD4+ T cells (**Fig. 2D, S2D**). However, subtle differences were observed in Spike-specific CD4+ T cells between vaccine- and infection-primed participants. For example, Infection-primed patients had higher frequency of *IFNG+GZMB+* cells than their vaccine-primed counterparts (P = 0.008, Wilcoxon test). Together, these data demonstrate largely similar Th subset distribution and polyfunctionality between the two cohorts that were mostly unaffected by booster vaccination, although there was a slightly greater expression of cytotoxic genes in Spike-specific CD4+ T cells from infection-primed individuals.

Different transcriptional profile among vaccine- and infection-primed memory CD4+ T cells

Given the subtle differences observed between the cohorts using a restricted analysis (Figure 2), we next asked whether the broader transcriptional profile of Spike-specific CD4+ T cells differed by mechanism of priming. To do this, we performed differential expression analysis of the AIM-reactive cluster before and after booster immunization. We found a total of 69 and 220 differentially expressed genes (DEGs) in Spike-specific CD4+ T cells between vaccine- and infection-primed subjects at the pre- and post-booster time points, respectively (**Fig. 3A, S3A**). Spike-specific CD4+ T cells from vaccine-primed individuals differentially expressed genes such as *NFKB2* and *REL,* which may reflect NF-kB signaling, whereas Spike-specific CD4+ T cells from infection-primed individuals differentially expressed genes such as *HLAB*, *GZMB*, *IFITM1*, *IFITM3*, and *IFI6*, which are known to be interferon-stimulated genes (ISGs) (*46*). These data revealed a heightened IFN and cytotoxic response in infection-primed Spike-specific CD4+ T cells.

Indeed, gene ontology analysis (*47*) of upregulated genes in Spike-specific CD4+ T cells from the infection-primed cohort at the post-booster time point demonstrated strong enrichment of terms for “interferon (IFN) alpha/beta signaling”, “cytotoxicity”, and “response to IFN gamma” (**Fig. 3B**). To test whether there were differences between cohorts at a pathway level, we evaluated the coordinated expression of genes using gene set enrichment analysis (GSEA) (*48*). GSEA revealed enrichment for IFN Alpha Response and IFN Gamma Response gene sets in Spike-specific CD4+ T cells from infection-primed individuals at the pre-booster time point (**Fig 3C**), consistent with the gene ontology analysis (**Fig. 3B**). Moreover, several other gene sets were differentially enriched, including Inflammatory Response, JAK/STAT3 signaling, and Complement in infection-primed individuals (**Fig S3B-C**). Furthermore, booster immunization did not substantially change gene set enrichment (**Fig. 3D, Fig S3C**). These findings suggest that Spike-specific CD4+ T cells are differentially imprinted at the time of priming and that the transcriptional profile during reactivation changes minimally following subsequent exposure by mRNA vaccination.

We next evaluated the coordinated gene expression of the vaccine-primed Spike-specific CD4+ T cells. Gene ontology analysis of DEGs in vaccine-primed Spike-specific CD4+ T cells (**Fig 3A, S3A**) revealed terms for “induction of NF-kappa B signaling” and “cell population proliferation” (**Fig 4A**). GSEA demonstrated strong enrichment for the NF-kB signaling gene set in the vaccine-primed participants at the post-booster time point (**Fig S3D**). Moreover, there was enrichment for Mitotic Spindle and G2M Checkpoint signatures in the

vaccine-primed Spike-specific CD4+ T cells at the pre- and post-booster time points (**Fig 4B, S3C-D**). These data suggested more robust expression of genes related to proliferation in the vaccine-primed Spike-specific CD4+ T cells and provoked the hypothesis that vaccine-primed Spike-specific CD4+ T cells may proliferate more robustly in response to stimulation by Spike peptides, compared to infection-primed Spike-specific CD4+ T cells. To test this hypothesis, we evaluated whether mRNA immunization induced similar frequencies of Spike-specific CD4+ T cells. From our prior study of CD4+ T cell responses in infection-primed and vaccine-primed adults (*12*), we evaluated the AIM-reactive population one month post second mRNA vaccination. Indeed, Spike-specific CD4+ T cell frequencies were 2-fold higher in the vaccine-primed participants than infection-primed participants (P = 0.05, Wilcoxon test, **Fig 4C-D**). These data support the notion that imprinting at the time of memory CD4+ T cell priming has durable effects on subsequent cellular activation and may affect proliferative potential.

