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Spike-specific T cells are enriched in breastmilk following SARS-CoV-2 mRNA vaccination

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The authors have no competing interests.
Abstract: Human breastmilk is rich in T cells; however, their specificity and function are largely unknown. We compared the phenotype, diversity, and antigen specificity of T cells in the breastmilk and peripheral blood of lactating individuals who received SARS-CoV-2 mRNA vaccination. Relative to blood, breastmilk contained higher frequencies of T effector and central memory populations that expressed mucosal-homing markers. T cell receptor (TCR) sequence overlap was limited between blood and breastmilk. Overabundant breastmilk clones were observed in all individuals, were diverse, and contained CDR3 sequences with known epitope specificity including to SARS-CoV-2 Spike. SARS-CoV-2 Spike-specific TCRs were more frequent in breastmilk compared to blood and expanded in breastmilk following a third mRNA vaccine dose. Our observations indicate that the lactating breast contains a distinct T cell population that can be modulated by maternal vaccination with potential implications for infant passive protection.
1. Introduction:

The breastfed human infant consumes up to 750,000 maternal leukocytes per day, 5-10% of which are T cells, the function of which is poorly understood\(^1\)\(^-\)\(^2\). Breastmilk lymphocytes are most abundant at delivery and decline over the first month post-partum to a steady state that persists for up to two years\(^1\)\(^-\)\(^4\). However, the infant’s exposure to breastmilk cells likely remains substantial throughout breastfeeding due to an increase in volume of breastmilk ingested as the infant grows\(^5\). Breastmilk T cells are phenotypically distinct from peripheral blood T cells, with higher expression of mucosal and effector memory markers\(^6\)\(^,\)\(^7\). Cytomegalovirus (CMV), Epstein-Barr virus (EBV), influenza, and HIV-specific T cell responses have been detected in breastmilk cells (BMC) at higher frequencies than in peripheral blood mononuclear cells (PBMC)\(^7\)\(^-\)\(^11\), and breastmilk T cells may expand in the setting of maternal or infant infection\(^2\)\(^,\)\(^12\)\(^,\)\(^13\).

The infant stomach pH\(^14\)\(^,\)\(^15\) and intestinal permeability\(^14\)\(^,\)\(^16\)\(^,\)\(^17\) are also highest in the first few weeks of life, and evidence from animal models demonstrates that breastmilk T cells can survive the offspring gastrointestinal tract and traffic into the mesenteric lymph nodes, liver, spleen, and lung as a form of maternal microchimerism\(^18\)\(^-\)\(^20\). In mice, breastmilk-derived maternal helminth-specific T cells were protective in the offspring upon challenge with the same helminth\(^18\), and in lambs, breastmilk-derived tetanus-specific T cells enhanced the response to tetanus vaccination in the offspring\(^21\). Human breastmilk maternal microchimerism has not been conclusively demonstrated, although we recently found in a cohort of infants that maternal microchimerism increased up to three months of age and was positively associated with breastfeeding\(^22\). These data emphasize the potential for breastmilk-acquired maternal T cells to become resident in the infant and provide an underrecognized form of passive protection.

The full repertoire of breastmilk T cells has not been described, however, and data on maternal vaccine-specific T cells in breastmilk are limited\(^23\). In the setting of the ongoing pandemic,
pregnant and lactating individuals are now widely receiving SARS-CoV-2 (SARS2) vaccines including the Spike protein mRNA-based vaccines mRNA1273 (Moderna) and BNT162b2 (Pfizer-BioNTech). SARS2 mRNA vaccines generate a robust T cell response in peripheral blood and induce the expansion of tissue resident T cell populations in the respiratory mucosa. A recent study reported Spike-reactive T cells in breastmilk of mRNA-vaccinated people, although the diversity of their TCR usage, relative clonality in breastmilk versus blood, and capacity to directly bind antigen were not described. To understand the breadth of maternal T cells consumed by the infant, we characterized the phenotype and diversity of paired breastmilk and peripheral T cells. We further investigated the hypothesis that Spike-specific T cells are present in the breastmilk of SARS2 mRNA-vaccinated individuals and expand upon antigen re-encounter.

2. Results:

2.1 Breastmilk is enriched for mucosal memory T cells

We collected paired blood and breastmilk from lactating individuals who had received two doses of BNT162b2 or mRNA1273, had no history of SARS2 infection, and were nucleocapsid protein seronegative (Fig. S1; Table 1). We characterized cell phenotype by flow cytometry in paired samples with at least 1,000,000 total BMC recovered and at least 100 cells in both the CD4+ and CD8+ T cell populations (n=17). Breastmilk contained a low but detectible frequency of T cells, and the frequency of CD4+ T cells was similar in BMC and PBMC, whereas the frequency of CD8+ T cells was somewhat lower in BMC than PBMC (24% vs. 30%, p=0.03; Fig. S2). Next, we identified naïve and memory T cell subsets in BMC and PBMC through surface expression of CD45RO and CCR7. We found a significant enrichment of CD45RO+/CCR7- effector memory (T_{EM}) and CD45RO+/CCR7+ central memory (T_{CM}) CD4+ T cell populations in BMC vs. PBMC (Fig. 1A, B). Within the CD8+ population, there was also a higher frequency of T_{EM} but
not \( T_{CM} \) in BMC relative to PBMC (Fig. 1A, B). These data emphasize that breastmilk is highly enriched for memory T cell populations relative to peripheral blood.

We next investigated the expression of mucosal-homing markers CCR9 and CD103 on T cells within BMC and PBMC. The CD4+ population within BMC versus PBMC had a higher frequency of CCR9+ and CD103+ cells, and a higher frequency of double positive CCR9+/CD103+ cells (Fig. 1C, D). Similarly, the CD8+ population within BMC versus PBMC had a higher frequency of CCR9+ and CD103+ cells, as well as a higher frequency of CCR9+/CD103+ cells (Fig. 1C, D). These data emphasize that the T cells in breastmilk express high levels of mucosal-homing markers.

