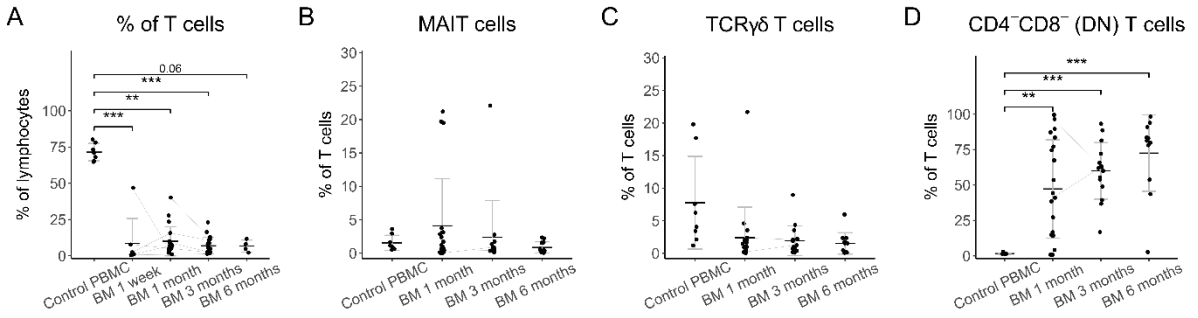


Figure S1: General T-cell composition (FACS) in human breastmilk of 1 week, 1, 3 and 6 months postpartum compared to PBMC of age-matched female control donors. **A)** Frequency of $CD3^+$ T cells as a percentage of the lymphocyte gate (PBMC n=7, BM 1 week n=7, BM 1 month n=19, BM 3 months n=20, BM 6 months n=4). **B)** Frequency of Mucosal Associated Invariant T cells ($TCRV\alpha7.2^+CD161^+$) as the percentage of live T cells. **C)** Frequency of T cells with a γδ-TCR as the percentage of live T cells. **D)** Frequency of $CD4^-CD8^-$ (DN) T cells as the percentage of T cells, excluding MAIT cells and $TCR\gamma\delta^+$ T cells. B-D) PBMC n=8, BM 1 month n=20, BM 3 months n=15, BM 6 months n=12. Significance stars denotate if **=p<0.01, ***=p<0.001 with pairwise comparisons among PBMC and BM across different time points using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Data represent mean±SD. Transparent lines connect datapoints of different time points postpartum from the same breastmilk donor. MAIT: musocal-associated invariant T cells ($TCRV\alpha7.2^+CD161^+$), BM: breastmilk, DN: double negative, TCR: T cell receptor.

