Expansion of mammary intraepithelial lymphocytes and intestinal inputs shape T cell dynamics in lactogenesis.

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ABSTRACT

Pregnancy brings about profound changes to the mammary gland in preparation for lactation. Changes in immunocyte populations that accompany this rapid remodeling are incompletely understood. We comprehensively analyzed mammary T cells through all parous stages, revealing a marked increase in CD4+ and CD8+ T effector cells in late pregnancy and lactation. T cell expansion was partly dependent on microbial signals and included an increase in TCRαβ+CD8αα+ cells with strong cytotoxic markers, located in the epithelium, that resemble intraepithelial lymphocytes of mucosal tissues. This relationship was substantiated by demonstrating T cell migration from gut to mammary gland in late pregnancy, by TCR clonotypes shared by intestine and mammary tissue in the same mouse, including intriguing gut TCR families. Putative counterparts of CD8αα+ IELs were found in human milk. Mammary T cells are thus poised to manage the transition from a non-mucosal tissue to a mucosal barrier during lactogenesis.

INTRODUCTION

The mammary gland is remarkable in its capacity to undergo multiple cycles of growth and regression during reproductive years. Mammary gland remodeling is largely driven by hormonal cues, resulting in stage specific tissue adaptations during puberty, pregnancy, lactation, and involution[1, 2, 3]. Pregnancy initiates proliferation of mammary epithelial cells, and ductal branching to support lactogenesis, a crucial process that ensures successful production and transfer of breast milk, essential for offspring health[4, 5]. The transition of the mammary gland into a secretory organ for the duration of lactation involves dramatic restructuring of cell composition and tissue enlargement, and increases its exposure to the outside environment, including microbes on maternal skin and offspring's oral cavity, rendering it a temporary barrier tissue[6, 7]. The end of lactation triggers mammary involution, a reversal to its non-secretory state marked by extensive apoptosis and tissue shrinkage[8, 9]. The physiological stress that accompanies the rapid transformation of the mammary gland across parous stages requires an extensive support network, including immunocytes, but the types of immunocytes involved and their functions in lactogenesis are unclear.

Innate and adaptive immunocytes are involved in immune regulation of the mammary gland at various developmental stages. Macrophages are required for mammary gland morphogenesis in puberty, alveologenesis in pregnancy, and tissue regression in involution[10, 11, 12, 13]. A specialized population of macrophages called ductal macrophages dominate the lactating mammary gland, and another subtype of macrophages, lactation-induced macrophages (liMacs), have been recently identified to support lactogenesis and milk production[11, 14]. B cells, specifically IgA and IgG producing plasma cells, are abundant in lactation and promote offspring health by shaping the antibody composition of milk. We and others have shown that IgA producing plasma cells can migrate from the intestine to the mammary gland in a CCL28 dependent manner[15, 16, 17, 18, 19]. Despite recent advances, our comprehension of various immunocyte types and their collaborative roles in facilitating mammary remodeling and regulating milk composition remains limited.

T cells play a fundamental role in tissue homeostasis by promoting defense, tolerance, tissue repair, and regulating cell proliferation and death. CD4+ T cells have been described in the mammary gland in puberty, and a subset of CD4+ T cells, RORγ+ regulatory T cells, increase

during involution[20, 21]. In addition, CD4+ and CD8+ T cells are present in milk[22]. Mammary γδ+ T cells with innate-like properties increase during lactation, and lack of these γδ + T cells can favor mammary oncogenesis[23]. During lactogenesis, several processes occur simultaneously, including epithelial cell stress from rapid expansion and milk production, and heightened exposure to the sudden increase in newly revealed self-antigens could create a conundrum for T cell tolerance, highlighting the need for T cell assistance. Yet, there is a large gap in our understanding of which T cell subsets are involved in lactogenesis, and how they contribute to tissue specific adaptations in the mammary gland during gestation, lactation, and involution.

We set out to define immunocyte changes in lactogenesis and uncovered novel T cell dynamics in the mammary gland. Gestation and lactation led to heightened activation of mammary gland T cells, including the expansion of CD8αα+ T cells with an intraepithelial location and with transcriptional characteristics of highly cytotoxic cells, thus closely mimicking the environment in mucosal tissues. We established the origin and microbe-dependency of T cell changes in lactation and identified human counterparts in milk, providing a detailed overview of T cell changes that accompany mammary gland remodeling across parous stages.

RESULTS

Lactation leads to increased T cell activation in the mammary gland.

During parous stages, mammary gland size and composition undergo substantial changes, yet there remains a gap in our understanding of how immunocytes adapt to, and perhaps facilitate, those transitions. We first quantified mammary immunocytes (CD45+) across parous stages by flow cytometry and found that pregnancy initiated a rapid increase in the total number of immunocytes, that was maintained in lactation, and involution (Fig 1A). To determine the expanding cell types and chart their adaptations, we performed a temporal analysis of immunocytes across different stages of parturition in female C57BL/6 (B6) mice; profiling mammary glands from nulliparous, gestation (G17), early lactation (day 3), and involution (day 21) stages, multiplexed into single cell RNAseq runs. These temporal datasets captured cells across all lineages with increased representation of T cell populations (Fig S1A). Validation by flow cytometry confirmed this expansion of T cells, while the proportion of myeloid cells, which dominated in the nulliparous, declined somewhat (while remaining the largest cell populations, as previously described[11, 14,

[21])(Fig 1B). Among myeloid cells, we observed the recently described lactation associated liMacs specifically during lactation[14](Fig S1B). To better understand the expanding T cell populations, we applied Louvain clustering[24], which parsed 6 clusters of T cells, which are annotated as naïve T cells (Tn), and effector T cells (Teff) that were either CD4+, CD8αβ+ or CD8α+CD8β- (CD8αα), and CD4-CD8-CD3+ T cells (Double negative, DN) (Fig 1C). There was a strong shift in the relative proportions of naïve vs activated states of CD4+ T cells and CD8+ T cells, from mostly naïve T cells before and during gestation to mostly activated states during lactation, with a particularly striking increase in CD8αα+ Teff in late pregnancy that persisted during involution (Fig 1D). In contrast to the CD8αβ+ Teff cells characterized by *Itgb1* and *Cxcr6* expression, CD8αα+ Teff cells expressed high levels of *Klrb1c*, *Itgae*, *Gzmb*, and *CD160*, suggesting increased cytotoxic potential (Fig 1E). The transcriptional signature of CD8αα+ cells was highly reminiscent of CD8αα+ T cells that have innate properties and reside in the epithelium of mucosal tissues, referred to as intraepithelial lymphocytes (IELs) (Fig 1C). CD8αα+ T cells mostly differentiate in the thymus in response to strong agonists and use either TCRαβ or TCRγδ, but mammary CD8αα+ T cells were mostly TCRαβ+ (Fig S2A). The changes in T cell populations across parous stages were consistent across multiple mice from three independent single cell experiments (Fig S3). Flow cytometric validation confirmed that gestating and lactating mammary glands displayed a significant increase in CD4+ Teff, CD8+ Teff, and DN populations (denoted by CD69+ CD44+ staining) (Fig 1F). CD8αα+ Teff cells were a major T cell population in early lactation compared to nulliparous mice (Fig 1F). Interestingly, while regulatory T cells (Tregs) increased during gestation, lactation led to a significant drop in Treg proportions and numbers (Fig S2B, C), followed by an increase in involution. Thus, mammary gland remodeling is accompanied by strong changes in T cell populations, including a hyper-activated state and increased CD8αα+ Teff cells in lactation, presumably in response to exposure to the outside environment.

CD8αα+ Teff cells in the mammary gland are intraepithelial.