### 2.2 Restricted T cell receptor repertoire overlap between peripheral blood and breastmilk

Because of the high frequency of memory populations, we next investigated the composition and diversity of the T cell receptor (TCR) repertoire in BMC. BMC were enriched for hematopoietic lineage cells (CD45+) using flow cytometry cell sorting, and sorted BMC for which >1,000 DNA genomic equivalents were recovered (n=16) underwent bulk TCR beta chain (TCR\( \beta \)) sequencing. Among BMC samples for which >1,000 TCR templates were recovered, we compared the degree of TCR overlap with matched peripheral blood (n=11) and surprisingly observed relatively low sequence similarity in all pairs, as measured by the Morisita and power geometric indices\(^29\) (Fig. 2, Table S1), indicating that BMC and PBMC have distinct TCR repertoires at the sequence level. In contrast, we observed a high degree of overlap in the PBMC TCR repertoire from one individual at two time points (9 and 17 days post-2nd vaccine dose) utilized as a positive control (Fig. 2, Table S1). Neither the Morisita index nor the power geometric index between paired BMC and PBMC was related to the number of productive templates in the BMC (\( R^2 = 0.4, p = 0.3; R^2 = 0.3, p = 0.4 \) respectively), suggesting that the low sequence overlap between BMC and PBMC was independent of sampling depth of the BMC. Within each participant, we compared the frequency of clonotypes across the two compartments.
using the immunoSEQ® Differential Abundance tool\textsuperscript{30}. In all individuals, there were select clonotypes that were statistically significantly overabundant in BMC relative to PBMC (Fig. 2; Table S1). In contrast, the Simpson Clonality and maximum clone frequency—two metrics of absolute clonality rather than relative clonality—did not differ in BMC and PBMC (Table S1), indicating that the two compartments were similarly diverse in each participant. These data indicate that while the TCR repertoire of BMC is as diverse as that of the PBMC, it is composed of a distinct TCR repertoire at the sequence level with select overabundant clonotypes relative to PBMC.

2.3 Characterization of breastmilk overabundant T cell clonotypes

We next explored the diversity of the overabundant T cell clonotypes in breastmilk. We analyzed each participant’s BMC T cell repertoire using a TCR distance metric, tcrdist\textsuperscript{31, 32}, which clusters TCRβs based on structural and functional similarities of amino acids within complementarity-determining regions (CDR). In all participants, overabundant clones were broadly distributed across the BMC TCRβ repertoire (Fig. 3A, Fig. S3). We additionally evaluated how overabundant clones clustered across all individuals. Most clones were unique to a single individual (i.e., they were “private”), and the clonotypes did not segregate by individual (Fig. 3B), reflecting the diversity of each participant’s overabundant clonotypes.

To determine potential antigen specificity, we compared CDR3 amino acid sequences of the overabundant breastmilk clones from all individuals to TCRβ sequence databases populated by validated epitope-specific TCRs. We identified five direct matches from two participants with CDR3 amino acid sequences and identical V gene usage, two of which were reported to bind SARS2 Spike epitopes (Fig. 3B, S4 Table S2). Notably, the two Spike-specific clones had also previously been reported to bind influenza M1\textsuperscript{33, 34}. Other direct matches included epitopes derived from influenza, \textit{Mycobacterium tuberculosis} lysate, and EBV (Fig. 3B, S4 Table S2). Additional clones with identical CDR3 sequences but non-identical V gene usage were reported.
to bind epitopes from SARS2, EBV, and M. tuberculosis (Fig. 3B, S4 Table S2). CDR3 sequences with previously published specificity clustered together, irrespective of individual (Fig 3B). Finally, we used the IEDB TCRMatch Too to predict TCR-epitope specificity based on sequence similarity to published TCR sequences, which identified TCR clones with potential specificity to a variety of viral epitopes (Table S3). These data indicate that overabundant breastmilk T cell clones are diverse and respond to a range of pathogen-specific epitopes, although the specificity of the vast majority of these clones is unknown.

2.4 SARS2 Spike-restricted TCRβs are present in breastmilk T cells

Because all participants had received SARS2 Spike mRNA vaccination, we next investigated the presence of Spike-specific clones in BMC T cells more broadly. We utilized the immunoSEQ® COVID Search Tool to identify candidate Spike-specific TCRβ in all sequenced BMC and PBMC samples (n=30). All PBMC (n=14) contained candidate Spike-specific TCRβ, though their predicted epitope specificity was distributed across the entire Spike protein with low frequency, suggesting that some of the TCRβ may represent clones in the naïve repertoire rather than expanded vaccine-specific populations (Fig. 4A). Thirteen of the 16 BMC samples contained TCRβ predicted to be Spike-specific. In the pairs where both BMC and PBMC were sampled and at least one clone in each compartment was predicted to be SARS2-specific (n=13 pairs), overall Spike-specific TCRβs were nearly 2-fold enriched in BMC versus PBMC (Fig. 4B). However, across participants there was heterogeneity in response with most individuals demonstrating enrichment (n=7), some with similar frequency (n=4), and two with lower frequency of Spike-specific T cells in the BMC relative to PBMC. The frequency of Spike-specific TCRβs were not strongly correlated with Spike-specific IgG or IgA in breastmilk or blood (Fig. S5).

To further validate the presence of Spike-specific T cells in breastmilk, we cross-referenced each participant’s BMC and PBMC TCRβ CDR3 amino acid sequences and V gene usage
against publicly available TCRβ datasets from Spike-epitope-loaded tetramer or multimer experiments\textsuperscript{36-40}. We identified high quality CDR3 sequence matches in half of all breastmilk samples, with five samples also containing hits with identical V gene usage (Table S4). All PBMC contained clones with identical CDR3 sequence and V gene usage to those published previously (Table S5). Consistent with prior studies of PBMC\textsuperscript{36-38, 41}, sequences specific to the Spike peptide YLQPRTFLL were prominent in the breastmilk and blood of individuals known to be HLA-A*02:01 positive. In addition, clones specific to the A*01:01-restricted Spike peptide LTDEMIAQY and the B*15:01-restricted Spike peptide NQKLIANQF were present in the breastmilk and blood of HLA concordant participants (Table S4, Table S5), suggesting shared epitope specificity following vaccination. Finally, we utilized the list of Spike-specific TCRβs in conjunction with the tcrdist3 algorithm\textsuperscript{31, 32} to identify novel potential Spike-specific T cell clones in the BMC (Table S6). Together, these observations demonstrate the presence of Spike-specific T cells in breastmilk following mRNA vaccination.