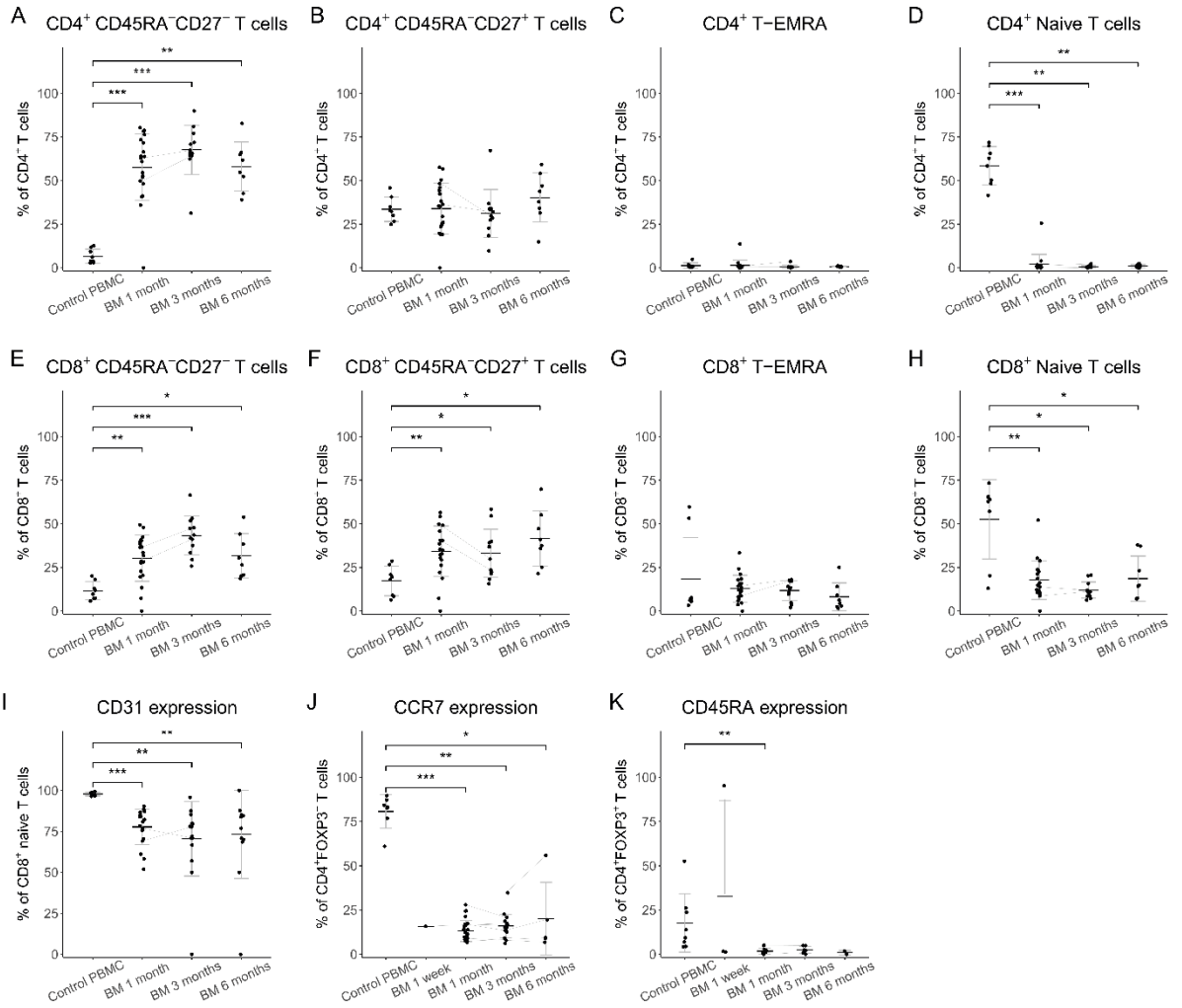


Figure S2: Naïve/Memory differentiation (FACS) of human breastmilk T cells of 1 week, 1, 3 and 6 months postpartum milk compared to PBMC of age-matched female control donors. Frequency of **A)** $CD45RA^{-}CD27^{-}$, **B)** $CD45RA^{-}CD27^{+}$, **C)** $CD45RA^{+}CD27^{-}$ 'T-EMRA' and **D)** $CD45RA^{+}CD27^{+}$ 'Naive' T cells as the percentage of $CD4^{+}$ T cells. Frequency of **E)** $CD45RA^{-}CD27^{-}$, **F)** $CD45RA^{-}CD27^{+}$, **G)** $CD45RA^{+}CD27^{-}$ 'T-EMRA' and **H)** $CD45RA^{+}CD27^{+}$ 'Naive' T cells as the percentage of $CD8^{+}$ T cells. **I)** Frequency of $CD31^{+}$ T cells as the percentage of $CD8^{+}CD45RA^{+}CD27^{+}$ 'Naïve' T cells. A-I) PBMC n=7, BM 1 week n=8, BM 1 month n=20, BM 3 months n=15, BM 6 months n=12. **J)** Frequency of $CCR7^{+}$ T cells as the percentage of $FOXP3^{-}CD4^{+}$ T cells (PBMC n=7, BM 1 week n=1, BM 1 month n=27, BM 3 months n=17, BM 6 months n=5). **K)** Frequency of $CD45RA^{+}$ T cells as the percentage of $FOXP3^{+}CD4^{+}$ 'Treg' T cells (PBMC n=7, BM 1 week n=2, BM 1 month n=13, BM 3 months n=7, BM 6 months n=2). Significance stars denotate if *=p<0.05, **=p<0.01, ***=p<0.001 with pairwise comparisons among PBMC and BM across different time points using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Data represent mean±SD. Transparent lines connect datapoints of different time points postpartum from the same breastmilk donor. BM: breastmilk.

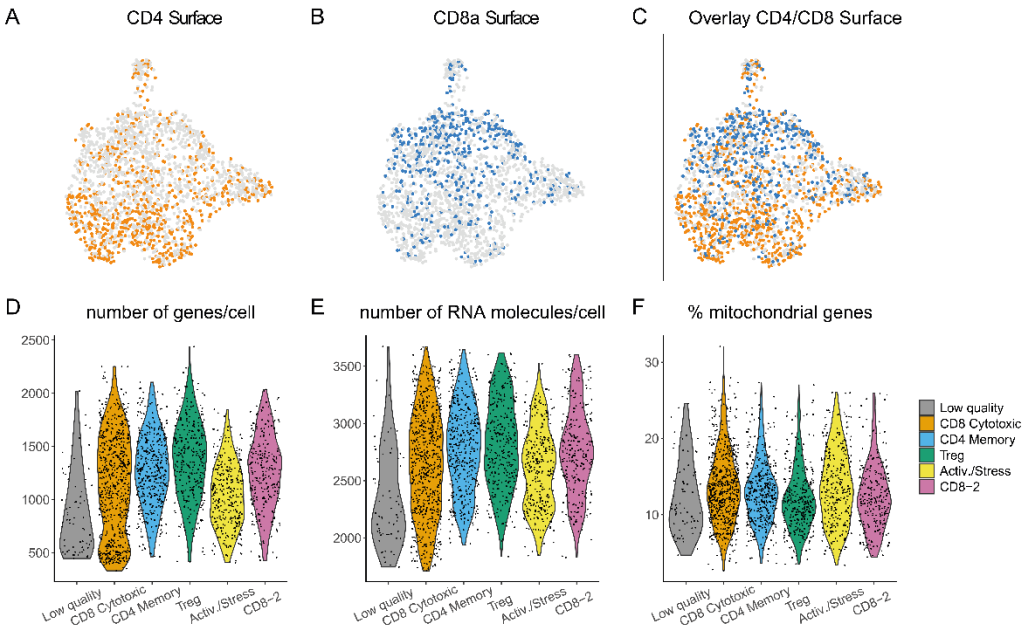


Figure S3: Descriptive figures of scRNAseq data of T cells from 1 month postpartum breastmilk (n=7). UMAP showing index sort data for **A)** surface CD4 (orange) and **B)** surface CD8α (blue), overlayed in **(C)**. Violinplots of quality control metrics, showing **D)** the number of genes (nFeature), **E)** number of RNA molecules (nCount) and **F)** percentage of mitochondrial genes per cell for each of the scRNAseq clusters.

