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# The Cellular Dynamics Of Early And Transitional Human Breast Milk

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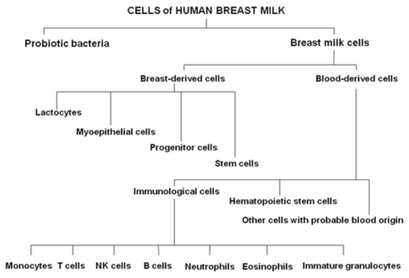
## Abstract

Breast milk (BM) is a complex fluid containing factors essential for infant nutrition and immunity. Breastfeeding has been shown to be protective against infections and other immune-mediated diseases during the lactation period and beyond in later childhood. This suggests that BM also imprints the neonatal immune system and influences long-term health. BM also contains populations of maternal-derived cells. Which factors in BM that are important for neonatal health and how they change during lactation have not been well-defined. In this study, we used a single-cell transcriptomic approach to identify and define cell types of early and transitional milk. We collected BM samples from mothers of infants 2-7 days (early milk) and 8-16 days (transitional milk) after delivery. We applied single-cell RNA sequencing on over 154,000 BM-derived cells. We identified 25 transcriptionally distinct populations of cells in the BM. As expected, the most abundant cells in BM were mammary epithelial cells and macrophages. Monocytes, T cells, dendritic cells, and neutrophils were also present and had a higher frequency in week 2, suggesting that some immune cells may remain abundant in the early days of lactation and slowly decline as milk matures. We also detected a small number of stem and progenitor, natural killer and B cells in the BM at a higher frequency in week 1. This work provides an atlas of the cellular component in human milk at two timepoints of lactation. In addition to cell identity and frequencies, we have also uncovered unique molecular pathways that are activated in BM cells. This work will lay the foundation for future studies of how these cells influence neonatal health.

## Introduction



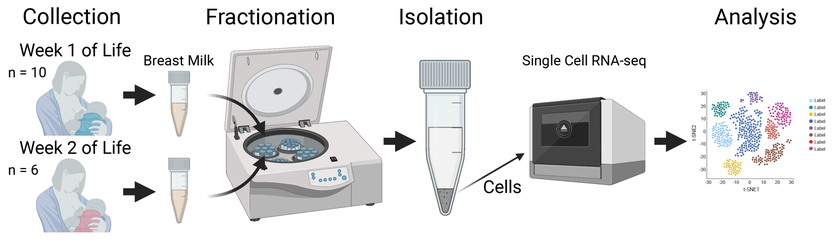

Witkowska-Zimny *et al*., 2019

Multiple studies have shown that human BM not only contains soluble bioactive factors, but also heterogeneous populations of maternal-derived epithelial, immune, and stem cell types. In our study, transcriptional profiling of BM cells revealed markers of inflammation, immune signaling and development, as well as cellular profiles and cellular subsets in BM that are dynamic over the first two weeks of infant life. We present how specific cellular factors can be clustered and identified as drivers of a shifting environment of development and inflammation.