Breakthrough infection alters the transcriptional profile of Spike-specific CD4+ T cells

Upon *in vitro* re-activation, a number of transcriptional differences between Spike-specific memory CD4+ T cells from infection-primed and vaccine-primed individuals was observed (**Fig. 3**). Of note, infection-primed individuals had all received three doses of mRNA vaccination post-booster, which suggested that imprinting due to initial SARS-CoV-2 viral infection was sustained through repeated mRNA vaccinations. This raised the question of whether vaccine-primed Spike-specific CD4+ T cells would transcriptionally resemble infection-primed Spike-specific CD4+ T cells following breakthrough infection with SARS-CoV-2.

To assess this, we examined Spike-specific CD4+ T cell responses one month after breakthrough infection with SARS-CoV-2, which had occurred in five of the seven vaccine-primed participants. The median time between booster immunization and PCR-confirmed breakthrough infection was 5 months (Table S1), and all breakthrough infections were mild and did not require hospitalization. To test whether vaccine-primed Spike-specific CD4+ T cells would shift to more closely resemble infection-primed state, we compared transcriptional profiles between the post-breakthrough and post-booster states, as these time points occurred one month after re-exposure to Spike. Differential gene expression analysis identified 166 genes differentially expressed between infection-primed and post-breakthrough Spike-specific CD4+ T cells (**Fig 5A**). Despite breakthrough infection, genes such as *DDIT4* continued to be differentially expressed by the vaccine-primed, post-breakthrough Spike-specific CD4+ T cells whereas genes such as *HLA-B*, *HLA-A*, and *GZMB* were differentially expressed by the infection-primed, post-booster Spike-specific CD4+ T cells (**Fig. S4A)**. These data suggest that breakthrough SARS-CoV-2 infection did not result in a complete shift to an infection-primed transcriptional profile.

We next considered whether post-breakthrough Spike-specific CD4+ T cells were transcriptionally distinct from post-booster vaccine-primed Spike-specific CD4+ T cells. To do this, we performed differential expression analysis and found 19 genes differentially expressed (**Fig 5A, S4B**), which included genes such as *IFITM1* and *IFI6* which are ISGs and were previously seen in the infection-primed Spike-specific CD4 T cells (**Fig. 3A**). Together, these results suggested breakthrough infection altered the transcriptional profile of Spike-specific CD4+ T cells, however only a small number of genes were differentially expressed.

Next, we considered whether there were differences induced in Spike-specific CD4+ T cells by breakthrough infection by pathway analysis. Consistent with prior observations (Fig. 3D), GSEA demonstrated that Interferon Alpha and Interferon Gamma Response were enriched in the infection-primed Spike-specific CD4+ T cells relative to the post-breakthrough vaccine-primed Spike-specific CD4+ T cells (**Fig 5B**), which suggested persistence of imprinting at time of initial priming. In contrast, GSEA comparison of post-booster vaccine-primed and post-breakthrough vaccine-primed conditions showed enrichment for Interferon Alpha Response, Mitotic Spindle, and TNF/NF-kB signaling in post-booster vaccine-primed Spike-specific CD4+ T

cells (**Fig 5C**), whereas there was enrichment for Myc targets in post-breakthrough Spike-specific CD4+ T cells.

To assess whether there was an enrichment in cytotoxic gene profiles following breakthrough infection as was seen with the infection-primed setting in **Fig. 2C**, we evaluated Spike-specific CD4+ T cells using clustering based on a restricted gene list. We observed no statistically significant expansion in Cluster 1, which had the cytotoxic gene signature, compared to post-booster vaccine-primed Spike-specific CD4+ T cells (**Fig S4C-D**). Thus, there was no enrichment for a cytotoxic gene signature following breakthrough infection relative to post-booster.

Lastly, principal component analysis (PCA) of the samples in pseudo bulk revealed strong separation between vaccine- and infection-primed cells driven by PC2, and post-breakthrough responses clustered with vaccine-primed samples (**Fig 5D**). Together, these data demonstrate that breakthrough SARS-CoV-2 infection in individuals resulted in subtle alterations in the transcriptional landscape of vaccine-primed Spike-specific CD4+ T cells but did not result in complete conversion to the transcriptional profile of infection-primed Spike-specific CD4+ T cells. These data support a model where memory CD4+ T cells retain a durable transcriptional imprint of the inflammatory context in which they were primed, changing minimally due to vaccination or subsequent viral infection.