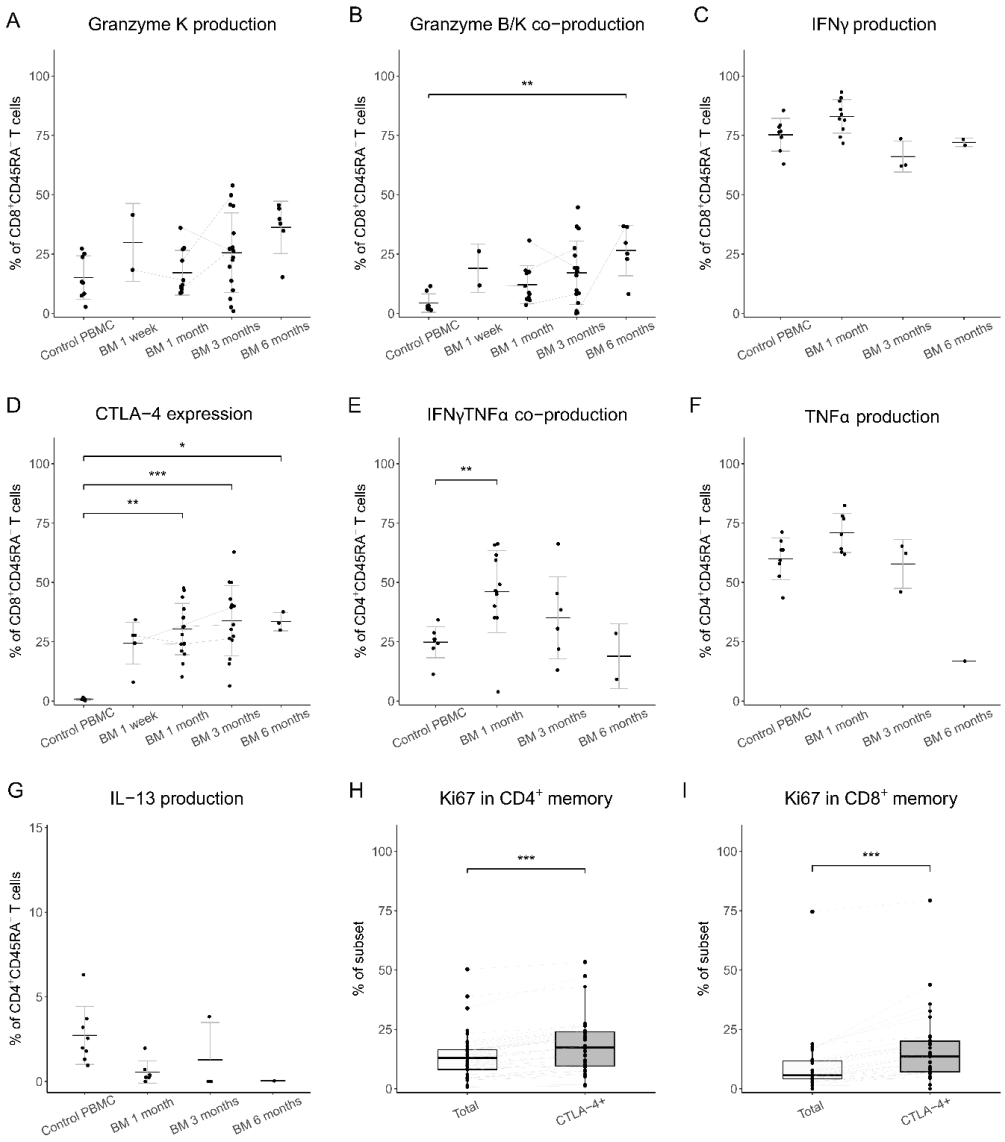


Figure S4: Functional profile of CD4$^+$ and CD8$^+$ memory T cells (FACS) in human breastmilk of 1 week, 1, 3 and 6 months postpartum compared to PBMC of age-matched female control donors. **A-D)** Frequency of granzyme K$^+$ (PBMC n=8, BM 1 week n=1, BM 1 month n=11, BM 3 months n=16, BM 6 months n=6) **(A)**, granzyme B$^+$K$^+$ (PBMC n=8, BM 1 week n=2, BM 1 month n=11, BM 3 months n=17, BM 6 months n=6) **(B)**, IFNγ$^+$ (PBMC n=8, BM 1 month n=10, BM 3 months n=3, BM 6 months n=2) **(C)** and CTLA-4$^+$ (PBMC n=7, BM 1 week n=6, BM 1 month n=17, BM 3 months n=15, BM 6 months n=3) **(D)** cells as the percentage of CD8$^+$CD45RA$^-$ T cells. **E-G)** Frequency of IFNγ$^+$TNFα$^+$ (PBMC n=8, BM 1 month n=12, BM 3 months n=7, BM 6 months n=2) **(E)**, TNFα$^+$ **(F)** and IL-13$^+$ **(G)** cells (E-G: PBMC n=8, BM 1 month n=7, BM 3 months n=3, BM 6 months n=2) as the percentage of CD4$^+$CD45RA$^-$T cells. **H-I)** Frequency of Ki67$^+$ cells within CD4$^+$CD45RA$^+$ (n=47) **(H)** and CD8$^+$CD45RA$^+$ (n=41) **(I)** breastmilk T cells, comparing the total population with the subset of cells positive for the immune-checkpoint receptor CTLA-4. Cytokine production was measured following stimulation with PMA/ionomycin. Significance stars denotate if *=p<0.05, **=p<0.01, ***=p<0.001. A-G) Testing pairwise comparisons between T cell subsets within each donor using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. H-I) Testing paired difference between expression in the total T-cell population and T-cell subset using Wilcoxon rank-sum test. Data represent mean±SD. Transparent lines connect datapoints from the same breastmilk donor. Only samples with more than 50 cells within the CTLA-4$^+$ populations are included. BM: breastmilk.