CD8αα+ IELs are highly abundant in the small intestine, and most of our understanding of this cell-type stems from intestinal studies. We compared the expression of classical CD8αα+ IEL genes such as *Tyrobp*, *Fcer1g*, *Itgae*, *Gzmb*, *Klrb1c*, and *Klra1* across mammary T cell subsets[25] and found increased representation of these genes in mammary CD8αα+ Teff cells but not their

CD8αβ+ Teff counterparts (Fig 2A). A similar signature has been described in TCRαβ+ innate-like T cells with high cytotoxic potential in mammary tumors called αβILTCKs, with high expression of *Fcer1g*, *Gzmb* and *Klrb1c*[26]. However, high expression of *Fcer1g* has also been reported in intestinal IEL populations[25, 27, 28], and in self-reactive thymic CD8αα+ T cells with innate characteristics[29]. We analyzed the expression of classical IEL genes in CD8 T cells from the mammary gland, small and large intestine, and spleen. Most IEL genes, *Tyrobp*, *Fcer1g*, *Itgae*, *Gzmb*, displayed similar expression in mammary gland, small intestine and large intestine but not the spleen (Fig 2B). The expression of killer cell lectin-like receptor (Klr) family genes such as *Klra1* and *Klrb1c*, were higher in mammary CD8 T cells compared to intestinal tissues (Fig 2B). We validated two of the classical IEL markers used above by flow cytometry, Ly49a (*Klra1*) – a killer cell lectin like receptor that binds MHC-I, and CD103 (*Itgae*), an integrin that mediates cell adhesion and tissue retention by binding to E-cadherin on epithelial cells. There was a significant increase in the proportion of Ly49a+CD103+ CD8αα+ T cells in lactating mammary glands compared to nulliparous mice (Fig 2C). Based on the gene expression profiles, we hypothesized that the CD8αα+ T cells in the mammary gland could be IELs. Since the defining characteristic of IELs is their residence in the epithelial layer, we surveyed the physical location of the CD8αα+ T cells in the mammary gland tissue. Indeed, we observed CD8α+ T cells adjacent to Epcam+ epithelial cells, in markedly increased numbers during late pregnancy and early lactation (Fig 2E). Overall, our results demonstrate that mammary CD8αα+ TCRβ+ cells are true IELs, with marked phenotypic similarity to intestinal IELs. IELs are associated with mucosal tissues, and the increase in mammary CD8αα+ IELs is consistent with the temporary mucosal state of the reconfigured mammary gland.

T cells migrate from the intestine during gestation.

Intestinal CD8αα+ IELs arise from thymic IEL progenitors (IELp) that are DN T cells expressing either PD-1 or T-bet[30]. IELps acquire the expression of gut-homing receptors in the thymus through agonist stimulation by self-antigens and express CD8αα upon seeding the intestinal epithelium[31]. To test whether the strong increase in mammary gland CD8αα+ IELs stemmed directly from the thymus, we thymectomized female mice before pregnancy (4 weeks of age) and assessed

mammary gland CD8αα+ IELs during lactation. Importantly, there were no differences between thymectomized and control mice in either proportions or numbers of CD8αα+ IELs in the lactating mammary gland (Fig 3A, S3A). There were also no differences in either PD-1+ or T-bet+ IELps in the lactating thymus when compared to nulliparous mice (Fig 3B). This raises two possibilities: either thymic progenitors seed the mammary gland before 4 weeks of age and these few T cells expand into CD8αα+ IELs during pregnancy, or mammary CD8αα+ IELs are of extrathymic origin, potentially from other mucosal sites, and migrate en masse to the mammary gland during late pregnancy. We recently used Kaede photoconvertible mice to track the migration of immunocytes from the intestines to other body locations[32]. Among migratory intestinal cells in the spleen was a small population of IEL-like CD8αα+ TCRαβ+ cells, which led us to hypothesize that mammary CD8αα+ IELs could be of intestinal origin. To test this hypothesis, intestinal sections (small and large intestine) of Kaede mice were photoconverted at different times of gestation and early lactation, and mammary glands were analyzed 24hrs later. Red T cells of intestinal origin had indeed migrated to the mammary gland, including all three CD8αα+, CD4+ and CD8αβ+ T cell types (Fig 3C). Thus, several types of intestinal effector T cells populate the mammary gland during late gestation and early lactation.

Shared T cell clones between intestinal IELs and mammary IELs.

To further establish the relationship between intestinal and mammary IELs, we analyzed the αβTCR clonotypes expressed by T cells in both locations. First, we used single-cell TCRseq to compare αβTCR pairs displayed by IELs (small and large intestine) and mammary gland T cells in the same mice (two virgin and two lactating, 709 and 2248 total mammary gland cells, respectively). Overall, the data showed unremarkable V and J region usage, CDR3 length and N region diversity frequencies. Canonical TRAV11/TRAJ18 TCRs were relatively abundant among lactating mammary T cells, mostly in CD4-CD8- DNs (2.2 and 8.1% of total cells, Fig. S4A). Rarefaction analysis revealed a notable degree of clonal amplification in different T cell-types from the lactating mammary gland (Fig S4B), with much mouse-to-mouse variation. We identified 13 instances of small intestine and mammary T cells sharing the exact same TCR clonotypes in T cells (Table S1). These shared clonotypes were defined by full nucleotide sequence identity, and were completely absent when comparing different mice, indicating that they stemmed from the

same T cell clones present in both mammary gland and small intestine (and large intestine, for some). The clonotypes shared with the intestines accounted for 4.3% and 0.6% of mammary T cells in the two lactating mice, certainly an underestimate given incomplete sampling. As indicated in Fig. 4A and Table S1, clonotypes present in both small intestine and mammary gland belonged to several cell types, indicating that the exchange between tissues involved different T lineages. Clonotypes shared between intestine and mammary gland were also observed in nulliparous females (2.2 and 0.9% of mammary T cells in the two mice profiled). Thus, exchanges of T cells between intestine and mammary gland pre-exist the onset of lactation.

These clonotypic analyses also revealed the sharing of a particular intriguing group of cells. For a broader comparison of small intestine cells, we leveraged TCR sequence data of intestinal IELs generated in the ImmGenT program[33], and used the TCRdist3 algorithm[34] to compute a matrix of distances between αβTCR clonotypes. This revealed two prominent TCR families within the small intestinal IEL compartment (Fig. 4B), whose over-representation was striking because of the recruitment of highly related TCRs with little clonal amplification, as denoted by subtly different nucleotide sequences. These TCR families were found in many independent samples, and one indeed corresponded to the previously reported "Revere" family[35] (we hereafter name the second family "Newbury" for consistency). Revere TCRs are mostly conserved in the CDR3b region, with exclusive usage of TRAJ22 and TRBJ1-4 (Fig. 4C, S5A), while the Newbury family is mostly conserved in the CDR3a region. Notably, these two families are almost exclusively represented in the intestinal CD8αα+ IELs, amounting to a few percent of T cells (Fig. S5B), and these highly identical TCRs of the CD8αα+ IELs are likely selected repeatedly by self-reactivity, in line with well-known selection of CD8αα differentiation by self-reactive transgenic TCRs[36, 37, 38]. Interestingly, Revere and Newbury TCRs were also observed in CD8αα+ IELs of the mammary gland (3 of each), with all the key sequence characteristics (Fig. 4D, S5C). Thus, peculiar TCR families of intestinal CD8αα+ IELs are found in IELs of the lactating mammary gland. Importantly, outside of the intestine and mammary gland, CD8αα+ IELs did not display Revere or Newbury family TCRs, as analyzed by the ImmGenT program (Fig. 4E, chisq.test p=6.10-4)). Together, CD8αα+ IELs in the lactating mammary gland displayed TCRs otherwise exclusive to intestinal IELs, as part of a broader exchange of T cell clones between the intestine and mammary gland.