DISCUSSION

Memory CD4+ T cell responses are an important feature of protective immunity, yet differentiation of high quality memory CD4+ T cells responses is not well understood. Using the AIM assay, we profiled the transcriptional landscape of Spike-specific CD4+ T cells among participants whose first exposure to Spike protein was via mRNA vaccination or by SARS-CoV-2 infection. First, we found a slight bias towards cytotoxic gene expression in Spike-specific CD4+ T cells from infection-primed individuals, based on expression of *GZMB*, *PRF1*, and chemokines such as *CCL3*, *CCL4*, and *CCL5*. Second, we uncovered a persistent signature of ISG expression in infection-primed Spike-specific CD4+ T cells that did not change following booster immunization. In contrast, there was enrichment of genes for several pathways including TNF/NFkB signaling pathway and Mitotic Spindle in the vaccine-primed Spike-specific CD4+ T cells that also did not change following booster immunization. Third, breakthrough infection of vaccine-primed individuals by SARS-CoV-2 did not dramatically alter the transcriptional profile of Spike-specific CD4+ T cells, although they did differentially express a small number of ISGs. These observations suggest that the inflammatory context at the time of CD4+ T cell priming may be durable and has implications for vaccination strategies that follow infection.

We observed a persistent signature of ISGs in infection-primed Spike-specific CD4+ T cells, suggesting that priming during acute infection can leave a durable imprint on antigen-specific CD4+ T cells, perhaps due to IFN signaling during acute viral infection. Indeed, we observed enrichment of Hallmark IFN Gamma and IFN Alpha Response gene sets in infection-primed memory CD4+ T cells compared to vaccine-primed CD4+ T cells. Prior studies of SARS-CoV-2 infection reported that IFN signaling plays a central role in COVID-19 (*49*, *50*). Recent studies have shown that through type I IFN signaling, plasmacytoid dendritic cells or neighboring SARS-CoV-2-infected cells cue macrophages to induce a cytokine storm, further augmenting IFN signaling and resulting in inflammation (*51*, *52*). Type I IFN signaling is important in T cell differentiation and proliferation, yet it is largely unknown what role high levels of type I IFN signaling play in determining antigen-specific memory T cell fates (*53*, *54*). Our results suggest that high levels of IFN signaling at the time of priming results in memory CD4+ T cells with an impaired proliferative capacity, consistent with other observations (*53*, *55*). Future

studies are needed to parse out the long-term consequences of IFN signaling, and the individual contributions of interferons, on the development and recall responses of memory CD4+ T cells.

Although mRNA vaccines also induce inflammation following administration, the level of inflammatory signaling is likely lower (*29*). We observed subtle differences in the cytokine production and polyfunctional measurements of Spike-specific CD4+ T cells in vaccine- and infection-primed participants. Additionally, we observed a possible proliferative advantage, demonstrated by enrichment of Mitotic Spindle and G2M Checkpoint gene sets, in vaccine-primed Spike-specific CD4+ T cells. These results are concordant with other studies showing that Spike-specific CD4+ T cell responses to second and third doses of mRNA vaccines against SARS-CoV-2 differ in frequency and phenotype among individuals initially exposed to Spike protein through infection versus vaccination (*12*, *13*, *15*, *40*). Furthermore, genes associated with NF-kappa B signaling were enriched in vaccine-primed Spike-specific memory T cells which suggest increased T cell activation. Aberrant NF-kappa B signaling has been associated with inflammatory diseases and responses which could explain why infection-primed memory pools exhibit decreased expression of this pathway (*59*). Since NF-kappa B family transcripts are controlled at the chromatin level, probing the underlying chromatin accessibility of Spike-specific memory CD4+ T cells will be needed to assess the regulation of the qualitative differences that we observed in vaccine-primed memory CD4+ T cells.