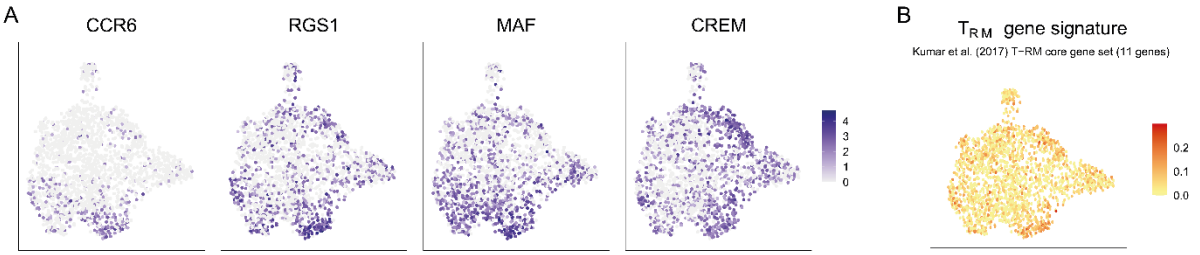


Figure S5: Tissue-related gene expression signature in human breastmilk T cells (n=7). **A)** UMAP showing gene expression (log-normalized) projections of tissue-related genes that ranked high among the DEG in cluster 3 (CCR6, RGS1, MAF, CREM) and cluster 6 (CREM). **B)** UMAP showing gene-set enrichment score (AUCell) of a core human T-RM gene set as published by Kumar et al. (2017). T-RM: tissue-resident memory T cell.