IEL-like cells are present in human mammary gland and human milk.

We assessed whether mammary IELs were conserved across species by profiling T cells in human breast tissue and human milk. First, we used previously published scRNAseq datasets[39] and found that human breast tissue contains CD8αα+ Teff-like cells (marked by expression of *ITGAE*, *CD94*, *CD160* and *NKG2D*) (Fig 5A, B). The presence of CD4 and CD8 T cells in milk has been reported before[22], but with no further characterization. To ask whether human milk contains CD8αα+ IEL-like cells, we analyzed fresh milk samples from lactating women. Multiparameter flow cytometry revealed both CD4+ and CD8+ T cells in all samples. In addition, we found CD8αβ+ T cells and CD8αα+ IEL-like cells in human milk, including cells that expressed CD103 (ITGAE), CD94, and NKG2D (Fig 5C-E). Overall, we identified human counterparts of mouse mammary CD8αα+ IELs in human breast and milk.

Microbiota influence activation states and gene expression patterns in mammary CD8aa+ IELs.

The classical function of IELs is to maintain barrier immunity, which raised the question of microbe-dependence of the CD8αα+ IELs in the lactating mammary gland, particularly because the transition involves exposure to environmental microbes. Lactating mammary glands in mice devoid of microbes, germ-free (GF), had much fewer total CD45+ cells compared to lactating specific pathogen free (SPF) mice (Fig 6A). To further determine population and transcriptional changes, we performed multiplexed scRNAseq on lactating mammary glands from GF or SPF mice. GF mice showed decreased proportions of CD4+ Teff and CD8αβ+ Teff cells compared to SPF mice (Fig 6B), and correspondingly increased proportions of cells with a naïve (*Sell*+, *Ccr7*+, *Il7r*+) phenotype. In contrast, CD8αα+ IELs were equally represented in the GF mammary glands (Fig 6B, C). However, the numbers of CD8αα+ IELs were lower in GF mice, given the decrease in total CD45+ cells (Fig 6D). The decrease in mammary CD8αα+ IELs was not due to decreased intestinal IELs, as GF mice displayed no differences in intestinal IEL numbers (Fig S6A). Validation by flow cytometry confirmed the decrease in CD4+ Teff and CD8αβ+ Teff populations observed by single cell sequencing (Fig 6D). Thus, the T cell populations that surge during the transformation of the mammary gland seem to include two components: one microbe-responsive, and one microbe independent (plausibly self-reactive). We then performed differential gene expression between SPF and GF CD8αα+ IELs and found several transcriptional differences,

where CD8αα+ IELs from GF mice displayed a significant decrease in *Il6ra*, *Cd5*, *Thy1*, *Cd160*, and *Klra1* (Fig 6E). Both *Cd5* and *Thy1* are classic thymic IELp markers and *Cd160* is associated with gut-homing and tissue retention of CD8αα+ IELs. Taken together with the total drop in CD45+ cells, this suggests that influx of immunocytes into the mammary gland may require microbial signals. It is plausible that the defect in mammary IELs may stem from migrating intestinal IELs that lack the same functionality, reflecting the role of intestinal microbes in priming mammary IELs as opposed to skin microbes. Nevertheless, our data points to the importance of commensal microbes in driving mammary gland T cell adaptation during lactation.

DISCUSSION

We report a dynamic atlas of T cells whose changes accompany the adaptation of the mammary gland to a mucosal state in lactation, with an increase in activated CD4+ and CD8+ T cells, and intraepithelial CD8αα+ T cells. Mammary CD8αα+ IELs were not dependent on thymic IELps during pregnancy, and shared TCR repertoires with intestinal CD8αα+ TCRαβ+ IELs, suggesting T cell migration from the intestine to the mammary gland. CD8αα+ IELs may be conserved across species since human breast tissue and milk contained T cells with a similar signature. Finally, we found that T cell activation and gene signature of CD8αα+ IELs in lactation were under microbial control, suggesting that the observed T cell changes could be geared towards promoting barrier immunity during lactation.

Intestinal CD8αα+ TCRαβ+ IELs regulate epithelial cell turnover and promote microbial tolerance[25], two functions that mammary CD8αα+ IELs could be involved in to promote lactogenesis. The TCR specificity of CD8αα+ IELs is directed towards self-antigens[36, 37, 38], and in line with that, we found two families of TCRs that are repeatedly generated and selected across mice, and were found in the small intestine and lactating mammary gland. These results suggest that mammary CD8αα+ IELs are poised to respond to the rapid epithelial cell proliferation, or to the plethora of self-antigens that are present in milk and as a result mammary gland. During pregnancy and lactation, the expression of gut-homing markers Ccl25, Ccl28, and MAdCAM1, increase in the mammary tissue which could lead to T cell migration to the mammary gland[19, 40, 41]. However, whether the expression of these homing markers change in the intestine during

pregnancy and lactation which mediate T cell egress, and whether hormones influence mammary homing markers needs to be further explored.

Apart from lactogenesis, CD8αα+ IELs could be involved in post-lactation oncogenesis. Similar populations of NK like unconventional T cells such as αβILTCKs and NK-like γδ T cells, have been shown to be important in suppressing mammary oncogenesis[23, 26]. Mammary CD8αα+ like T cells are present in human milk but their function in milk is unclear. One possibility is that they are passive, and their presence is linked to epithelial cell sloughing in lactogenesis, which increases epithelium-associated CD8αα+ T cells in milk. Maternal T cells have been previously shown to colonize neonatal intestines[42], raising the other possibility that milk CD8αα+ IELs could migrate into and colonize the neonatal intestine to promote barrier protection.

Pregnancy induces an immunosuppressive state to maintain maternal-fetal tolerance and support successful pregnancy outcomes[43]. However, in the mammary gland, we observe an increase in T cell activation in late pregnancy and early lactation presumably to prepare for increased microbial exposure in lactation. Consistent with this idea, activated T cells decrease in the GF mammary gland, and the drop in mammary Tregs in lactating SPF mice is not observed in GF mice (Fig S6B). Interestingly, liMacs were also reduced in lactating GF mammary glands[14], suggesting that microbes or microbe-derived signals can influence multiple immunocyte-types involved in mammary remodeling.

In summary, we have characterized novel T cell changes in lactogenesis and provide evidence for T cell migration potentially along the entero-mammary axis. Our results could provide new strategies to improve maternal defense and tolerance during and post-lactation.

METHODS

Mice

C57BL/6 (B6) mice were purchased from Jackson Labs and maintained in specific pathogen free conditions at Harvard Medical School and Salk Institute for Biological Sciences. Germ free mice were purchased from the University of California San Diego. Kaede reporter mice were obtained from O. Kanagawa (RIKEN, Wako, Japan) and maintained on the B6 background (Tomura et al., 2008; Morton et al., 2014). Nulliparous mice were littermate controls of mice profiled at

pregnancy, lactation, or involution. For timed pregnancies, female B6 mice were set up at 7-8 wks of age with male B6 mice, female mice with plugs were separated and housed individually for the duration of pregnancy, and mammary glands were profiled at day 12 (G12), day 17 (G17) of pregnancy, lactation day 3-5 (L3), and involution day 1 (I), which was one day after weaning of pups at day 21. All experiments were performed following guidelines listed in animal protocols (IS00001257, Harvard Medical School) and (23-00007**,** Salk Institute for Biological Studies) approved by the Institutional Animal Care and Use Committee.

Preparation of lymphocytes and flow cytometry.