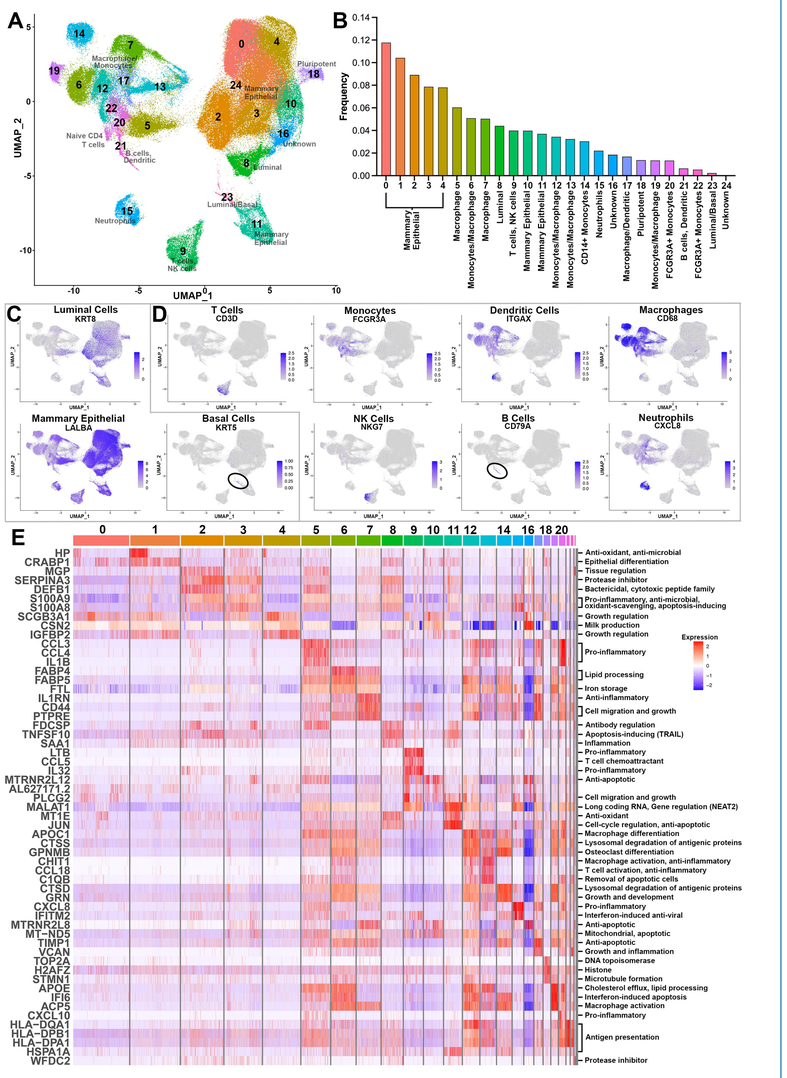
## Goal

To determine the complete cellular composition of human breast milk using single-cell RNA sequencing, and how these compositions change during the first two weeks of life. Understanding of human BM cells as milk production begins will provide a foundation for the importance and impact of BM cells on infant development.

## Approach

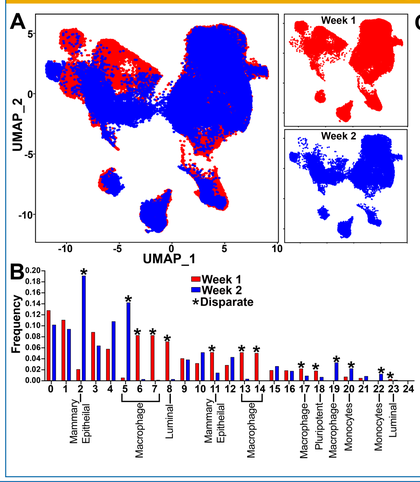



## Human breast milk contains a diverse array of cell types




(**A**) UMAP of a 16-sample aggregate clustering of human milk 154,132 cells labeled by cluster number (0-24) and dominant cell types. (**B**) Bar graph of the frequency of total cells within each cluster. Dominant cell type is listed below the bar. (**C**) UMAPS of cells expressing luminal cell marker *Krt8*, mammary epithelial cell marker *Lalba* and basal cell marker *Krt5*. (**D**) UMAPs of cells expressing T cell marker *Cd3d*, monocyte marker *Fcgr3a*, dendritic cell marker *Itgax,* macrophage marker *Cd68*, NK cell marker *Nkg7*, B cell marker *Cd79a*, neutrophil marker *Cxcl8*. (**E**) Heatmap of the top variable genes within each cluster and their functionality.

## Disparities in cell populations over time are driven by macrophage and epithelial cells




**C**

**Week 1**

| Cell type | Adjusted P-value |
|---|---|
| Microfold Cells | 3.38054E-13 |
| Osteoclasts | 9.38954E-07 |
| Monocytes | 4.93006E-06 |
| Dendritic Cells | 9.94123E-05 |
| Langerhans Cells | 0.000114009 |
| Macrophages | 0.000568012 |
| Alveolar Macrophages | 0.000686091 |
| Sebocytes | 0.000686091 |
| Osteoclast Precursor Cells | 0.002162355 |
| Platelets | 0.002890841 |