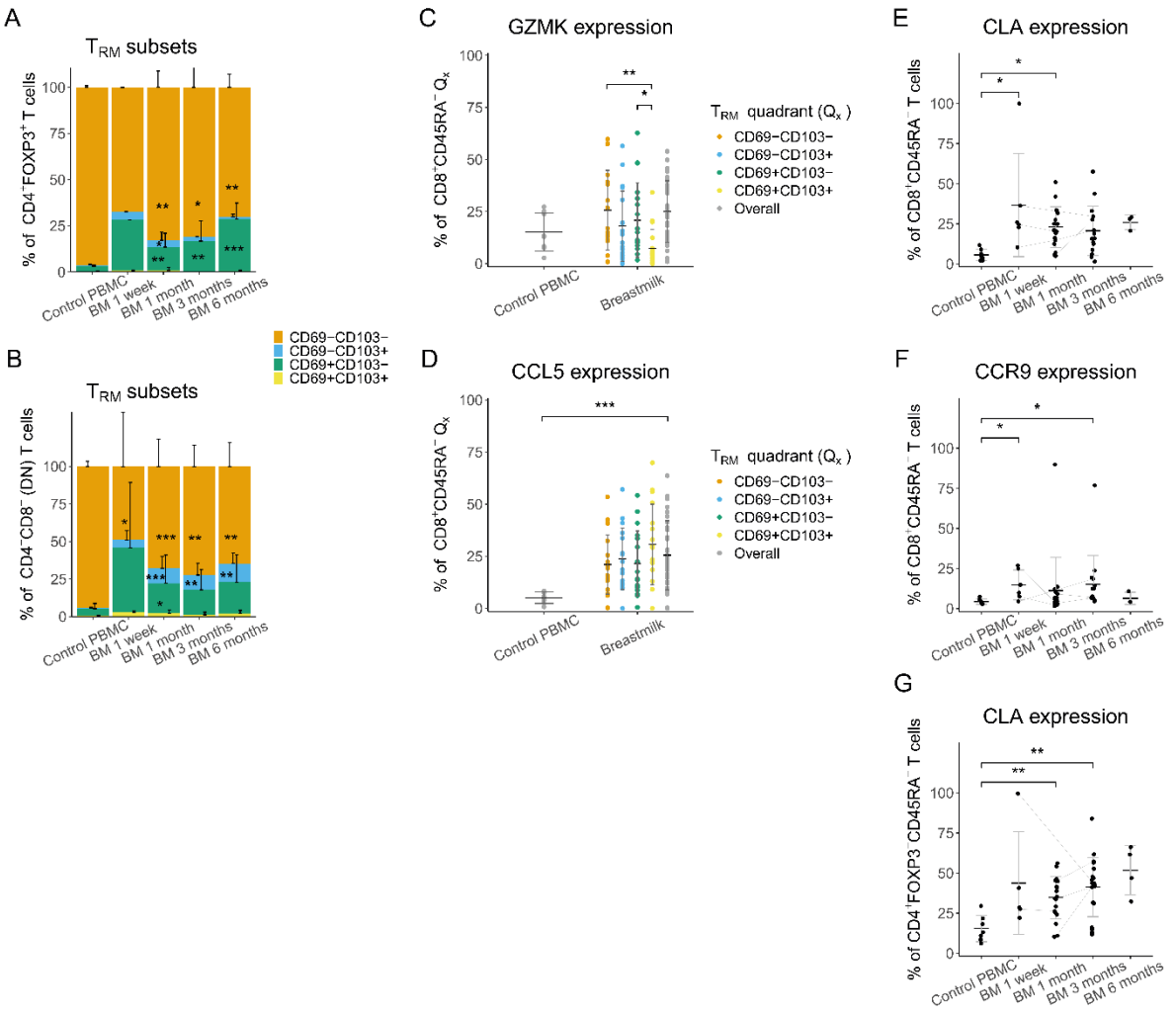


Figure S6: Tissue-residency and tissue homing T cell profiles (FACS) in human breastmilk of 1 week, 1, 3 and 6 months postpartum compared to PBMC of age-matched female control donors. **A-B)** Distribution of different tissue-resident memory T cell (T-RM) subsets based on CD69 and CD103 expression (FACS) as the percentage of $CD4^+FOXP3^+$ T cells (PBMC n=7, BM 1 week n=1, BM 1 month n=23, BM 3 months n=4, BM 6 months n=3) **(A)** and $CD4^-CD8^-$ (DN) T cells (PBMC n=8, BM 1 week n=2, BM 1 month n=28, BM 3 months n=18, BM 6 months n=5) **(B)**. **C)** Frequency of $CCL5^+$ (PBMC n=9, BM n=18) and **D)** granzyme K^+ cells (PBMC n=8, BM n=34) expressed as the percentage of $CD8^+CD45RA^-$ T cells compared between the four different $CD8^+$ T-RM subsets within breastmilk (coloured dots) and with the total population in control PBMC (grey dots). **E)** Frequency of CLA^+ and **F)** $CCR9^+$ cells as the percentage of $CD8^+CD45RA^-$ T cells (PBMC n=7, BM 1 week n=6, BM 1 month n=17, BM 3 months n=15, BM 6 months n=3) and **G)** CLA^+ cells as the percentage of $CD4^+FOXP3^-CD45RA^-$ T cells (PBMC n=7, BM 1 week n=5, BM 1 month n=19, BM 3 months n=19, BM 6 months n=4). Significance stars denotate if *=p<0.05, **=p<0.01, ***=p<0.001. A-B,E-G) Testing pairwise comparisons among PBMC and BM timepoints using the Kruskal-Wallis test followed by Dunn's test for multiple comparisons. D) Testing comparisons between breastmilk T-cell subsets using the Friedman test followed by Bonferroni-corrected pairwise Wilcoxon rank sum post-hoc testing. Data represent mean±SD. Transparent lines connect datapoints of different time points from the same breastmilk donor. BM: breastmilk, T-RM: tissue-resident memory T cell.

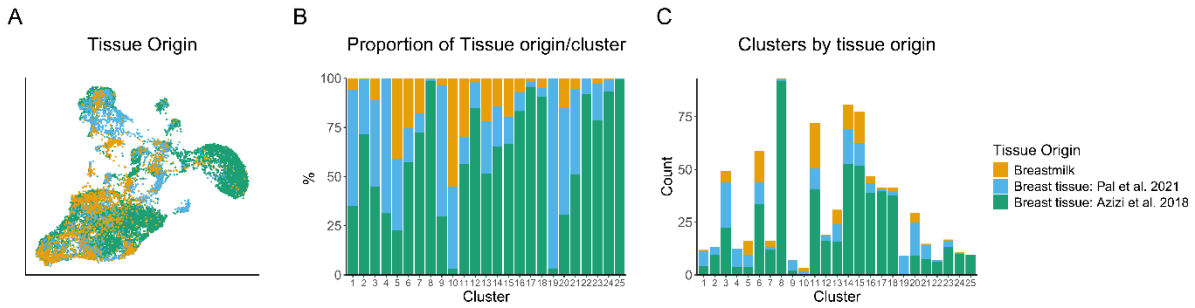


Figure S7: Clustering of breastmilk T cells with the immune cell subsets from healthy breast tissue extracted from two publicly available scRNAseq datasets, displaying breastmilk T cells in orange (n=7), breast tissue cells from Pal et al. (2021) in blue (n=12) and breast tissue CD45^{+} immune cells from Azizi et al (2018) in green (n=10). **A)** UMAP showing localization of the cells originating of each of the three datasets. **B)** Relative and **C)** Absolute frequency of each of the three datasets per cluster.