Mammary gland: Inguinal lymph nodes were removed and mammary glands 3, 4 and 5 were collected, minced and dissociated in collagenase solution (3mg/mL collagenase type II (Sigma C6885) and 2% FBS in DMEM) in a 37˚C shaking water bath for 20 min with manual shaking every 5 min, followed by red blood cell lysis. Single cell suspensions were filtered and washed with 2% DMEM solution.

Thymus and LN: Lymphocytes from thymus and inguinal lymph nodes were obtained by mechanical disruption, filtered and washed with 10% RPMI solution.

Intestines: Small and large intestinal tissues were measured, cleaned, and treated with RPMI containing 1mMDTT, 20mMEDTA and 2% FBS at 37˚C for 15 min to remove epithelial cells, minced and dissociated in collagenase solution (1.5mg/mL collagenase II (GIBCO), 0.5mg/mL dispase and 1%FBS in RPMI) with constant stirring at 37˚C for 40min. Single cell suspensions were filtered and washed with 10% RPMI solution.

Staining: Single cell suspensions of cells resulting from tissue dissociations were stained with different panels of antibodies with surface markers for CD45, CD4, CD8a, CD8b, TCRβ, TCRδ, NK1.1, Ly49, CD103, Thy1, PD-1, CD122, CD5, CD69, CD44, and CD62L, Zombie UV Fixable Viability and intracellular markers for T-bet and Foxp3. For intracellular staining of transcription factors, cells were stained for surface markers and fixed in eBioscience Fix/Perm buffer overnight, followed by permeabilization in eBioscience permeabilization buffer at room temperature for 45 min in the presence of antibodies. Cells were acquired with a BD LSRII or BD FACSymphony A3 and analysis was performed with FlowJo 10 software.

Single cell RNA and TCR sequencing

T cells (DAPI-CD3+CD44+TCRβ+) were sorted from the mammary gland, intestines, spleen and thymus (DAPI-CD3+ TCRβ+CD122+Thy1+) using a BD FACSAria after hashtagging with Biolegend TotalSeq-C reagents, and samples were pooled for encapsulation (10X Chromium). Libraries were prepared using Chromium Single cell 3' reagents kit v2, TCR and hashtag libraries were processed as described (49).

Imaging and Microscopy

Mammary tissues were harvested and fixed in 20% Sucrose 4% Paraformaldehyde (PFA) solution in PBS overnight at 4°C. They were then embedded in OCT compound for cross sectioning and stored at -80°C. Sections (10µm) were blocked in 10% Fetal Bovine Serum, 2% Normal Mouse Serum, 1:100 TruStain FcX (CD16/32), 0.1% Tween-20 TBS for 1 hour at RT and incubated for 3 hours at RT or overnight at 4°C with primary antibodies. Tissue was washed and, when needed, incubated with fluorescently labelled streptavidin for 2 hours before nuclear staining with DAPI. Images were acquired using a THUNDER (Leica) inverted widefield microscope with HC PL APO 63x/1.40-0.60 OIL objective. Signal from DAPI, FITC, TRITC, Cy5 and Cy7 was obtained by illuminating the sample with 390 nm, 475 nm, 555 nm, 575 nm, 635 nm, 747 nm excitations wavelengths respectively. Signal detector was composed of 2 QUAD cubes DFT5 for DAPI/FITC/TRITC/Cy5 and CYR7 for DFP/YFP/Texas Red/Cy7. Images for each experiment were collected under identical imaging conditions using Leica LAS X Software. All images were analyzed using FiJi (v2.0.0-rc-69/1.52i) (Schindelin, J., et al., 2012).

Human Milk

5 mL of human milk were received from UC San Diego HMB Biorepository and diluted 1:1 with PBS. Milk samples were centrifuged to remove lipid and whey layers. Remaining cells were stained using viability dye, CD4, CD94, CD8β, CD3, TCRγδ, CD44, CD45, CD8α, CD103, and NKG2D. Cells were analyzed using BD LSRFortessa or BD FACSymphony A3 and analysis was performed with FlowJo10 software.

Quantification and Statistical Analysis

Data are presented as mean ± SEM. Unless stated otherwise, significance was assessed by Student's t-test or one-way Anova in GraphPad Prism 8.0.

Data availability

Single cell sequencing data are available in NCBI with accession number XXXXX.