2.5 Spike-specific T cells in breastmilk expand after SARS2 mRNA vaccine

To understand whether Spike-specific clones in breastmilk were responsive to antigen re-exposure, we took advantage of a natural restimulation experiment in which participants (n=8) donated breastmilk pre- and approximately 1-week post-3rd vaccine dose for in-depth phenotyping and staining with HLA class I tetramers loaded with immunodominant Spike epitopes, specifically HLA-A*02_YLQ, HLA-A*01_LDT, and HLA-A*24_NYN (Table 2, Fig. 5A, B, C)\textsuperscript{36-38, 41}. The proportion of Spike tetramer-positive CD8+ T cells of all CD8+ T cells significantly increased in breastmilk between the pre- and post-3\textsuperscript{rd} dose samples (0.8% to 2.8%, p=0.03), whereas tetramer-positive CD8+ T cells in PBMC showed minimal response (0.04% to 0.04%, p=0.9) (Fig. 5D). The expression of activation markers on tetramer+ CD8+ T cells was consistently high but did not vary between pre- and post-3\textsuperscript{rd} dose samples including CD69 (51% vs. 50%, p=0.9), CD137 (61% vs. 42%, p=0.3), and CCR5 (89% vs. 84%, p=0.4). The
proportion of tetramer+ CD8+ T cells expressing CCR9 and CD103 was similar to our earlier bulk analysis and did not vary between pre- and post-3rd dose (CCR9: 50% vs. 31%, p=0.3; CD103: 18% vs. 7%, p=0.4; CCR9/CD103: 1.4% vs. 3.4%, p=0.3). A proportion of tetramer+ cells pre and post-3rd dose also expressed the lymphocyte integrin α4β7, which regulates T cell migration to the intestine (21% vs. 16%, p=0.7). These data demonstrate that SARS2 Spike-specific cells in breastmilk respond in vivo upon antigen restimulation.

3. Discussion:

We present a comprehensive comparison of the T cells present in breastmilk relative to peripheral blood, considering phenotype, diversity, and antigen specificity. We find that T cells in breastmilk are nearly uniformly memory populations and have high expression of mucosal-homing markers. Their TCRβ repertoire is diverse, yet distinct from paired PBMC, and with select overabundant clones. Further, we identify SARS2 Spike-specific clones in the breastmilk of mRNA vaccinated individuals, emphasizing that vaccine-specific T cells are present at mucosal sites such as the breast, with important implications for both maternal and infant health.

Breastmilk T cells were enriched for effector memory populations, indicating that breastmilk T cells may be poised to rapidly respond following antigen re-encounter. Further, breastmilk T cells displayed high levels of mucosal-homing markers, consistent with earlier reports in individuals living with or without HIV. These data suggest that breastmilk T cells may be derived from a tissue-resident population in the breast, rather than vessel microtrauma and contamination by peripheral blood. The high expression of both CCR9 and CD103 by breastmilk T cells also supports the notion of an entero-mammary axis. Future studies should investigate whether breastmilk T cells traffic to the infant respiratory and gastrointestinal tracts when consumed and enhance cellular immunity to pathogens, as has been shown in helminthic
infection in mice\textsuperscript{18}, or amplify infant response to homologous vaccination, as has been shown with tetanus vaccination in lambs\textsuperscript{21}.

The TCR\(\beta\) repertoire in BMC was diverse and had uniquely expanded clonotypes relative to paired PBMC. To date, studies have focused on T cell responses to specific pathogens\textsuperscript{7-12}, rather than capturing the full diversity of the compartment. The low degree of TCR\(\beta\) repertoire overlap between the BMC and PBMC may reflect a difference in the distribution of naïve versus antigen-experienced T cells. However, the absolute clonality was similar and there was evidence of high frequency clonotypes, likely memory cells, that were differentially abundant, suggesting distinct T cell responses in the two compartments independent of the naïve population. The observation of overabundant clones in the BMC is consistent with prior reports of an enrichment of virus-specific responses in breastmilk relative to PBMC\textsuperscript{7-10}, although in our paired TCR\(\beta\) analysis only one individual had CMV-specific TCR\(\beta\) and none reported HIV infection, emphasizing that this breast-specific enrichment is not restricted to clones specific to these viruses. In addition, within each individual, overabundant clones were diverse.

To identify antigen specificity of BMC T cells, we used a combination of prior published TCR\(\beta\) specificities and predictive algorithms. Although the specificity of the vast majority of BMC overabundant clones remained elusive, we were able to detect SARS2 Spike-specific clones with high confidence in most individuals. Consistent with previous observations of convergent epitope specificity across HLA-concordant individuals following SARS2 mRNA vaccination\textsuperscript{36}, we found identical Spike-specific CDR3 sequences in BMC of several individuals. This observation, along with the approval of 3\textsuperscript{rd} mRNA vaccine doses, provided a unique opportunity to observe the dynamics of Spike-specific CD8\textsuperscript{+} T cells in the breastmilk after antigen re-encounter using well-validated HLA Class I tetramers loaded with Spike epitopes\textsuperscript{36-38, 41}. In contrast to blood-derived CD8\textsuperscript{+} T cells, the frequency of tetramer-positive CD8\textsuperscript{+} T cells in breastmilk significantly increased after the 3\textsuperscript{rd} SARS2 mRNA vaccine dose, suggesting that T cells in the lactating
breast may be particularly poised to respond to maternal mRNA vaccination. These expanded populations may reflect increased trafficking from the periphery or local antigen re-exposure, consistent with recent reports of detectable SARS2 mRNA vaccine in breastmilk\(^4\). Tetramer+ CD8+ T cells in breastmilk had high expression of activation and mucosal homing markers, emphasizing their functional potential\(^2\).

In addition to the potential benefit provided to the infant, the recognition of the breast as a site of mucosal immunity distinct from peripheral immunity has important implications for the study of vaccine responses. Prior work on the response to SARS2 mRNA vaccines has primarily focused on both CD4+ and CD8+ T cell responses present in peripheral blood\(^2\),\(^5\) with limited study of T cell responses at mucosal sites. One recent study found that SARS2 mRNA vaccination induces the expansion of resident CD8+ T cells in the upper respiratory tract\(^2\), suggesting that SARS2 mRNA vaccines promote robust T cell responses in the mucosa. In addition, a recent study reported an increase in Spike peptide-reactive T cells in breastmilk following mRNA vaccination\(^2\), consistent with our observations. Notably, lower respiratory tract responses are difficult to access in human populations, whereas the collection of breastmilk in lactating individuals is non-invasive. While the association between respiratory tract and breastmilk T cell responses merits further investigation, measuring immune responses in breastmilk may allow for characterization of mucosal immunity more broadly following vaccination.

Our study had several limitations. BMC T cells were low frequency, and although we designed our experimental approach to maximize the information obtained from each sample, the sampling depth between BMC and PBMC differed. However, we took advantage of several computational solutions to overcome this challenge, including repertoire analysis utilizing tools less susceptible to bias with differences in sampling depth and down-sampling for clonality analysis. We were further limited to bulk TCR\(\beta\) sequencing of combined CD4+ and CD8+
populations, as the number of each sub-population was too small to meet technical requirements to analyze separately. Future work should consider the use of single cell RNAseq to investigate the two populations separately. The number of T cells recovered from each breastmilk limited our ability to conduct functional analyses. However, we took advantage of individuals who received a 3rd dose of SARS2 mRNA vaccine to demonstrate expansion of Spike-specific T cells with tetramer staining. We utilized previously frozen and thawed BMC and PBMC, which may have biased recovery of cell populations, particularly in the breastmilk. Future studies should consider detailed analysis of fresh BMC if possible. Finally, initiation and support for breastfeeding varies by demographic groups including socioeconomic status, and future work should address a more diverse population of lactating people.