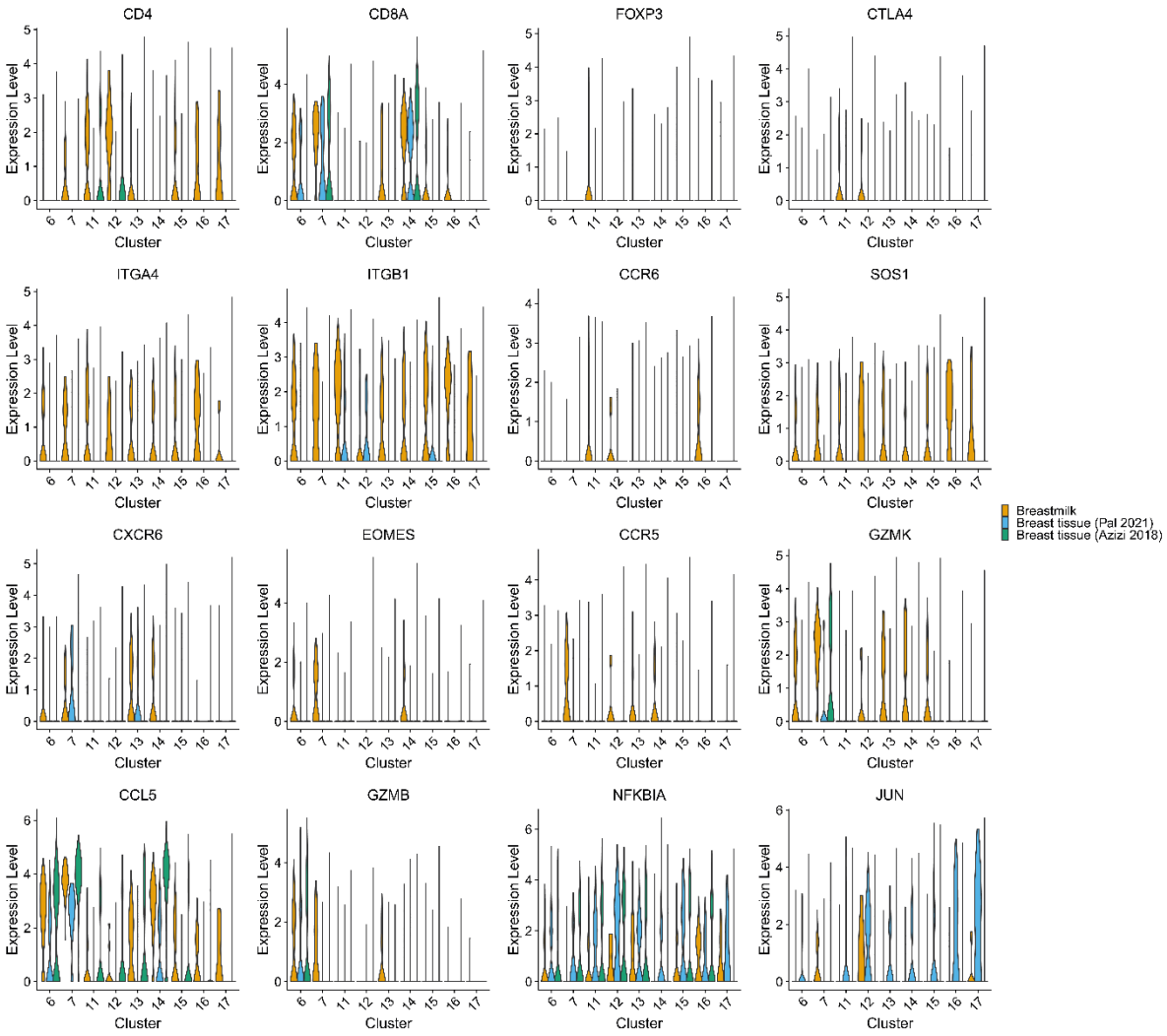


Figure S8: Comparison of gene expression levels of different T-cell markers across breastmilk and breast tissue. Showing violin plots of gene expression (log-normalized) from the clusters enriched for T cells upon clustering of breastmilk T cells together with breast tissue immune cells from two publicly available scRNAseq datasets, with breastmilk T cells in orange (n=7), breast tissue cells from Pal et al. (2021) in blue (n=12) and breast tissue CD45$^+$ immune cells from Azizi et al (2018) in green (n=10).

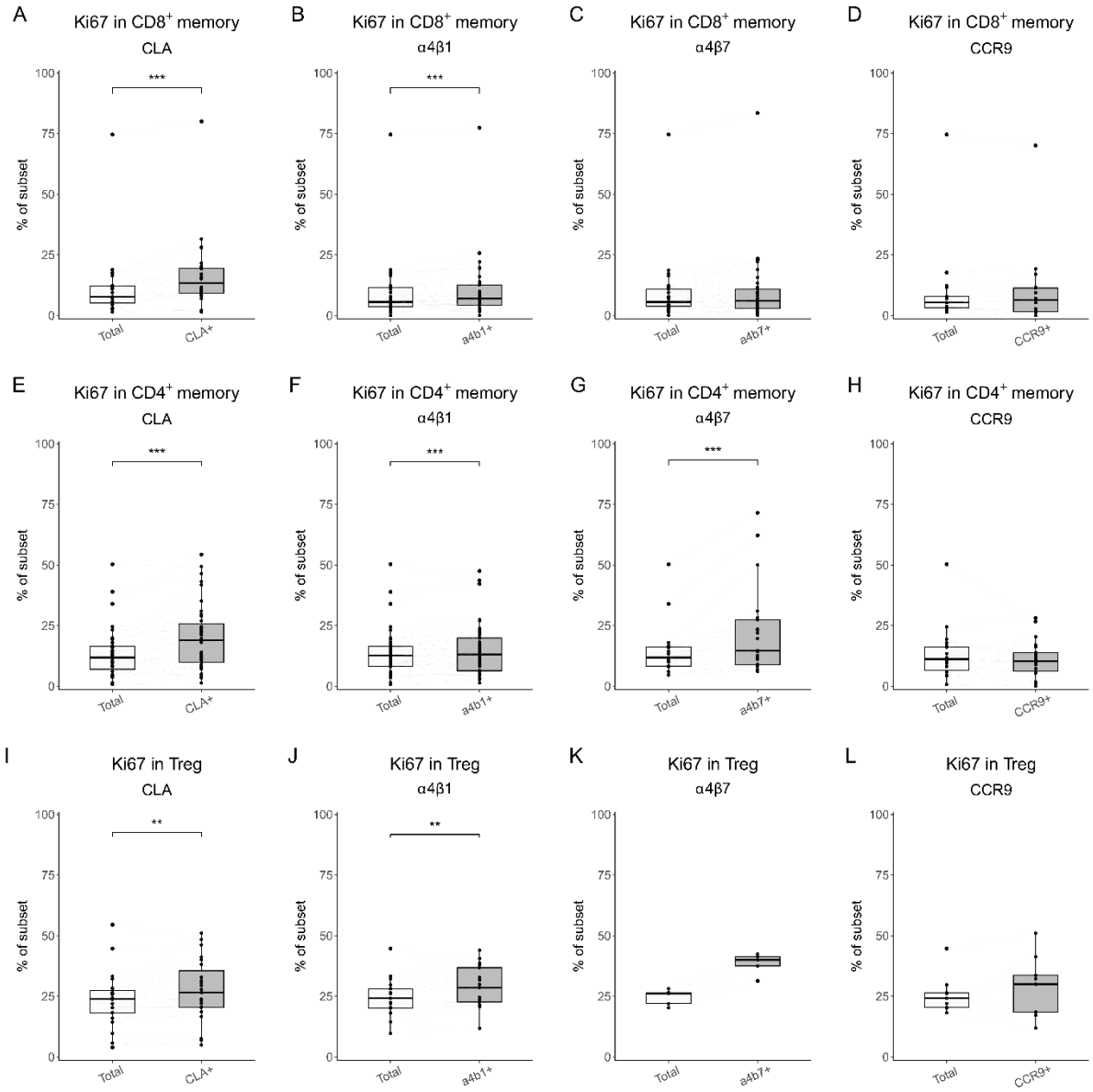


Figure S9: Proliferation of breastmilk T cells, assessed by expression of Ki-67, comparing T cells expressing homing receptors with total T cells (FACS, n=26). Frequency of Ki67$^+$ cells within CD8$^+$CD45RA$^+$ **(A-D)**, CD4$^+$CD45RA$^+$ **(E-H)** and regulatory **(I-L)** breastmilk T cells comparing the total population with the subset of T cells positive for the homing receptors CLA **(A, E, I)**, α4β1 **(B, F, J)**, α4β7 **(C,G,K)** and CCR9 **(D,H,L)**. Significance stars denotate if **=p<0.01, ***=p<0.001, testing paired difference between expression in the total T-cell population and T-cell subset using Wilcoxon rank-sum test. Transparent lines connect datapoints from the same breastmilk donor. Only samples with more than 50 cells within the homing receptor-positive populations were included.