Breakthrough SARS-CoV-2 infection after three doses of mRNA vaccine resulted in transcriptional differences when compared to vaccine derived responses to Spike protein. Our data support that breakthrough infection likely induced de novo CD4+ T cell responses, introducing a hybrid immune memory pool. All five breakthrough infections occurred during the Omicron wave, so pre-existing Spike-specific CD4+ T cells must have been cross-reactive to mount a protective response. Many have reported that CD4+ T cell epitopes are largely conserved across wild-type Spike (Wuhan strain) and Omicron, and that this cross-reactivity is maintained up to 6 months post SARS-CoV-2 mRNA vaccination (*18–20*, *60*). Others have reported a profound drop in T cell responses against Omicron compared to Wuhan Spike protein (*61*). Vaccination improves breakthrough infection outcomes (*62*), yet the exact number of vaccine-derived CD4+ T cells that participate in a protective response remains unknown. Comprehensive T cell repertoire studies are needed to examine whether memory CD4+ T cells primed by vaccination respond to Omicron infection in this cohort of patients.

Nonetheless, a robust IFN response remained upregulated in infection-primed Spike-specific CD4+ T cells compared to post-breakthrough samples. These results suggested that initial immune priming by vaccine attenuated the capacity of infection to reprogram memory pools. However, patients infected with the Omicron variant experience milder disease than those infected with earlier variants, consistent with findings in animal models confirming lower pathogenicity of the Omicron variant (*63*). Despite overall mild SARS-CoV-2 infections in our infection-primed cohort, it is likely that breakthrough infection with the Omicron variant did not induce a similar inflammatory immune milieu during acute disease. Direct comparison of inflammatory cytokines and IFN levels in patient sera during acute infection with Wuhan or Omicron SARS-CoV-2 is needed to address this question.

Although we found robust transcriptional differences between infection- and vaccine-primed memory CD4+ T cells, several caveats should be considered. First, we focused on CD4+ T cells due to their role in long-term immunity (*3*), but we did not examine the effects of inflammation on priming of non-Spike-specific CD4+ or memory CD8+ T cells or B cells, all of which play important roles in immunity to SARS-CoV-2. Second, we assessed circulating measured memory CD4+ T cell responses, yet we know that tissue resident memory CD4+ T cells are crucial in other settings protective immunity (*64*). Exploring memory CD4+ T cells in human tissue, particularly secondary lymphoid organs, would clarify whether similar transcriptional changes accrue during priming of other non-circulating cells. Third, better understanding is needed of the relevance of the transcriptional differences observed here for

immunity, given the multifaceted nature of the immune response to re-exposure to SARS-CoV-2. Although our data suggest reduced proliferative ability of infection-primed Spike-specific CD4+ T cells, it is unclear whether this manifests in differences in susceptibility or worse outcomes with re-infection. Animal models with SARS-CoV-2 challenge may help to determine the differences in immunity associated with memory CD4+ T cells that were infection- or infection-primed. Lastly, further studies will be needed to understand the extent to which our findings generalize across age, sex, racial, and ethnic groups.

Together, these data suggest that the imprint of inflammation during Spike-specific memory CD4+ T cell formation resulted in persistent transcriptional alterations which were sustained despite mRNA vaccination, relative to memory CD4+ T cells primed during vaccination, and that SARS-CoV-2 breakthrough infection was not associated with dramatic alterations to the transcriptional profile of vaccine-primed memory CD4+ T cells. Our results provide insight into factors that harm quality and functionality of memory CD4+ T cell responses, which will inform optimization of CD4+ T cell responses and future vaccine design.

MATERIALS AND METHODS

Study design

We examined T cell responses in adults receiving a third dose of BNT162b2 vaccine at the time points indicated in **Fig.1A.** Following written informed consent, peripheral blood was drawn by standard phlebotomy longitudinally from 14 adults (7 vaccine-primed and 7 infection-primed) in observational studies in accordance with NYU Institutional Review Board protocols (s18-02035 and s18-02037). Participant demographics are summarized in **Tables S1-3**.

Blood samples processing and storage

Blood draws occurred around eight months after second vaccination (“pre-booster”) and approximately one month post third vaccination (“post-booster”), as depicted in **Fig. 1A**. Peripheral blood mononuclear cells (PBMC) were isolated from CPT vacutainers (BD Biosciences) within four hours of the blood draw and cryopreserved in liquid nitrogen.