We demonstrate that breastmilk T cells are highly diverse and enriched for mucosal memory populations, emphasizing that the lactating breast represents a key site of mucosal immunity that warrants additional study. Further, we identify SARS2 Spike-specific T cells in mRNA vaccinated individuals, a critical demonstration of vaccine-specific T cells in breastmilk. This observation may have important implications for both the study of vaccine-induced T cell responses in the vaccinated individual as well as for infant passive protection.

4. Methods:

4.1 Cohort

The initial cohort was comprised of lactating individuals (n = 26, Fig. S1) who were recruited as part of the Center for Global Infectious Disease Research Biorespository, approved by the Seattle Children’s Institutional Review Board (IRB) (STUDY00002048). Participants self-reported as healthy, not pregnant, and weighing > 110 pounds. Demographic data was collected including age, history of SARS or other serious infections, SARS2 vaccination (brand and dates of doses), sex of infant(s), and parity. All participants reported receipt of two doses of
a SARS2 Spike mRNA vaccine. No participants reported SARS2 infection, and all were negative for anti-nucleocapsid IgG (Abbott Architect SARS-CoV-2 IgG assay). Breastmilk was pumped by the participants using the method of their choice and transferred fresh to the study team. Blood was obtained from 23 individuals. 20 individuals were included for experimental analysis based on at least $10^6$ BMC recovered (Fig S1). For the pre- and post-3rd mRNA vaccine dose studies, blood and breastmilk were collected before and ~1 week after 3rd mRNA vaccine dose (n=7 PBMC and BMC, n=1 BMC only). Collection interval was based on T cell responses observed in peripheral blood\textsuperscript{24, 25}. Five participants from the original cohort returned to provide additional specimens, and samples from 3 additional individuals were added from the Maternal Immunizations in Low and High-Risk Pregnancies, approved by the University of Washington IRB (STUDY00008491). Participants from both studies provided written informed consent.

4.2 Isolation of PBMCs from whole blood

Whole blood was collected in EDTA Vacutainer tubes (BD). Within 4 hours of collection, tubes were centrifuged at 400 x g for 10 minutes. The plasma fraction was removed, centrifuged at 800 x g for 15 minutes, aliquotted into cryovials, and stored at -80°C. The remaining blood was diluted in sterile phosphate buffered saline (PBS), layered onto Lymphocyte Separation Medium (Corning), and centrifuged at 800 x g for 20 minutes at room temperature with no brake. The resulting buffy coat layer was removed and washed two to three times in PBS. Cells were counted using a C-Chip hemocytometer (INCYTO), resuspended in freezing medium (50% fetal bovine serum [FBS], 40% RPMI with L-glutamine, 10% DMSO (Millipore Sigma)) at 5 to 10 million cells/mL, and aliquotted into cryovials for storage in liquid nitrogen.

4.3 Isolation of BMC
Milk was centrifuged at 400 x g for 15 minutes at 4°C, and the aqueous fraction was aliquoted into cryovials and stored at -80°C. The cell pellet was washed three times in 40 mL sterile PBS with 2% FBS. As above, cells were counted using a C-Chip hemocytometer (INCYTO), resuspended in freezing medium at 1 to 3 million cells/mL, aliquoted into cryovials, placed in a 1°C cryogenic freezing container at -80°C overnight, and then transferred to liquid nitrogen.

4.4 Phenotyping and cell sorting by flow cytometry

PBMC or BMC were thawed at 37°C and added to pre-warmed thaw medium (RPMI with L-glutamine, 20% FBS) and centrifuged at 400 x g for 5 minutes. Cell pellets were resuspended in 5 mL complete medium (RPMI with L-glutamine, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin), counted, and assessed for viability. Cells were stained with 100 µL of master mix containing fluorophore-conjugated antibodies and viability dye (Table S7; Panel 1) in Brilliant Stain Buffer (BD) for 30 minutes at room temperature. Stained cells were washed and resuspended FACS buffer. Cells were run on a FACSMelody Cell Sorter (BD) or a FACSAria II Cell Sorter (BD), and for BMC CD45+ cells were collected. Single stained CompBeads (ThermoFisher) were used as compensation controls, and unstained or fluorescence-minus-one stained cells were used to set fluorescence gates. Data were analyzed on FlowJo version 10 (BD). CD45RO+/CCR7- T cells were designated as effector memory (T_{EM}), CD45RO+/CCR7+ T cells were designated as central memory (T_{CM}), and CD45RO-/CCR7+ T cells were designated as naïve-like (T_{N-like}). Gating strategy is shown in Fig. S6. In addition, data were used to anticipate the frequency of T cells (CD3+) in the collected CD45+ population from BMC to optimize subsequent genomic DNA extraction.

4.5 Tetramer generation

YLQPRTFLL (i.e. YLQ peptide), LTDEMIAQY (i.e. LTD peptide), and NYNYLYRLF (i.e. NYN peptide) were synthesized by GenScript Biotech (Piscataway, NJ). YLQ peptide (400 µM) was
mixed 1:1 (v/v) with 200 μg/mL Flex-T HLA-A*02:01 ultraviolet exchange UVX monomer (BioLegend) and treated with UV irradiation (368 nm) for 30 minutes using a UV crosslinker (Fisher Scientific) to remove the UV-labile peptide. The mixture was incubated at 37°C for 30 minutes to form YLQ monomers, which were then tetramerized through the addition of 200 μg/mL streptavidin-APC (BD) and incubation on ice for 30 minutes. Excess streptavidin was blocked with PBS + 0.4 μM D-Biotin + 0.3% (w/v) NaN₃ overnight at 4°C. HLA-A*01_LTD-APC and HLA-A*24_NYN-APC tetramers were generated by the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA).