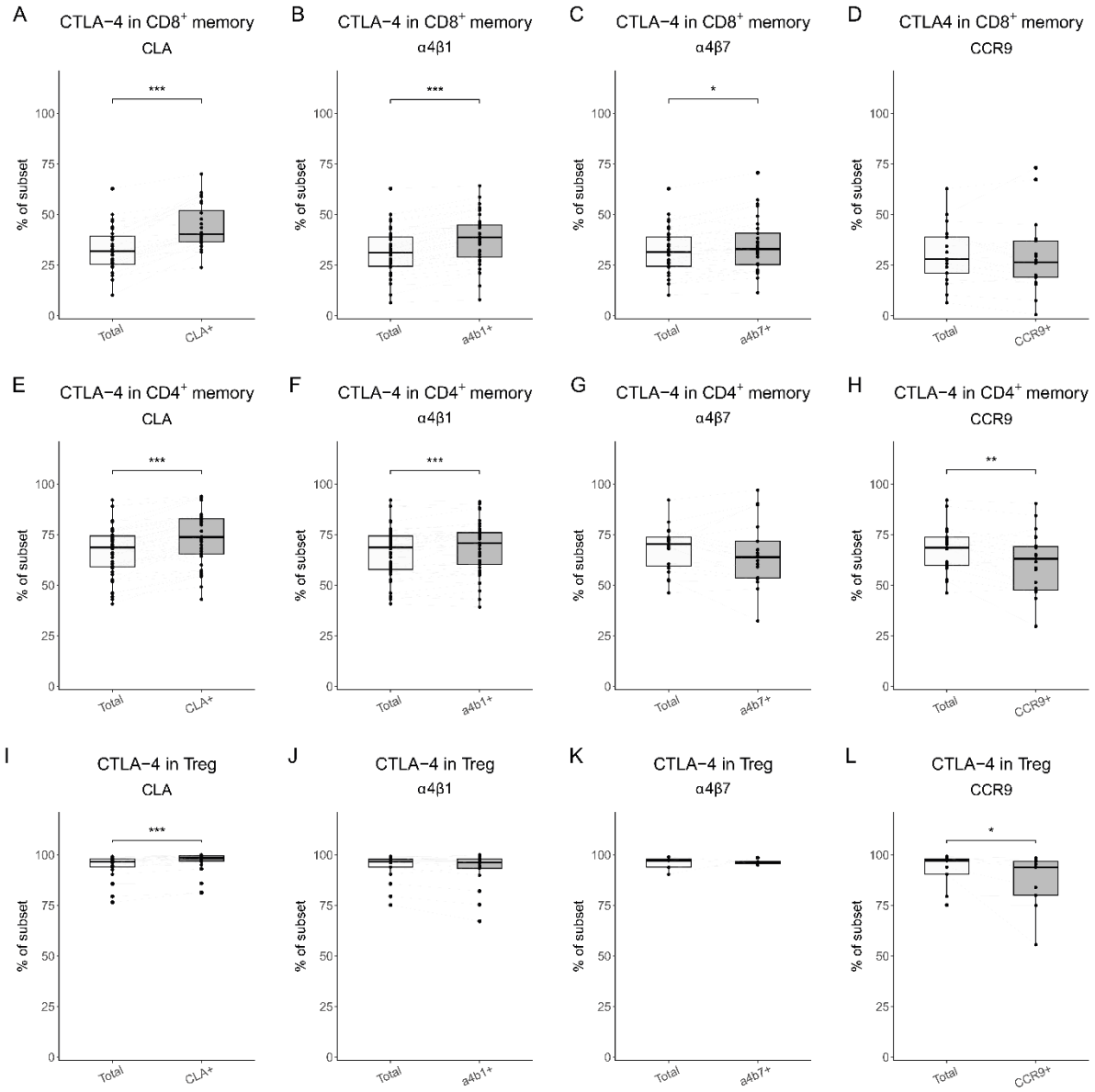


Figure S10: CTLA-4 expression in breastmilk T cells expressing homing receptors compared with total T cells (FACS, n=26). Frequency of CTLA-4$^+$ cells within CD8$^+$CD45RA$^+$ **(A-D)**, CD4$^+$CD45RA$^+$**(E-H)** and regulatory **(I-L)** breastmilk T cells comparing the total population with the subset of cells positive for the homing receptors CLA **(A, E, I)**, α4β1 **(B, F, J)**, α4β7 **(C,G,K)** and CCR9 **(D,H,L)**. Significance stars denotate if *=p<0.05, **=p<0.01, ***=p<0.001, Testing paired difference between expression in the total T-cell population and T-cell subset using Wilcoxon rank-sum test. Lines connect datapoints from the same breastmilk donor. Only samples with more than 50 cells within the homing receptor-positive populations were included.