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Figure 1

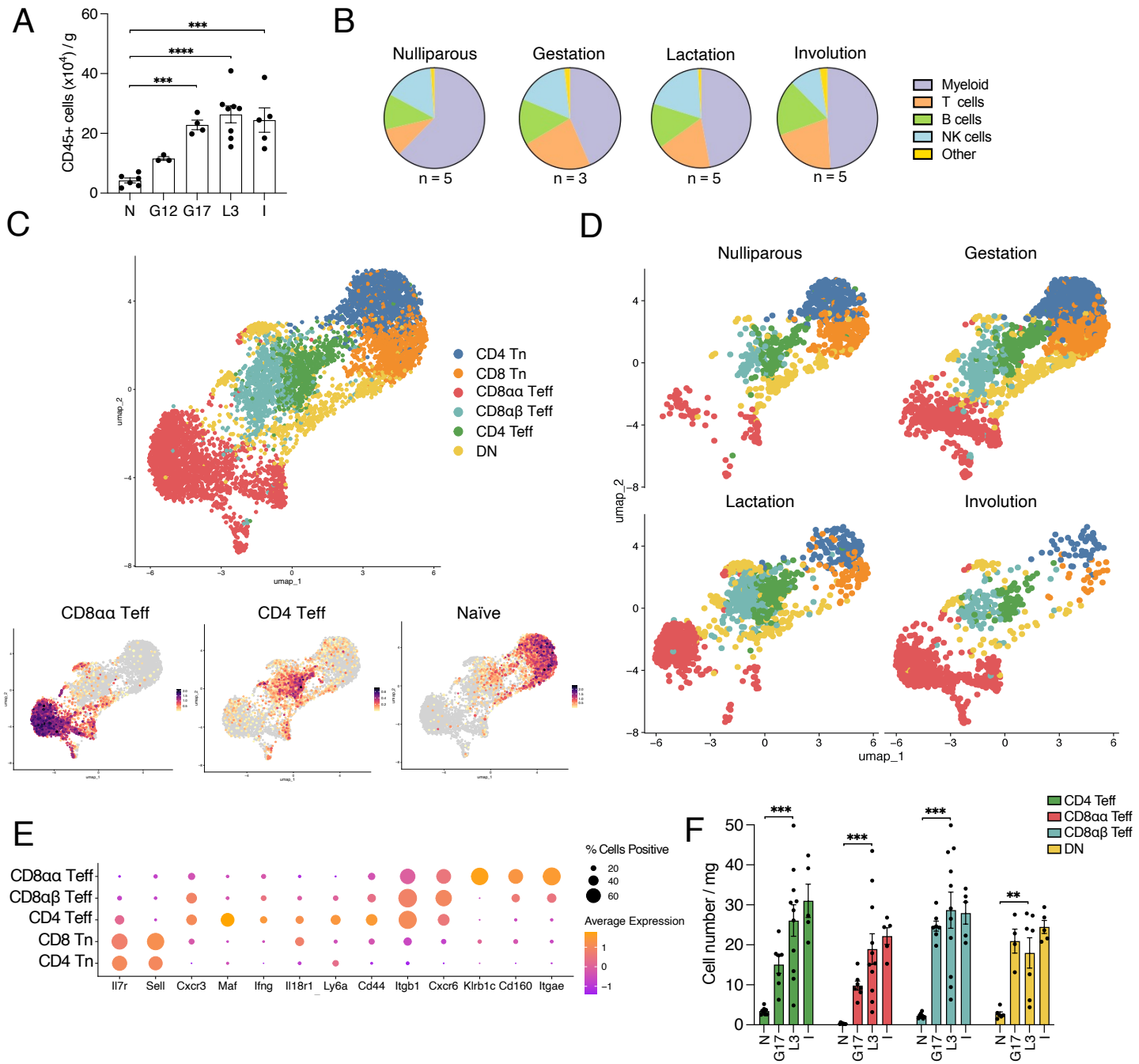

A
CD45+ cells (x10^4) / g
60
40
20
0
***
****
***
N
G12
G17
L3
I
B
Nulliparous
Gestation
Lactation
Involution
n = 5
n = 3
n = 5
n = 5
Myeloid
T cells
B cells
NK cells
Other
C
umap_1
umap_2
CD4 Tn
CD8 Tn
CD8αα Teff
CD8αβ Teff
CD4 Teff
DN
CD8αα Teff
CD4 Teff
Naïve
D
Nulliparous
Gestation
Lactation
Involution
umap_1
umap_2
E
CD8αα Teff
CD8αβ Teff
CD4 Teff
CD8 Tn
CD4 Tn
Il7r
Sell
Cxcr3
Maf
Ifng
Il18r1
Ly6a
Cd44
Itgb1
Cxcr6
Klrb1c
Cd160
Itgae
% Cells Positive
20
40
60
Average Expression
1
0
−1
F
CD4 Teff
CD8αα Teff
CD8αβ Teff
DN
Cell number / mg
50
40
30
20
10
0
***
***
***
**
N
G17
L3
I

Figure 1. Late gestation and lactation lead to increased Teff populations in the mammary gland.

A) Quantification of total number of CD45+ cells normalized to weight across parous stages by flow cytometry.

B) Representative proportions of major immune cell types in the mammary gland across parous stages quantified by flow cytometry.

C) UMAP projection of mammary T cells from mammary glands of all stages with feature plots of specified T cell gene signatures.

D) UMAP projection of mammary T cell populations in (C) split by stages: nulliparous, gestation (G17), lactation (L3), and involution (I).

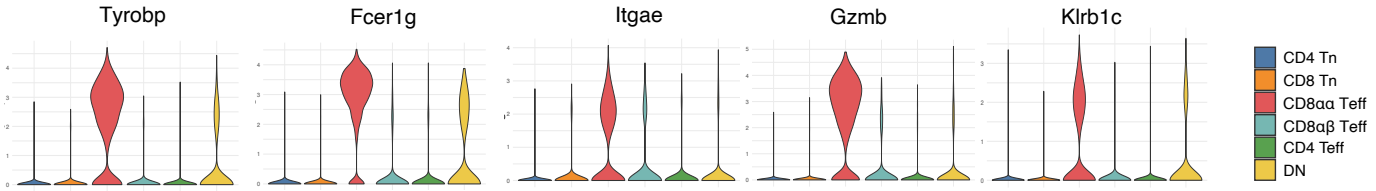
E) Dot plot of selected highly expressed genes in T cell clusters identified in (C).

F) Quantification of total cell numbers normalized to weight, of different T cell populations identified in (C) by flow cytometry. Teff populations were determined as CD4 Teff: CD4+ CD44+ CD62L-. CD8αα Teff: CD8α+ CD8β- CD44+ CD62L-. CD8αβ Teff: CD8α+ CD8β+ CD44+CD62L-, DN: TCRβ+ CD4- CD8α-.

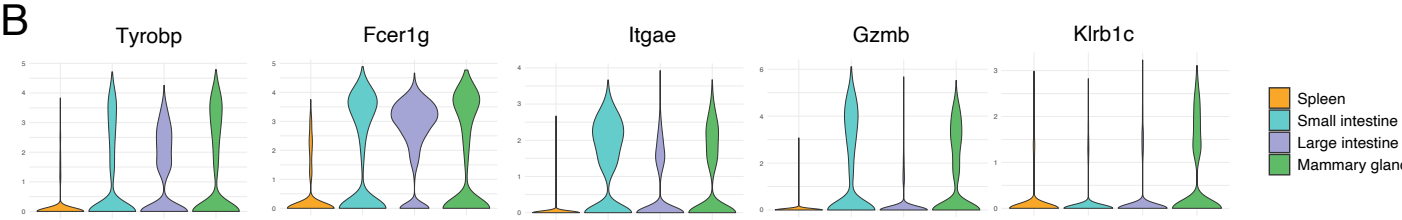
**$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by one-way ANOVA (A) and student's t-test (F).

Figure 2

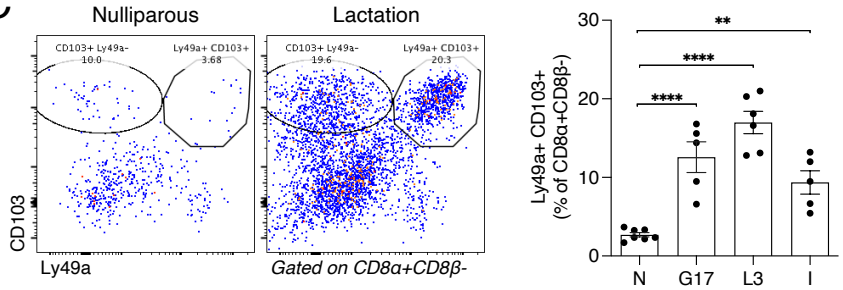
A



B



C



D

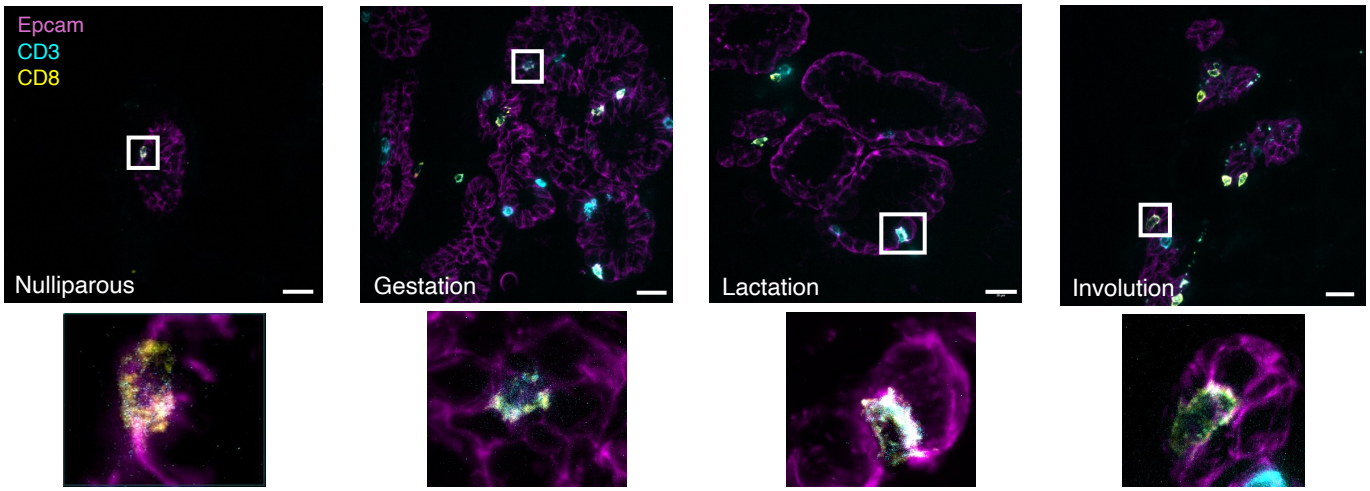


Figure 2. Mammary CD8aa+ T cells are intraepithelial lymphocytes.