4.6 Tetramer staining and flow cytometry analysis

Cells were thawed and washed as described above and then stained with HLA-A*02_YLQ-APC, HLA-A*01_LTD-APC, or HLA-A*24_NYN-APC tetramer (1:100) for 30 minutes at 4°C. Cells were washed with FACS buffer and then stained with antibody mix containing the optimal dilutions of all antibodies and viability dye in Brilliant Staining Buffer (Table S8; panel 2) for 30 minutes at room temperature, protected from light. Stained cells were washed in FACS buffer and run on a FACS aria II Cell Sorter (BD) with single stained CompBeads (ThermoFisher) as compensation controls. Data were analyzed on FlowJo version 10 (BD). HLA-A*02_YLQ tetramer performance was validated with YLQ-specific CD8+ T cells expanded from an HLA-A*02:01 SARS2 convalescent donor spiked into an HLA-A*02:01 negative donor with PBMC collected prior to 2019 (Fig. S7). HLA-A*01_LTD and HLA-A*24_NYN tetramer performance was validated using HLA-matched PBMC from donors predicted to have TCRs specific to these epitopes from TCRβ sequencing data and HLA-unmatched PBMC as negative controls. Tetramer positive cells from BMC and PBMC were gated as follows: lymphocyte cloud (FSC-A by SSC-A) → single cells (FSC-A by FSC-H) → live cells (live/dead Aqua by FSC-H) → CD45+/CD3+ → CD8+ → tetramer+. 
4.7 HLA typing

Genomic DNA was extracted from participants’ PBMC with the QIAamp® DNA Blood Mini Kit per manufacturer instructions, and HLA class I genotyping was performed via direct sequencing (Sisco Genetics, Seattle, WA).

4.8 TCRβ sequencing

Flow-sorted BMC (CD45+) expected to contain T cells underwent genomic DNA extraction using a protocol modified to recover low-yield DNA. Briefly, sorted BMC were pelleted in their collection tubes at 400 x g for 10 minutes. Cell pellets were processed in collection tubes using 30 µL of Qiagen Protease (Qiagen) and incubated at 70°C for 20 minutes. 1.5 µg of carrier RNA (Qiagen) and 300 µL of Buffer AL (Qiagen) was added. Samples were incubated for another 20 minutes at 70°C. To recover DNA, 300 µL of 100% ethanol was added to the sample and transferred to a QIAamp® Mini spin column followed by the manufacturer’s standard protocol from the QIAamp® DNA Blood Mini Kit. DNA was eluted in water. To assess DNA yield, a qPCR assay targeting the β-globin gene was performed, and only samples anticipated to contain at least 1,000 T cells were sent for TCRβ sequencing. Maternal PBMC underwent genomic DNA extraction using the standard protocol provided in the QIAamp® DNA Blood Mini Kit (Qiagen), and 3.4 µg of total genomic DNA was sent for TCRβ sequencing. Samples were sent in batches to Adaptive Biotechnologies (Seattle, WA, USA) and assayed using their ImmunoSEQ® hsTCRB46 service pipeline at a survey level.

4.9 TCR sequence analysis

TCRβ sequence data were analyzed using the immunoSEQ® Analyzer software and/or exported to R for analysis with the package immunoArch or divo. Repertoire overlap between blood and breastmilk was assessed using the Morisita index (immunoSEQ Analyzer®) and the power geometric index (divo), which are relatively protected from differences in sampling.
To identify overabundant clones in the breastmilk, frequencies of TCR clonotype nucleotide sequences in the breastmilk were compared to those in peripheral blood using the Differential Abundance tool in immunoSEQ® Analyzer using the binomial statistical method with Benjamini Hochberg correction and a lower limit of detection of 10.

The CDR3β amino acid sequence of each overabundant clone was compared to that of all other overabundant clones within each participant using tcrdist3. Similarly, CDR3β amino acid sequences of overabundant clones across all participants were compared to one another using tcrdist3. To identify epitope specificity of overabundant breastmilk clones, TCRβ sequences were matched by CDR3 amino acid and V gene identification against several public databases of TCRβ epitope specificity, namely ImmuneCODE (https://clients.adaptivebiotech.com/pub/covid-2020), VDJdb (available at https://vdjdb.cdr3.net), TCRBdb (http://bioinfo.life.hust.edu.cn/TCRdb/#/), McPAS-TCRB (http://friedmanlab.weizmann.ac.il/McPAS-TCRB/), and Immune Epitope Database and analysis Resource (IEDB) (http://www.iedb.org/home_v3.php). Epitope matches were considered a direct match if the CDR3 amino acid and TRBV gene were identical and were considered a predicted match if the CDR3 amino acid sequences were identical but V gene usage was mismatched. All overabundant breastmilk clones were also queried using the IEDB TCRMatch Tool (http://tools.iedb.org/tcrmatch/) with a score threshold of 0.97 to identify closely related epitope restrictions.

TCRβ sequences from both breastmilk and PBMC were evaluated for candidate Spike-specific restriction using the COVID Search Tool in immunoSEQ® Analyzer, which utilizes TCRβ sequences assigned as specific for SARS-CoV-2 from the ImmuneCODE database. For comparison of clonality metrics only, the full dataset of paired breastmilk and PBMC TCRβ sequences was down-sampled to the lowest productive template frequency. Candidate spike-
specific TCRβ in breastmilk and PBMC were further validated by comparing CDR3β amino acid sequences, V gene usage, epitope HLA restriction, and participant HLA concordance to an internally vetted dataset of published TCRβ sequences obtained via Spike-epitope-loaded tetramer or multimer-based experiments\textsuperscript{36-40}. An exact match at the TCRβ CDR3 sequence was required for these analyses. The internally vetted dataset\textsuperscript{36-40} was used to train the tcrdist3 algorithm\textsuperscript{31, 32} with a distance unit threshold of 10 to identify additional potential Spike-specific TCRβ in breastmilk.

4.10 ELISA
ELISAs were conducted as previously described\textsuperscript{50}. Briefly, 384-well plates were coated with SARS-CoV-2 spike protein (Institute for Protein Design, University of Washington). Wells were blocked and washed, and samples were added according to the parameters in Table S9. Aqueous breast milk fractions were diluted 1:2 and plasma samples were diluted 1:20 in assay diluent. Each sample was then serially diluted 1:5 across the plate. The average optical density value for all dilutions of the negative sample was used to set a minimum cutoff value for each plate, and each sample’s end point titer was calculated.

4.11 Statistical approach
To assess the primary difference between cellular phenotypic frequency (e.g. % CCR9+ of CD4+ T cells) in BMC versus PBMC, we built a linear regression model for each outcome with sample type as the predictor, adjustment for time since delivery, and clustering by individual to account for the correlation of individuals contributing paired BMC and PBMC. Due to the difference in sampling depth in the two compartments, negative binomial models were used to compare the frequency of Spike-specific templates in BMC versus PBMC, accounting for both the number of total SARS2-specific templates identified (viral genome wide) and the number of Spike-specific templates identified to determine the enrichment of Spike above background
reactivity to non-SARS2 coronaviruses and/or TCRs present in the naïve repertoire, and adjusting for time since delivery. The negative binomial model generates an incidence rate ratio (IRR) which represents the number of Spike-specific templates found in the experimental group (e.g. BMC) for every one template identified in the control group (e.g. PBMC). The phenotype and frequency of tetramer positive T cells in the BMC of individuals pre- and post-3rd dose was compared with paired t tests. A p-value less than 0.05 was considered significant.