Activation-induced marker analysis

Cryopreserved PBMCs were thawed and rested overnight at 37°C in RPMI 1640 with L-glutamine (Fisher) containing 10% FBS (Fisher), 2 mM L-glutamine (Fisher) and supplemented with DNase and $MgCl_2$. The next day, cells were stimulated with 15-mer peptide pools encompassing the SARS-CoV-2 Spike protein (S1, S, and S+ PepTivators, Miltenyi). Sterile water was used for the unstimulated controls. After stimulation for 20 hours at 37°C, cells were washed with PBS containing 10 mM EDTA for 5 minutes. Cells underwent Fc blockade with Human TruStain FcX (BioLegend) and NovaBlock (Thermo Fisher Scientific) for 10 min at room temperature, followed by surface staining antibody cocktail at room temperature for 20 min in the dark, followed by resuspension in 1% para-formaldehyde (Electron Microscopy Sciences) prior to acquisition on a five-laser Aurora cytometer (Cytek Biosciences).

Expanded cellular indexing of transcriptomes and epitopes by sequencing

Following overnight stimulation in the AIM assay (above), cells were stained for 30 minutes at room temperature in the dark with antibodies against CD69 and CD137 conjugated to PE-Dazzle 594 and PE, respectively (Biolegend). Stained cells were passed through EasySep Human PE Positive Selection Kit (STEMCELL Technologies) to enrich stimulated cells. Cells were then put on ice and processed for exCITE-seq. Cells were processed for expanded cellular indexing of transcriptomes and epitopes by sequencing (exCITE-seq) using Chromium Next GEM Single Cell 5’ HT Kit v2 (10X Genomics). Cells were stained with hashtag oligos, to allow

multiplexing and doublet detection, and a panel of barcoded antibodies targeting surface epitopes (Biolegend) as previously described (*42*, *65–67*). Cells were pooled and loaded onto Chromium HT Chips and ran on a Chromium controller (10X Genomics). Gene expression, V(D)J, and surface protein expression libraries were made using the 5' Feature Barcode Kit, Chromium Single Cell V(D)J Amplification Kit, and Chromium Next GEM Single Cell 5' Library Kit (10X Genomics) following the protocols recommended by the manufacturer. Libraries were pooled at desired concentrations and sequenced using the NovaSeq 6000. FASTQ files were aligned to the human genome (GRCh38 ensemble), antibody reference barcodes, and demultiplexed using the Cellranger software v7 (10X Genomics).

exCITE-seq data processing

Primary data analysis and statistical analysis were then performed using the R environment (version 4.1.2). Seurat v4.2.0 (*43*) was used to process single cell libraries and integrate all the exCITE-seq modalities. HTOs were demultiplexed using a combination of HTODemux from Seurat and scDblFinder (*68*). RNA was normalized across batches using SCTransform and filtered counts were integrated using integration functions in Seurat. TCR sequences were processed and analyzed using scRepertoire v1.3.5 (*69*). SPICE was used to analyze polyfunctionality (version 6.1) (*70*). GO was performed using Metascape (*47*). Gene set enrichment analysis (GSEA) was performed with 10,000 permutations of pre-ranked gene sets (http://software.broadinstitute.org/gsea/downloads.jsp).

Statistics

Data were assumed to have non-Gaussian distribution, and nonparametric tests were preferentially used throughout using two-tailed tests at α=0.05. Genes were considered differentially expressed at a false discovery rate of 0.05 or less. Prism 9.0 was used to perform statistical analyses. Study schematic was made using BioRender.

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Competing interests

MJM reported potential competing interests: laboratory research and clinical trials contracts with Lilly, Pfizer (exclusive of the current work), and Sanofi for vaccines or MAB vs SARS-CoV-2; contract funding from USG/HHS/BARDA for research specimen characterization and repository; research grant funding from USG/HHS/NIH for SARS-CoV-2 vaccine and MAB clinical trials; personal fees from Meissa Vaccines, Inc. and Pfizer for Scientific Advisory Board service. RSH has received research support from CareDx for SARS-CoV-2 vaccine studies. RSH is a consultant for Bristol-Myers-Squibb.