A) Violin plots depicting gene expression of classical IEL markers in different T cell populations from figure 1C.

B) Violin plots depicting gene expression of classical IEL markers in CD8+ T cells from spleen, small intestinal epithelium, large intestinal epithelium and mammary gland.

C) Representative flow cytometry plots and quantification of CD103+Ly49+CD8αα+ T cells (gated on TCRαβ+CD8α+CD8β-) across parous stages in the mammary gland.

D) Representative immunoflourescence images of the mammary gland at nulliparous, gestation (G17), lactation (L3), and involution (I) of the epithelium (Epcam+ in magenta), and T cell markers, CD8α (yellow) and CD3 (cyan). Selected insets show the presence of CD8α+ CD3+ cells within the epithelial layer. Scale bar = 20μm.

**$p<0.01$, ****$p<0.0001$ by student's t-test.

Figure 3

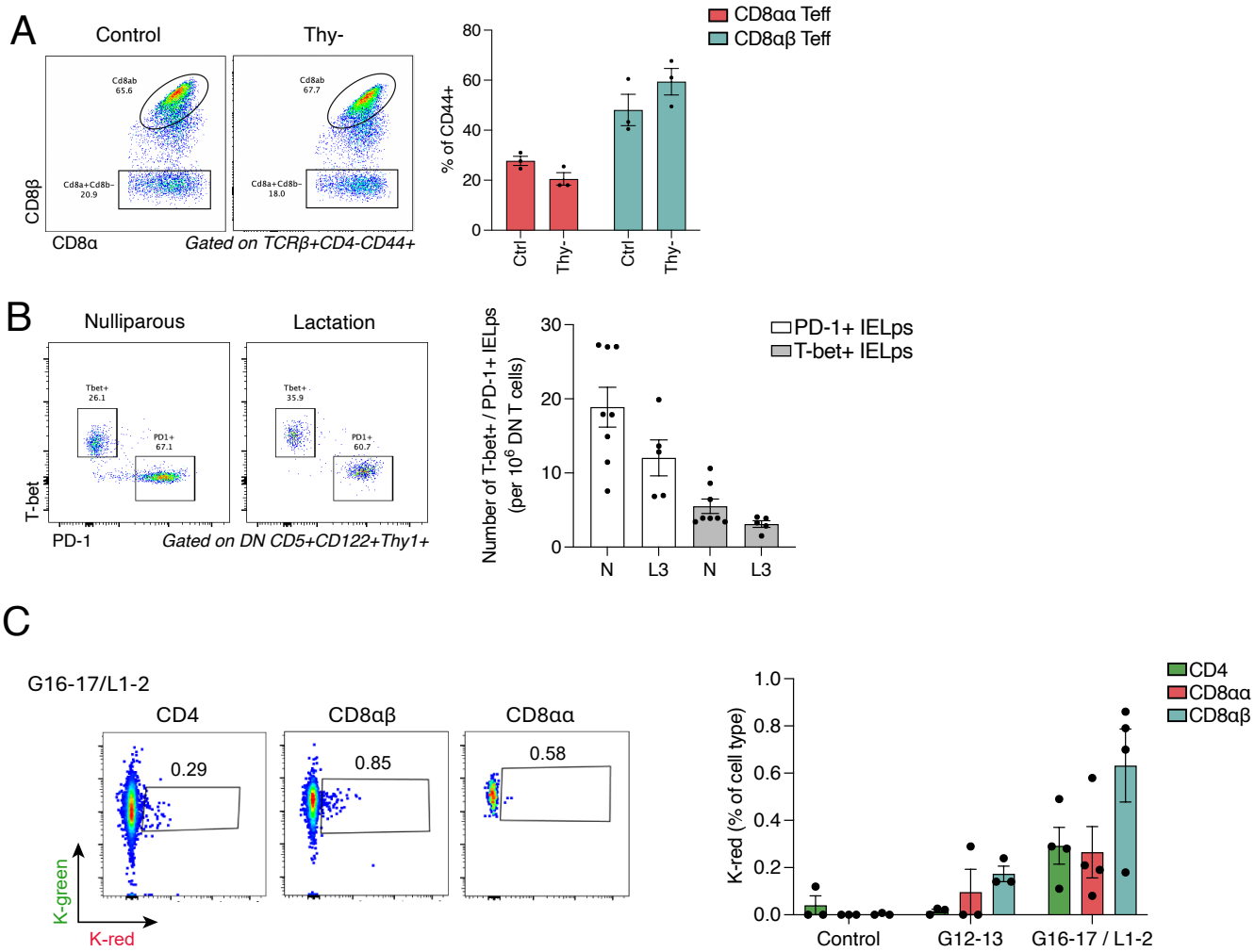

A
Control
Thy-
Cd8ab
65.6
Cd8a+Cd8b-
20.9
Cd8ab
67.7
Cd8a+Cd8b-
18.0
CD8β
CD8α
Gated on TCRβ+CD4-CD44+
CD8αα Teff
CD8αβ Teff
% of CD44+
80
60
40
20
0
Ctrl
Thy-
Ctrl
Thy-
B
Nulliparous
Lactation
Tbet+
26.1
PD1+
67.1
Tbet+
35.9
PD1+
60.7
T-bet
PD-1
Gated on DN CD5+CD122+Thy1+
PD-1+ IELps
T-bet+ IELps
Number of T-bet+ / PD-1+ IELps
(per 10^6 DN T cells)
30
20
10
0
N
L3
N
L3
C
G16-17/L1-2
CD4
CD8αβ
CD8αα
0.29
0.85
0.58
K-green
K-red
CD4
CD8αα
CD8αβ
K-red (% of cell type)
1.0
0.8
0.6
0.4
0.2
0.0
Control
G12-13
G16-17 / L1-2

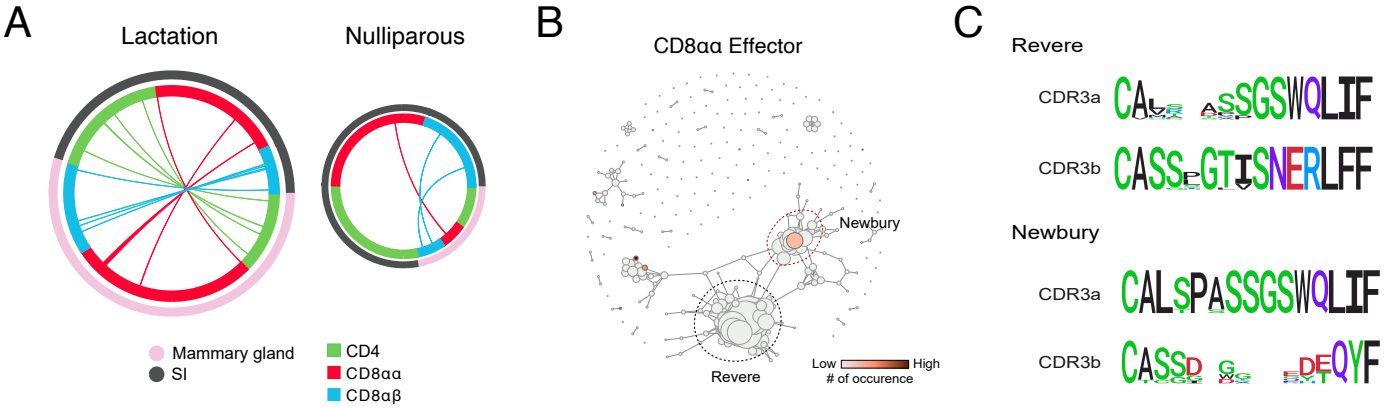
Figure 3. Intestinal T cells migrate to the mammary gland during late gestation and lactation.