**Data Availability:** All flow cytometry data are available in the Primary Data Table (Table S1), and all TCR sequencing data will be deposited in immuneACCESS®.
5. References:

1. Hassiotou F, Geddes DT, Hartmann PE. Cells in human milk: state of the science. *J Hum Lact* 2013; 29(2): 171-182.

2. Hassiotou F, Hepworth AR, Metzger P, Tat Tai C, Trengove N, Hartmann PE et al. Maternal and infant infections stimulate a rapid leukocyte response in breastmilk. *Clin Transl Immunology* 2013; 2(4): e3.

3. Goldman AS, Garza C, Nichols BL, Goldblum RM. Immunologic factors in human milk during the first year of lactation. *J Pediatr* 1982; 100(4): 563-567.

4. Trend S, de Jong E, Lloyd ML, Kok CH, Richmond P, Doherty DA et al. Leukocyte Populations in Human Preterm and Term Breast Milk Identified by Multicolour Flow Cytometry. *PLoS ONE* 2015; 10(8): e0135580.

5. Kent JC. How breastfeeding works. *J Midwifery Womens Health* 2007; 52(6): 564-570.

6. Kourtis AP, Ibegbu CC, Theiler R, Xu YX, Bansil P, Jamieson DJ et al. Breast milk CD4+ T cells express high levels of C chemokine receptor 5 and CXC chemokine receptor 4 and are preserved in HIV-infected mothers receiving highly active antiretroviral therapy. *The Journal of infectious diseases* 2007; 195(7): 965-972.

7. Sabbaj S, Ghosh MK, Edwards BH, Leeth R, Decker WD, Goepfert PA et al. Breast milk-derived antigen-specific CD8+ T cells: an extralymphoid effector memory cell population in humans. *J Immunol* 2005; 174(5): 2951-2956.

8. Sabbaj S, Edwards BH, Ghosh MK, Semrau K, Cheelo S, Thea DM et al. Human immunodeficiency virus-specific CD8(+) T cells in human breast milk. *J Virol* 2002; 76(15): 7365-7373.

9. Moylan DC, Pati SK, Ross SA, Fowler KB, Boppana SB, Sabbaj S. Breast Milk Human Cytomegalovirus (CMV) Viral Load and the Establishment of Breast Milk CMV-pp65-Specific CD8 T Cells in Human CMV Infected Mothers. *The Journal of infectious diseases* 2017; 216(9): 1176-1179.

10. Lohman BL, Slyker J, Mbori-Ngacha D, Bosire R, Farquhar C, Obimbo E et al. Prevalence and magnitude of human immunodeficiency virus (HIV) type 1-specific lymphocyte responses in breast milk from HIV-1-seropositive women. *The Journal of infectious diseases* 2003; 188(11): 1666-1674.

11. Ruben FL, Holzman IR, Fireman P. Responses of lymphocytes from human colostrum or milk to influenza antigens. *Am J Obstet Gynecol* 1982; 143(5): 518-522.

12. Bryan DL, Hart PH, Forsyth KD, Gibson RA. Immunomodulatory constituents of human milk change in response to infant bronchiolitis. *Pediatr Allergy Immunol* 2007; 18(6): 495-502.
13. Riskin A, Almog M, Peri R, Halasz K, Srugo I, Kessel A. Changes in immunomodulatory constituents of human milk in response to active infection in the nursing infant. *Pediatr Res* 2012; 71(2): 220-225.

14. Moles JP, Tuaillon E, Kankasa C, Bedin AS, Nagot N, Marchant A et al. Breastmilk cell trafficking induces microchimerism-mediated immune system maturation in the infant. *Pediatr Allergy Immunol* 2018; 29(2): 133-143.

15. Miller RA. Observations on the gastric acidity during the first month of life. *Arch Dis Child* 1941; 16(85): 22-30.

16. Catassi C, Bonucci A, Coppa GV, Carlucci A, Giorgi PL. Intestinal permeability changes during the first month: effect of natural versus artificial feeding. *J Pediatr Gastroenterol Nutr* 1995; 21(4): 383-386.

17. Kalach N, Rocchiccioli F, de Boissieu D, Benhamou PH, Dupont C. Intestinal permeability in children: variation with age and reliability in the diagnosis of cow’s milk allergy. *Acta Paediatr* 2001; 90(5): 499-504.

18. Darby MG, Chetty A, Mrjden D, Rolot M, Smith K, Mackowiak C et al. Pre-conception maternal helminth infection transfers via nursing long-lasting cellular immunity against helminths to offspring. *Sci Adv* 2019; 5(5): eaav3058.

19. Ma LJ, Walter B, Deguzman A, Muller HK, Walker AM. Trans-epithelial immune cell transfer during suckling modulates delayed-type hypersensitivity in recipients as a function of gender. *PLoS ONE* 2008; 3(10): e3562.

20. Zhou L, Yoshimura Y, Huang Y, Suzuki R, Yokoyama M, Okabe M et al. Two independent pathways of maternal cell transmission to offspring: through placenta during pregnancy and by breast-feeding after birth. *Immunology* 2000; 101(4): 570-580.

21. Tuboly S, Bernath S, Glavits R, Kovacs A, Megyeri Z. Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. *Acta Vet Hung* 1995; 43(1): 105-115.

22. Balle C, Armistead B, Kiravu A, Song X, Happel AU, Hoffmann AA et al. Factors influencing maternal microchimerism throughout infancy and its impact on infant T cell immunity. *J Clin Invest* 2022; 132(13).

23. Goncalves J, Juliano AM, Charepe N, Alenquer M, Athayde D, Ferreira F et al. Secretory IgA and T cells targeting SARS-CoV-2 spike protein are transferred to the breastmilk upon mRNA vaccination. *Cell Rep Med* 2021; 2(12): 100468.

24. Jackson LA, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN et al. An mRNA Vaccine against SARS-CoV-2 - Preliminary Report. *N Engl J Med* 2020; 383(20): 1920-1931.

25. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *N Engl J Med* 2020; 383(27): 2603-2615.
26. Collier AY, McMahan K, Yu J, Tostanoski LH, Aguayo R, Ansel J et al. Immunogenicity of COVID-19 mRNA Vaccines in Pregnant and Lactating Women. *JAMA* 2021.

27. Skelly DT, Harding AC, Gilbert-Jaramillo J, Knight ML, Longet S, Brown A et al. Two doses of SARS-CoV-2 vaccination induce robust immune responses to emerging SARS-CoV-2 variants of concern. *Nat Commun* 2021; 12(1): 5061.