Supplementary Table S2

Homing/Treg - 1 (figures: 1C; 4B-C; 5C; 6E-G; S1A; S2K; S5D,H-I; S6E-G; S9; S10)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	Ki67	Intracellular	Mip1	Agilent
	Blue 3	PerCP-Cy5.5	Integrin B7	Surface	FIB27	Biolegend
640	Red 1	APC	CCR9	Surface	LO53E8	Biolegend
	Red 1/1	AF700	Integrin B1	Surface	TS2/16	Biolegend
	Red 2	APC-Cy7	CD8	Surface	SK1	BD
405	Violet 1	PB	CLA	Surface	HECA-452	Biolegend
	Violet 2	BV510	TCRgd	Surface	B1	Biolegend
	Violet 3	BV605	CD3	Surface	UCHT1	Biolegend
	Violet 4					Biolegend
	Violet 5	BV711	CD45RA	Surface	HI100	Biolegend
	Violet 6	BV785	CD4	Surface	OKT4	Biolegend
561	Y/Gr 1	PE	CTLA4	Intracellular	BNI3	BD
	Y/Gr 2	PE-Cy594	FOXP3	Intracellular	259D/C7	BD
	Y/Gr 3					
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	Integrin A4	Surface	9F10	Biolegend

Cytokines (PMA/ionomycin stimulated) (figures: 4A; S4C, E-G)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	GZMB	Intracellular	GB11	BD
	Blue 3	PerCP-Cy5.5	IL13	Intracellular	JES10-5A2	Biolegend
640	Red 1	AF647	TNFa	Intracellular	MAB11	Biolegend
	Red 1/1					
	Red 2	APC-Cy7	CD8	Surface	SK1	BD
405	Violet 1	PB	CLA	Surface	HECA-452	Biolegend
	Violet 2	eFluor506	Live/dead			eBioscience
	Violet 3	BV605	CD3	Surface	UCHT1	Biolegend
	Violet 4					
	Violet 5	BV711	CD45RA	Surface	HI100	Biolegend
	Violet 6	BV785	CD4	Surface	OKT4	Biolegend
561	Y/Gr 1	PE	CD69	Surface	FN50	BD
	Y/Gr 2					
	Y/Gr 3					
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	IFNg	Intracellular	4S.B3	BD

CD8/T-RM (figures: 3B-D; 6A-B, D; S4A-B, S6C-D)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	CD11a	Surface	Clone 38	Biorad
	Blue 3	PerCP-Cy5.5	Granzyme K	Intracellular	GM26E7	Biolegend
640	Red 1	APC	KLRD1	Surface	HP-3D9	BD
	Red 1/1	AF700	CD3	Surface	UCHT1	Biolegend
	Red 2	APC-eF780	EOMES	Intracellular	WD1928	eBioscience
405	Violet 1	PB	Granzyme B	Intracellular	GB11	BD
	Violet 2	eFluor506	Dead cell stain			eBioscience
	Violet 3	BV605	CD103	Surface	Ber-ACT8	Biolegend
	Violet 4	BV650	CD8	Surface	RPA-T8	BD
	Violet 5	BV711	CD45RA	Surface	HI100	Biolegend
	Violet 6	BV785	CD4	Surface	OKT4	Biolegend
561	Y/Gr 1	PE	CCL5	Intracellular	VL1	Biolegend
	Y/Gr 2	PE-Dazzle594	CXCR6	Surface	K041E5	Biolegend
	Y/Gr 3					
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	CD69	Surface	FN50	BD

T cell subsets (figures: 1D-E; S1B-D; S2A-I)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	TCRgd	Surface	IMMU510	Beckman-Coultier
	Blue 3	PerCP-Cy5.5	CD8	Surface	RPA-T8	Biolegend
640	Red 1					
	Red 1/1	AF700	CD3	Surface	UCHT1	Biolegend
	Red 2	APC-eF780	CD27	Surface	O323	eBioscience
405	Violet 1					
	Violet 2	eFluor506	Live/dead			eBioscience
	Violet 3	BV605	CD31	Surface	WM59	BD
	Violet 4					
	Violet 5	BV711	CD45RA	Surface	HI100	Biolegend
	Violet 6	BV785	CD4	Surface	OKT4	Biolegend
561	Y/Gr 1					
	Y/Gr 2					
	Y/Gr 3	PE-Cy5	CD161	Surface	DX12	BD
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	TCR Vα7.2	Surface	3C10	Biolegend

Homing/Treg - 2 (figures: 5B,D; S2J; S6A-B)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	CD103	Surface	2G5	Beckman-Coultier
	Blue 3	PerCP-Cy5.5	PD-1	Surface	EH12.2H7	Biolegend
640	Red 1	APC	CCR7	Surface	G043H7	Biolegend
	Red 1/1					
	Red 2	APC-Cy7	CD8	Surface	SK1	BD
405	Violet 1	eF450	FOXP3	Intracellular	PCH101	eBioscience
	Violet 2	BV510	CD3	Surface	OKT3	Biolegend
	Violet 3	BV605	CXCR3	Surface	G025H7	Biolegend
	Violet 4					
	Violet 5	BV711	CD25	Surface	2A3	BD
	Violet 6					
561	Y/Gr 1	PE	CCR5	Surface	eBioT21/8	eBioscience
	Y/Gr 2	PE-Cy594	CXCR4	Surface	12G5	BD
	Y/Gr 3	PE-Cy5	CD4	Surface	RPA-T4	BD
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	CD69	Surface	FN50	BD

Sort staining (scRNAseq)

Laser	Channel	Fluorochrome	Antibody		Clone	Company
488	Blue 1	FITC	CD103	Surface	2G5	Beckman-Coultier
	Blue 3	PerCP-Cy5.5	CD25	Surface	BC96	Sony Biotechnology
640	Red 1	AF647	CD45RO	Surface	UCHL1	Biolegend
	Red 1/1		CD3	Surface	UCHT1	Biolegend
	Red 2	APC-Cy7	CD8a	Surface	SK1	BD
405	Violet 1	Sytox blue	Dead cell stain			Invitrogen
	Violet 2	BV510	TCRgd	Surface	B1	Biolegend
	Violet 3	BV605	CD127	Surface	A019D5	Sony Biotechnology
	Violet 4					
	Violet 5	BV711				
	Violet 6		CD4	Surface	OKT4	Biolegend
561	Y/Gr 1	PE	CD69	Surface	FN50	BD
	Y/Gr 2	PE-Cy594				
	Y/Gr 3	PE-Cy5				
	Y/Gr 4					
	Y/Gr 5	PE-Cy7	TCRVa7.2	Surface	3C10	Biolegend

Supplemental Acknowledgements

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