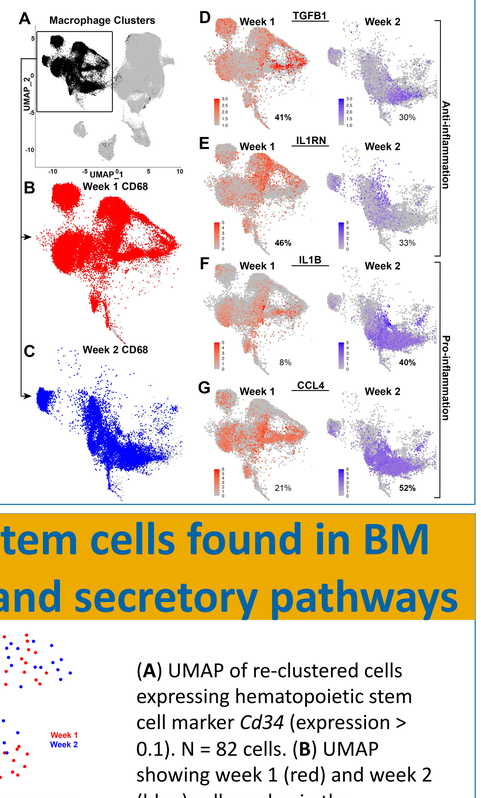
**Week 2**

| Cell type | Adjusted P-value |
|---|---|
| Ductal Cells | 2.88886E-07 |
| Mammary Epithelial Cells | 2.88886E-07 |
| Cholangiocytes | 6.17503E-06 |
| Basal Cells | 6.25787E-06 |
| Acinar Cells | 6.72726E-05 |
| Salivary Mucous Cells | 0.000680094 |
| Epithelial Cells | 0.000728958 |
| Goblet Cells | 0.000779234 |
| Langerhans Cells | 0.000779234 |
| Sebocytes | 0.000779234 |

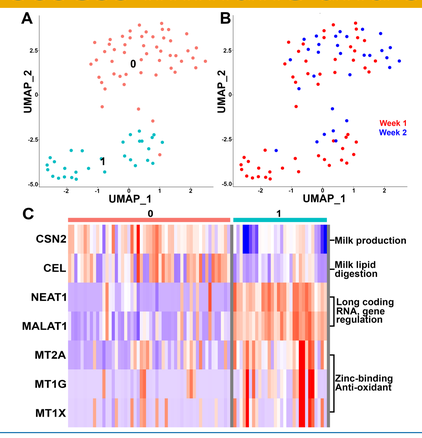
(**A**) UMAPs of the contribution from 92,395 week 1 (red) and 61,737 week 2 (blue) cells in the aggregate clustering profile. (**B**) Bar graph of the frequency of cells from each timepoint within each cluster. Disparate clusters have a higher frequency (> 50% different) of cells from one time point over the other. Dominant cell type is listed below the bar for clusters considered disparate. (**C**) Enrichment table of the cell pathway classifications for genes positively expressed in the entire aggregate at each time point. Classifications are ranked by significance of genes occurring in a classification pathway.

## Macrophage shift from an anti- to pro-inflammatory priority between weeks

(**A**) UMAP highlighting of the macrophage-rich clusters with disparities between timepoints (black). (**B**) UMAP of macrophage-rich clusters highlighting week 1 only cells expressing macrophage marker *Cd68* (red, expression > 0.5). (**C**) UMAP of macrophage-rich clusters highlighting week 2 only cells expressing macrophage marker *Cd68* (blue, expression > 0.5). (**D**) UMAP of macrophage-rich clusters showing week 1 cells expressing anti-inflammatory marker *Tgfb1*. (**E**) UMAP of macrophage-rich clusters showing week 1 cells expressing anti-inflammatory marker *Il1rn*. (**F**) UMAP of macrophage-rich clusters showing week 2 cells expressing pro-inflammatory marker *Il1b*. (**G**) UMAP of macrophage-rich clusters showing week 2 cells expressing pro-inflammatory marker *Ccl4*.




## Hematopoietic stem cells found in BM possess immune and secretory pathways




(**A**) UMAP of re-clustered cells expressing hematopoietic stem cell marker *Cd34* (expression > 0.1). N = 82 cells. (**B**) UMAP showing week 1 (red) and week 2 (blue) cell overlap in the clustering. (**C**) Heatmap of the expression level of the most variable genes for each cluster shown in (A). Genes are listed to the left of the rows; their functional involvement is listed to the right of the rows.

## Conclusions

- Single cell sequencing of over 154,000 breast milk-derived cells identified a diverse array of cell types.
- Epithelial cell clusters differed in milk production, anti-microbial, differentiation, and growth regulating genes.
- Immune cell clusters were defined by their inflammatory, antigen processing, activation, and remodeling genes.
- The abundance of macrophage in week 1 and epithelial cells in week 2, suggests a move from an immune-centric repertoire into a milk production repertoire as more epithelial cells differentiate into secretory mammary glands.
- Transition into milk production parallels a change in macrophage function from anti- to pro-inflammatory signaling.
- Hematopoietic stem cells present in BM at both weeks clustered into two groups, one possessing immune pathways and the other epithelial pathways. This suggests that, in the BM environment, HSCs may have broader differentiative capabilities that are governed by a shift into milk production.

Further study will implement a systems approach involving the soluble bioactive component, such as cytokines and growth factors.

## Acknowledgements

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