28. Ssemaganda A, Nguyen HM, Nuhu F, Jahan N, Card CM, Kizayk S et al. Expansion of cytotoxic tissue-resident CD8(+) T cells and CCR6(+)CD161(+) CD4(+) T cells in the nasal mucosa following mRNA COVID-19 vaccination. *Nat Commun* 2022; 13(1): 3357.

29. Rempala GA, Seweryn M. Methods for diversity and overlap analysis in T-cell receptor populations. *J Math Biol* 2013; 67(6-7): 1339-1368.

30. Rytlewski J, Deng S, Xie T, Davis C, Robins H, Yusko E et al. Model to improve specificity for identification of clinically-relevant expanded T cells in peripheral blood. *PLoS ONE* 2019; 14(3): e0213684.

31. Dash P, Fiore-Gartland AJ, Hertz T, Wang GC, Sharma S, Souquette A et al. Quantifiable predictive features define epitope-specific T cell receptor repertoires. *Nature* 2017; 547(7661): 89-93.

32. Mayer-Blackwell K, Schattgen S, Cohen-Lavi L, Crawford JC, Souquette A, Gaevert JA et al. TCR meta-clonotypes for biomarker discovery with tcrdist3: identification of public, HLA-restricted SARS-CoV-2 associated TCR features. *bioRxiv* 2021.

33. Gil A, Yassai MB, Naumov YN, Selin LK. Narrowing of human influenza A virus-specific T cell receptor alpha and beta repertoires with increasing age. *J Virol* 2015; 89(8): 4102-4116.

34. Chen G, Yang X, Ko A, Sun X, Gao M, Zhang Y et al. Sequence and Structural Analyses Reveal Distinct and Highly Diverse Human CD8(+) TCR Repertoires to Immunodominant Viral Antigens. *Cell Rep* 2017; 19(3): 569-583.

35. Nolan S, Vignali M, Klinger M, Dines JN, Kaplan IM, Svejnoha E et al. A large-scale database of T-cell receptor beta (TCRbeta) sequences and binding associations from natural and synthetic exposure to SARS-CoV-2. *Res Sq* 2020.

36. Minervina AA, Pogorelyy MV, Kirk AM, Allen EK, Allison KJ, Lin CY et al. Convergent epitope-specific T cell responses after SARS-CoV-2 infection and vaccination. *medRxiv* 2021.

37. Shomuradova AS, Vagida MS, Sheetikov SA, Zornikova KV, Kiryukhin D, Titov A et al. SARS-CoV-2 Epitopes Are Recognized by a Public and Diverse Repertoire of Human T Cell Receptors. *Immunity* 2020; 53(6): 1245-1257 e1245.

38. Agerer B, Koblischke M, Gudipati V, Montano-Gutierrez LF, Smyth M, Popa A et al. SARS-CoV-2 mutations in MHC-I-restricted epitopes evade CD8(+) T cell responses. *Sci Immunol* 2021; 6(57).
39. Dykema AG, Zhang B, Woldemeskel BA, Garliss CC, Cheung LS, Choudhury D et al. Functional characterization of CD4+ T cell receptors crossreactive for SARS-CoV-2 and endemic coronaviruses. *J Clin Invest* 2021; **131**(10).

40. Nguyen THO, Rowntree LC, Petersen J, Chua BY, Hensen L, Kedzierski L et al. CD8(+) T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope display high naive precursor frequency and TCR promiscuity. *Immunity* 2021; **54**(5): 1066-1082 e1065.

41. Snyder TM, Gittelman RM, Klinger M, May DH, Osborne EJ, Taniguchi R et al. Magnitude and Dynamics of the T-Cell Response to SARS-CoV-2 Infection at Both Individual and Population Levels. *medRxiv* 2020.

42. Lefrancois L, Marzo AL. The descent of memory T-cell subsets. *Nat Rev Immunol* 2006; 6(8): 618-623.

43. Goff SL, Danforth DN. The Role of Immune Cells in Breast Tissue and Immunotherapy for the Treatment of Breast Cancer. *Clin Breast Cancer* 2021; **21**(1): e63-e73.

44. Ramanan D, Sefik E, Galvan-Pena S, Wu M, Yang L, Yang Z et al. An Immunologic Mode of Multigenerational Transmission Governs a Gut Treg Setpoint. *Cell* 2020; **181**(6): 1276-1290 e1213.

45. Hanna N, Heffes-Doon A, Lin X, Manzano De Mejia C, Botros B, Gurzenda E et al. Detection of Messenger RNA COVID-19 Vaccines in Human Breast Milk. *JAMA Pediatr* 2022.

46. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood* 2009; **114**(19): 4099-4107.

47. Bagaev DV, Vroomans RMA, Samir J, Stervbo U, Rius C, Dolton G et al. VDJdb in 2019: database extension, new analysis infrastructure and a T-cell receptor motif compendium. *Nucleic Acids Res* 2020; **48**(D1): D1057-D1062.

48. Chen SY, Yue T, Lei Q, Guo AY. TCRdb: a comprehensive database for T-cell receptor sequences with powerful search function. *Nucleic Acids Res* 2021; **49**(D1): D468-D474.

49. Tickotsky N, Sagiv T, Prilusky J, Shifrut E, Friedman N. McPAS-TCR: a manually curated catalogue of pathology-associated T cell receptor sequences. *Bioinformatics* 2017; **33**(18): 2924-2929.

50. Larsen SE, Berube BJ, Pecor T, Cross E, Brown BP, Williams BD et al. Qualification of ELISA and neutralization methodologies to measure SARS-CoV-2 humoral immunity using human clinical samples. *J Immunol Methods* 2021; **499**: 113160.
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Figure Legends:

Fig. 1. Antigen-experienced and mucosal-homing T cells are enriched in breastmilk. PBMC and BMC were analyzed by flow cytometry. Comparisons made with linear regression and clustering by individual, ***p< 0.001, **p< 0.01, *p< 0.05, n.s. (not significant). (A) Scatter plots showing expression of CD45RO and CCR7 in CD4+ and CD8+ T cells from PBMC and BMC in one representative participant. (B) Frequencies of T_{EM}, T_{CM}, and T_{N-like} CD4+ and CD8+ T cells in PBMC and BMC (n=17): CD4+ T_{EM}: 41% vs. 84%, p<0.001, CD4+ T_{CM}: 6% vs. 11%, p=0.006, CD4+ T_{N-like}: 11% vs. 1%, p<0.001; CD8+ T_{EM}: 38% vs.73%, p< 0.001, CD8+ T_{CM}: 1% vs. 1%, p=n.s., CD8+ T_{N-like}: 12% vs. 0.2%, p<0.001. (C) Scatter plots showing expression of CCR9 and CD103 in CD4+ and CD8+ T cells from PBMC and BMC in one representative participant. (D) Expression of CCR9 and CD103 within CD4+ and CD8+ T cell populations in PBMC and BMC: CD4+/CCR9+ (n=11): 4% vs. 38%, p<0.001, CD4+/CD103+: 0.4% vs. 7%, p <0.001, CD4+/CCR9+/CD103+: 0.1 vs. 5%, p<0.001, CD8+/CCR9+: 3% vs. 12%, p=0.005; CD8+/CD103+: 3% vs. 32%, p<0.001, CD8+/CCR9+/CD103+: 0.4% vs. 3%, p<0.001.