A) Representative flow cytometry plots and quantification of CD8αα+ Teff and CD8αβ+ Teff cell populations (gated on TCRβ+ γδ- CD4- CD8α+ CD44+ CD62L-) in control and thymectomized lactating mice.

B) Representative flow cytometry plots and quantification of total number of T-bet+ and PD-1+ IELps (gated on TCRβ+ CD4- CD8α- CD5+ CD122+ Thy1+) in nulliparous and lactating (L3) thymus.

C) Representative flow cytometry plots and quantification of kaede red cells within T cell populations (gated on TCRβ+ followed by either CD4+ or CD8β+ or CD8α+ CD8β-) in the mammary gland of mice 24 hours post-photoconversion of the intestine. Controls are non-photoconverted mice.

Figure 4



D

	Mouse	Tissue	Cell type	Vα	Jα	CDR3a	Vβ	Jβ	CDR3b
Newbury	m1	SI IEL	CD8αα	TRAV12-2	TRAJ22	CALSASSGSWQLIF	TRBV13-1	TRBJ2-7	CASSDFGGFHEQYF
	m2	SI IEL	CD8αα	TRAV12-3	TRAJ22	CALTPASSGSWQLIF	TRBV13-1	TRBJ2-7	CASSDTGGEYEQYF
	m2	SI IEL	CD8αα	TRAV12-3	TRAJ22	CALSPASSGSWQLIF	TRBV13-1	TRBJ2-5	CASSDADREDTQYF
	m3	Lact MG	CD8αα	TRAV12-3	TRAJ22	CALTPASSGSWQLIF	TRBV3	TRBJ2-4	CASSPTASQNTLYF
	m3	Lact MG	CD8αα	TRAV12-2	TRAJ22	CALSASSGSWQLIF	TRBV13-2	TRBJ2-3	CARTGTEAETLYF
	m3	Lact MG	DN	TRAV12-2	TRAJ22	CALSASSGSWQLIF	TRBV13-1	TRBJ2-7	CASSEGLSSYEQYF
Revere	m2	SI IEL	CD8αα	TRAV6-2	TRAJ22	CVLGASSGSWQLIF	TRBV3	TRBJ1-4	CASSPGTISNERLFF
	m2	SI IEL	CD8αα	TRAV16N	TRAJ22	CAMRASSGSWQLIF	TRBV14	TRBJ1-4	CASSPGTISNERLFF
	m1	SI IEL	CD8αα	TRAV7-6	TRAJ22	CAVASSGSWQLIF	TRBV12-1	TRBJ1-4	CASSSGTISNERLFF
	m4	Lact MG	CD8αα	TRAV6D-5	TRAJ22	CVLSSGSWQLIF	TRBV12-1	TRBJ1-4	CASSPGTISNERLFF
	m3	Lact MG	CD8αα	TRAV6-3	TRAJ22	CAMRALSSGSWQLIF	TRBV13-1	TRBJ1-4	CASSPGTISNERLFF
	m4	Lact MG	DN	TRAV7-2	TRAJ22	CAASISGSWQLIF	TRBV3	TRBJ1-4	CASSSGTISNERLFF

E

Tissue	CD8αα+ counts	Revere + Newbury counts	Percentage
SI	6577	264	4.01
Peritoneal cavity	28	1	3.57
Colon	2324	3	0.13
Mammary gland	857	4	0.47
Blood	45	0	0.00
Bone marrow	239	0	0.00
Liver	205	0	0.00
LN	54	0	0.00
Lung	108	0	0.00
Salivary gland	45	0	0.00
Spleen	158	0	0.00
Tumor	80	0	0.00
Uterus	258	0	0.00
All other tissues	3544	4	0.11

Figure 4. Mammary T cells share peculiar TCR repertoires with small intestinal T cells.

A) Chord diagrams in lactating (L3) and nulliparous mice representing clonotype sharing in different T cell populations (inner ring) between the small intestine and mammary gland (outer ring). Each line represents a TCR clonotype.

B) Distance matrix between αβTCR clonotypes in intestinal IELs. Red circle denotes "Newbury TCR" and black circle denotes "Revere TCR".

C) CDR3 sequence of Revere and Newbury TCRs.

D) Table representing instances of Revere and Newbury TCRs in CD8αα+ T cells in mammary gland and small intestine across different mice.

E) Counts of Revere and Newbury TCRs in CD8αα+ T cells across multiple tissues.