FIGURE LEGENDS

Figure 1. Spike-specific CD4+ T cells form a distinct cluster in the Activation-Induced Marker assay
(**A**) Study schematic. (**B**) Example flow plots for activation-induced markers (AIM) assay with Spike peptide pool showing differences for CD69+ CD200+ CD4+ in unstimulated and stimulated conditions ($P = 0.03$, Wilcoxon matched-pairs signed rank test). (**C**) UMAP projection of all CD4+ and CD8+ T cells pooled across samples, clustered for gene expression, and split by unstimulated and stimulated conditions. (**D**) Scaled expression of *TNFRSF4* (OX40), *TNFRSF9* (CD137), *TFRC* (CD71), and *LTA* demonstrating localized signal in AIM Reactive clusters. (**E**) Gene ontology analysis for differentially genes expressed at nominal $P < 0.05$ for AIM Reactive CD4+ T cell compared to CD4+ T cell cluster.

Figure 2. Infection-primed Spike-specific CD4+ T cells have a cytotoxic phenotype
A) Spike-specific CD4+ T cells projected onto UMAP and clustered for gene expression of the following 27 parameters; *IFNG*, *TNF*, *IL2*, *IL12A*, *CXCR3*, *CCR5*, *STAT4*, *TBX21*, *RUNX3*, *IL4*, *IL5*, *IL13*, *CXCR4*, *GATA3*, *STAT6*, *CCR4*, *IL21*, *IL17A*, *RORC*, *STAT3*, *CCR6*, *IL10*, *IL2RA*, *FOXP3*, *CCR7*, *TGFB1*, *CXCR5*. Expression of 12 genes visualized to the right. (**B**) Scaled expression of top 3 differentially expressed genes at nominal $P < 0.01$ for each cluster. (**C**) Graphs of vaccine- versus infection- primed Spike-specific CD4+ T cell cluster distribution at pre- and post- booster timepoints (two-way ANOVA). (**D**) Polyfunctionality analysis performed with cytokines shown.

Figure 3. Infection-primed Spike-specific CD4+T cells are enriched for IFN response hallmark gene sets
(**A**)Volcano plot showing 220 differentially expressed genes at nominal $P < 0.05$ post-booster. (**B**) Gene ontology analysis for differentially genes expressed at nominal $P < 0.05$ for infection-primed compared to vaccine-primed Spike-specific CD4+ T cells at post-booster time point. (**C-D**) GSEA for Interferon Gamma and Alpha Responses gene sets for Spike-specific CD4+ T cells pre- and post- booster for vaccine- and infection- primed participants. Positive enrichment scores denote enrichment towards the infection-primed cohort.

Figure 4. Vaccine-primed Spike-specific CD4+ T cells demonstrate high proliferative potential
(**A**) Gene ontology analysis for differentially genes expressed at nominal $P < 0.05$ for vaccine-primed compared to vaccine-primed Spike-specific CD4+ T cells post-booster. (**B**) GSEA results exhibited strong enrichment of Mitotic Spindle and G2M Checkpoint gene sets in vaccine-primed Spike-specific CD4+ T cells at pre- and post- booster time points. Negative enrichment scores denote enrichment towards the vaccine-primed cohort. (**C**) Example flow plots for AIM assay with Spike peptide pool showing differences for CD69+ CD200+ CD4+ in vaccine- and infection- primed cohorts at one month post 2nd vaccine dose. (**D**) Frequency differences of CD69+ CD200+ CD4+ ($P = 0.05$, Wilcoxon test).

Figure 5. Breakthrough infection alters the transcriptional profile of Spike-specific CD4+ T cells
(**A**) Number of differentially expressed genes (DEGs) (nominal $P < 0.05$) at post-booster time points for infection-primed (left) and vaccine-primed (right) Spike-Specific CD4+ T cells compared to post-breakthrough responses. (**B**) GSEA results for Hallmark gene sets enriched at $FDR < 0.05$ where positive enrichment scores denote enrichment towards the post-breakthrough samples and negative enrichment scores signify enrichment towards infection-

primed samples. (**C**) Paired analysis between vaccine-primed post-booster and post-breakthrough Spike-specific CD4+ T cells for enrichment of Hallmark gene sets. Negative enrichment scores signify enrichment towards post-booster responses. (**D**) PCA plots of post-booster infection- (purple), vaccine- (orange) primed, and post-breakthrough (green) Spike-specific CD4+ T cells in pseudo bulk.

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