Fig. 2. The T cell receptor (TCR) repertoires in breastmilk and peripheral blood are distinct. Bulk TCRβ sequencing from BMC and PBMC individuals who had paired samples available and at least 1,000 TCRβ templates in the BMC sample (n=11). Each clone is represented as a dot, with the relative frequency in BMC represented on the X axis and relative frequency in PBMC on the Y axis. TCRβ repertoire overlap was analyzed using the Morisita index (M.I., value inset), with a value of 0 representing no repertoire overlap and a value of 1 representing complete overlap of the repertoires. The frequencies of each individual TCRβ
clonotype in the two compartments were statistically compared using the immunoSEQ®
Differential Abundance Tool. Gray dashed line indicates frequency equality in the two
compartments. Blue dots represent clonotypes that are significantly more abundant in BMC
relative to PBMC. Orange dots represent clonotypes that are significantly more abundant in
PBMC relative to BMC. Gray dots represent clonotypes that were not significantly different in
frequency between the two compartments. Pale gray dots represent clonotypes that fell below
the frequency for valid statistical comparison. As a control, TCRβ clonotypes from an
individual’s PBMC obtained 9 days and 17 days after 2nd mRNA vaccine dose were compared
(upper left plot) demonstrating a high degree of repertoire overlap and only a few clones
expanded at the second time point relative to the first.

Fig. 3. Overabundant TCR clones in breastmilk are diverse. (A) For each individual
participant with paired PBMC available (n=11) all TCRβ CDR3 amino acid sequences obtained
from the BMC were compared to one another using tcrdist3; representative plots from two
individuals are displayed. Black ticks denote TCRβ sequences significantly overabundant in
BMC relative to PBMC, demonstrating that overabundant clones were distributed across the full
breastmilk TCR repertoire for each individual (i.e., did not cluster by sequence). (B)
Overabundant BMC TCRβ CDR3 amino acid sequences from 11 participants were compared to
one another across participants using tcrdist3. Overabundant clones did not cluster by
individual and most clones were private, emphasizing the diversity of overabundant clones
across individuals. Overabundant TCRβ clones were further compared by CDR3 amino acid
sequence and V gene usage against available public databases of known TCR epitope
specificity. Clones matching pathogen-specific epitopes are marked with colored ticks. Epitope
matches were considered a direct match if the CDR3 amino acid and TRβV gene were identical
and were considered a predicted match if the CDR3 amino acid sequences were identical but V
gene usage was mismatched. Only the minority of clone specificity could be assigned, and characterized clones were tightly clustered across all individuals. For Participant 10, only CDR3 amino acid sequences enriched by a factor ≥50 relative to PBMC or with epitope specificity were included to reduce data skewing from this participant.

**Fig. 4.** Candidate SARS2 Spike-specific T cells are enriched in the breastmilk relative to the peripheral blood of vaccinated individuals. The frequency of candidate Spike-specific clones in all sequenced PBMC and BMC (n=14 paired, n=2 BMC only) was expressed relative to all clones predicted to bind to SARS2 antigens. Spike template frequency was compared using a negative binomial model, **p<0.01.** (A) TCRβ sequences predicted to bind to SARS2 were identified in BMC and PBMC using the ImmunoSEQ® T-MAP COVID Search Tool and are mapped by their epitope binding location on the Spike protein. Gold line indicates the amino acid position of the Spike peptide pool that includes the YLQPRTFLL epitope (HLA-A*02 restricted), green line indicates the position of the NYNYLYRLF epitope (HLA-A*24 restricted), and purple line indicates the position of the LTDEMIAQY epitope (HLA-A*01 restricted). (B) Spike-specific TCRβ templates are enriched in BMC relative to PBMC, incident rate ratio (IRR)=1.66, p=0.004.

**Fig. 5.** SARS2 Spike-specific T cells expand in breastmilk following 3rd mRNA vaccine dose. BMC and PBMC from before and ~ 1 week after receipt of the 3rd dose of SARS2 mRNA vaccine were stained with SARS2 Spike epitope-loaded class I tetramers and analyzed by flow cytometry to quantify Spike-specific CD8+ T cells. Comparisons made with paired t tests, *p<0.05, n.s. (non-significant). Scatter plots of (A) HLA-A*02_YLQ-positive (n=5), (B) HLA-A*01_LTD-positive (n=2), and (C) HLA-A*24_NYN-positive (n=1) CD8+ T cells in breastmilk obtained before (top) and after (bottom) 3rd mRNA vaccine dose are shown from HLA
concordant individuals. Frequencies of tetramer+ cells of CD8+ T cells inset. (D) Frequencies of tetramer+ cells of CD8+ T cells in BMC (n=8, blue) and PBMC (n=7, red). Triangle=HLA-A*02_YLQ, circle=HLA-A*01_LTD, square=HLA-A*24_NYN. BMC: 0.8% to 2.8%, p=0.03; PBMC: 0.04% to 0.04%, p=0.9.

Tables:
**Demographics**

**Total (n=20)**

Maternal age, years, median (range)  
37 (29-39)

Maternal vaccine type, n (%)  
- Pfizer-BioNTech: 17 (85%)
- Moderna: 3 (15%)

Time since delivery, weeks, median (range)  
26 (2-56)

Time since second SARS2 mRNA dose, weeks, median (range)  
11 (0.7-24)

Parity, median (range)  
1.5 (1-3)

Infant sex, n (%)  
- Female: 7 (33%)
- Male: 14 (67%)

**Table 1. Cohort characteristics.**

| SARS2 Spike epitope sequence | Sequence position within Spike protein | HLA class I restriction |
|-----------------------------|---------------------------------------|-------------------------|
| YLQPRTFLL                  | 269-277                               | HLA-A*02:01             |
| NYNYLYRLF                  | 448-456                               | HLA-A*24:02             |
| LTDEMI AQY                 | 865-873                               | HLA-A*01:01             |

**Table 2. Spike epitope-loaded tetramers.**

**Supplementary Material:**
Fig. S1 to S8
Tables S1 to S8