Figure 5

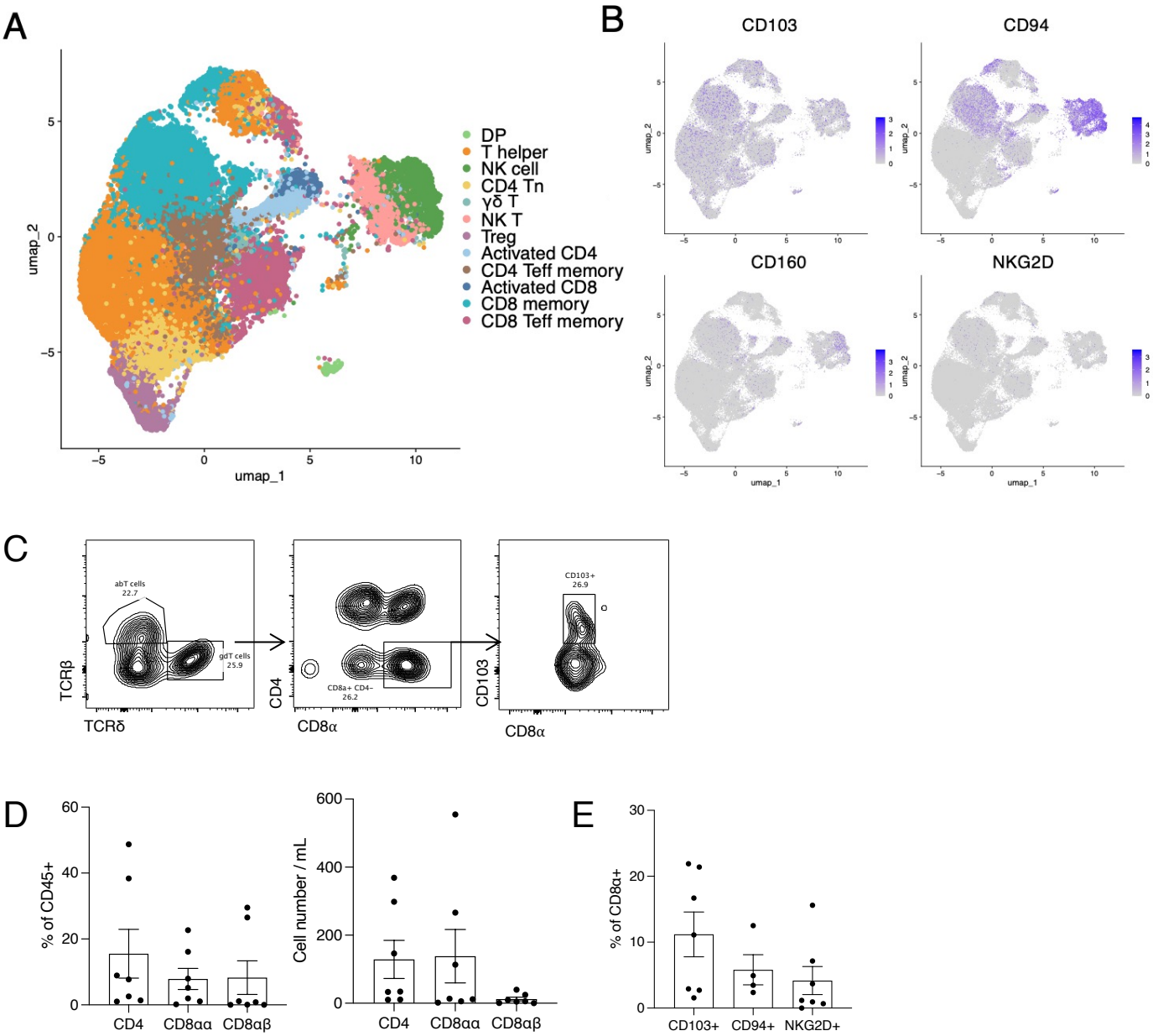

A
DP
T helper
NK cell
CD4 Tn
γδ T
NK T
Treg
Activated CD4
CD4 Teff memory
Activated CD8
CD8 memory
CD8 Teff memory
umap_1
umap_2
B
CD103
CD94
CD160
NKG2D
C
abT cells
22.7
gdT cells
25.9
TCRβ
TCRδ
CD8a+ CD4−
26.2
CD4
CD8α
CD103+
26.9
CD103
D
% of CD45+
CD4
CD8αα
CD8αβ
Cell number / mL
E
% of CD8α+
CD103+
CD94+
NKG2D+

Figure 5. Mammary CD8aa+ IEL-like cells are found in human breast and milk.

A) UMAP projection of mammary immunocytes from human breast tissue (sourced from Kumar et al).

B) Feature plots of CD8αα+ Teff markers projected on UMAP from (A).

C) Representative flow cytometry gating of CD8αα+ IEL-like cells in human milk samples.

D) Quantification of CD4+, CD8αα+ and CD8αβ+ cells as percent of CD45+ cells (left) and cell number normalized to volume (right) in human milk samples.

E) Quantification of human CD8αα IEL markers CD103, CD94, and NKG2D within CD8αα IEL-like cells in human milk samples.

Figure 6

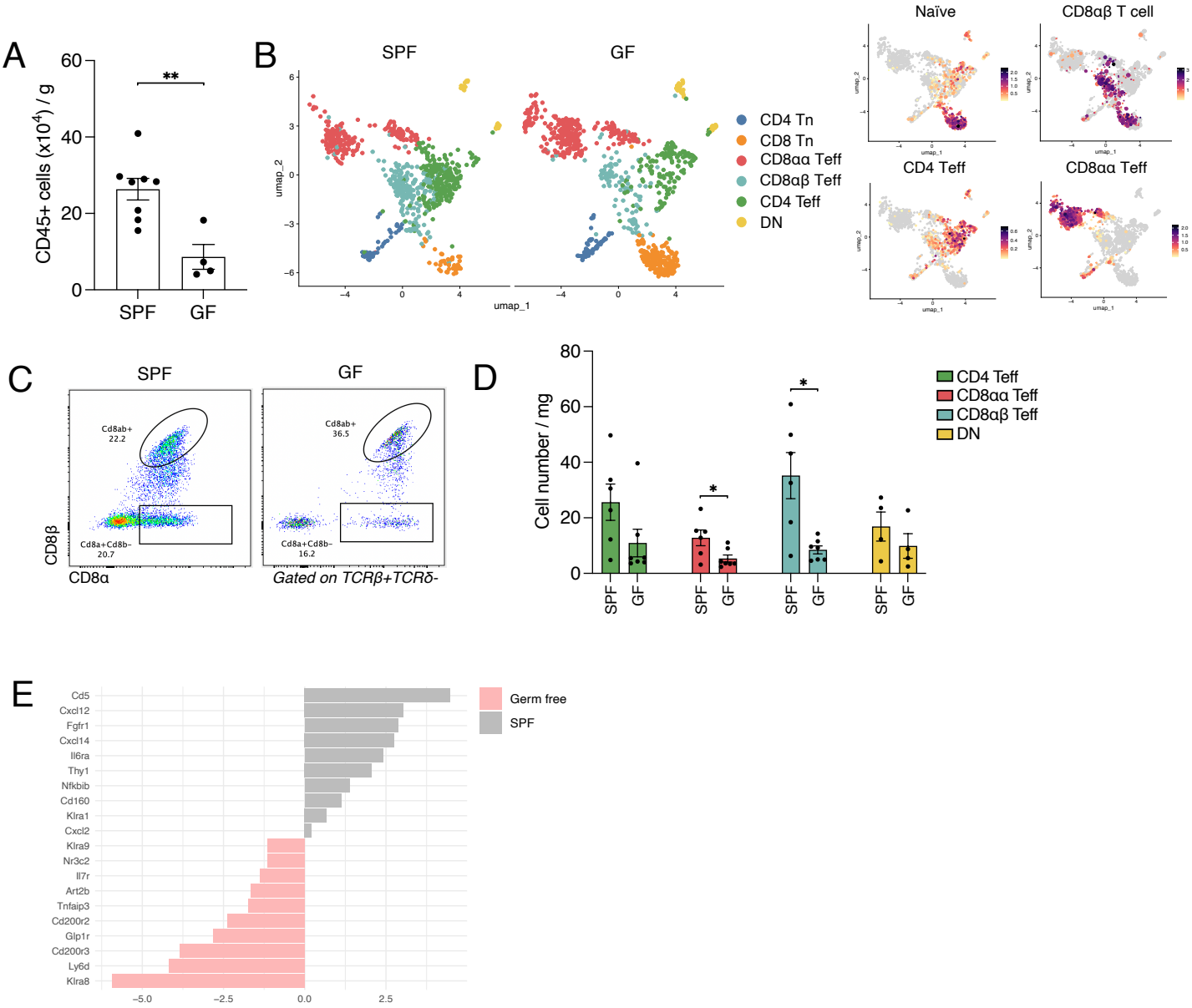

A
CD45+ cells (x10^4) / g
60
40
20
0
**
SPF
GF
B
SPF
GF
umap_2
umap_1
CD4 Tn
CD8 Tn
CD8αα Teff
CD8αβ Teff
CD4 Teff
DN
Naïve
CD8αβ T cell
CD4 Teff
CD8αα Teff
C
SPF
GF
Cd8ab+
22.2
Cd8a+Cd8b−
20.7
Cd8ab+
36.5
Cd8a+Cd8b−
16.2
CD8β
CD8α
Gated on TCRβ+TCRδ-
D
Cell number / mg
80
60
40
20
0
*
*
CD4 Teff
CD8αα Teff
CD8αβ Teff
DN
SPF
GF
SPF
GF
SPF
GF
SPF
GF
E
Cd5
Cxcl12
Fgfr1
Cxcl14
Il6ra
Thy1
Nfkbib
Cd160
Klra1
Cxcl2
Klra9
Nr3c2
Il7r
Art2b
Tnfaip3
Cd200r2
Glp1r
Cd200r3
Ly6d
Klra8
−5.0
−2.5
0.0
2.5
Germ free
SPF

Figure 6. T cell expansion in the lactating mammary gland is partially dependent on microbes.

A) Quantification of total number of CD45+ cells normalized to weight in SPF and GF lactating mammary glands by flow cytometry.

B) UMAP projection of mammary T cells from lactating SPF and GF mice with feature plots of naïve and effector T cell gene signatures.

C) Representative flow cytometry plots of CD8αα+ and CD8αβ+ cells (gated on live CD45+ TCRβ+ TCRδ-) in lactating SPF and GF mice.

D) Quantification of total cell numbers normalized to weight, of different T cell populations identified in (B) by flow cytometry. Cell types determined as in Fig 1F.

E) Differentially expressed genes in mammary CD8αα+ IELs between lactating SPF and GF mice.

*$p<0.05$, **$p<0.01$ by student's t-test.