SPECIFIC AIMS

Human cytomegalovirus (HCMV) is the most frequent cause of congenital infection and a leading cause of sensorineural hearing loss. Although vaccine development to prevent this intrauterine infection is a high priority, a major hurdle in HCMV vaccine development is an incomplete understanding of adaptive immune responses that limit virus transmission.

Infants of seropositive mothers are passively immunized by transplacentally acquired and breast milk (BrM) derived antiviral antibodies (Abs), yet are consistently infected following epithelial exposure to virus in BrM. In addition to virus neutralizing Abs, BrM contains HCMV-specific T cells. Since only ~50% of infants exposed to HCMV in BrM will become infected by 6 months of age, investigating this in vivo human model of natural HCMV acquisition provides a unique opportunity to define protective antiviral immune responses. In humans, studying in vivo virus transmission at a mucosal surface is virtually impossible. However, BrM transmission of HCMV is one the few situations where this can be done given that BrM transmission does not result in a negative outcome for the baby and treatment is not warranted, allowing for the study of “natural” transmission in humans. Moreover, given the paucity of information on T cell responses in BrM, this model allows us to more fully understand T cells’ role in BrM immunity.

Memory T cells provide immediate or long-term protective immunity in tissues, depending on the type of memory cell that is established. In HCMV infection, it is known that short lived effector memory T cells (Tem) as well as long-lived tissue resident memory T cells (TrM) are generated after infection. Moreover, both types of memory subsets have been shown in animal models to provide immediate protection at tissue sites, thus an understanding of the type of cell that are generated and the protection they provide in the tissue compartment is crucial to develop vaccines. However, a direct comparison between these two memory subsets has not been studied in order to answer the following question: Which memory T cell is better at providing local protection against HCMV? Our preliminary data and published data demonstrate that both of these memory T cells are present in BrM. However, their function in BrM and their role in HCMV transmission to infants via BrM is not known. In addition, whether they are distinct T cell populations is not clear. Thus, comparing different memory T cells of the same specificity in the same compartment allows us to determine if these two distinct memory cell populations play a differential role at preventing HCMV transmission to the infant. The study of BrM HCMV infection is well suited to answer these questions.

We hypothesize that BrM HCMV transmission occurs due to inadequate control of viral replication by HCMV-specific T cells and the predominance of TrM over Tem T cells in breast milk results in better control and therefore decreased transmission. The goals of the project are determining the type of peripheral memory T cell population present in BrM as a model for studying tissue immunity, in controlling virus, and therefore responsible for reduced transmission to the infant. In order to accomplish these goals, we have designed a longitudinal cohort study of mother-infant pairs and propose the following specific aim:

Specific Aim: Define the differences in the memory phenotypes and functional profiles of HCMV-specific T cells in BrM of HCMV infected women. The development of T cell immunity in BrM is a desirable defense mechanism against HCMV transmission to the infant, and while the role of TrM cells in the control of local infections has been established for tissues, such as the skin, there is no current description of TrM in breast milk. Moreover, it is unknown whether these cells limit HCMV transmission. We propose to study the subsets of memory T cells; Tem, terminally differentiated effector memory (Temra), and central memory (Tcm), with a special focus on the relatively novel TrM T cell subset as important components of the HCMV-specific T cell response, to understand their role in BrM immunity. Our goal is to assess whether the presence of any given memory subset in BrM is associated with viral control leading to a decrease in HCMV transmission to the infant. We will map responses to 19 HCMV proteins using the IFN-γ ELSIPOT, followed by functional characterization using flow cytometry to the responsive antigens. Spade and t-SNE analysis will be used to compare the various memory T cell subsets. Using molecular characterization of memory cells guided by RNA-seq analysis we will examine and define the transcriptional regulators of the memory subsets present in BrM. This analysis will define differences among T cells present in transmitters vs non-transmitters and determine their unique transcriptional signature. Once specific markers have been identified, we will test their expression on the various T cell subsets from BrM. Analysis of these markers may shed light into the processes required for reduced transmission. Lastly, we will determine whether the TrM phenotype can be recapitulated in PBMC with the addition of cytokines, as has been demonstrated in murine studies.

In summary, we will define memory T cells in BrM and their relationship to HCMV transmission. This information is crucial for the development of HCMV vaccines and treatment modalities in the setting of transplantation for the prevention of HCMV infection. This data will not only help understand the role memory T cells have in HCMV control in BrM, but the information can more broadly inform vaccine trials that use CMV as vectors in experimental vaccines for the generation of T cell mediated immune responses. Studies in BrM will not only advance our understanding of breast milk immunity that can be harnessed to protect breast fed infants, the studies can also add to the understanding of mucosal immunity and what T cell subsets are protective during mucosal infections in humans.
RESEARCH STRATEGY

A. Background/Significance

Human cytomegalovirus (HCMV) is an important cause of invasive disease in immunocompromised individuals\(^1\)-\(^4\). HCMV is also the most frequent cause of congenital infection and a leading non-genetic cause of hearing loss and neurologic disabilities in children\(^5\)-\(^9\). Post-natal transmission of HCMV is predominately via breast milk (BrM), despite infants of seropositive mothers passively immunized by antiviral antibodies including virus neutralizing antibodies acquired transplacentally and via BrM\(^{10, 11}\). Most HCMV seropositive women (>90%) shed virus in BrM\(^{12}\) and therefore, breastfed infants of seropositive mothers are continuously exposed to HCMV. Since only about 50% of infants exposed to HCMV in BrM will become infected by 6 months of age, investigating this in vivo model of natural HCMV acquisition at the mucosal surface provides a unique opportunity to define protective antiviral immune responses.

Protective immune response against acquisition of HCMV have not been defined. Our understanding of the role of cell mediated immunity in controlling HCMV infections especially within mucosal tissues is very limited. Studies in the murine CMV (MCMV) model have provided evidence that virus-specific T cell responses are important in the control of primary MCMV infection\(^{13-16}\). However, the role of T cell immunity in protection and control of CMV infection in humans has been much harder to determine. Nevertheless, virus control has been demonstrated after adoptive transfer of HCMV-specific T cells to patients undergoing stem cell transplantation\(^{17-19}\). Moreover, adoptive transfer has been shown to reduce the risk of HCMV infection and restoring HCMV immunity in individuals undergoing solid organ transplantation\(^{20-23}\). A recent study of HCMV-specific immune response in tissues from human organ donors demonstrated compartmentalization whereby, responses in peripheral blood mononuclear cells (PBMC) did not reflect those in tissue. In addition, there was no relationship between the frequency of HCMV-specific CD8 T cells and viral load (VL) in the tissues\(^{24}\). Studies that have examined the role of T cell responses in peripheral blood (PB) and their relationship with systemic HCMV VL in maternal and congenital infections has yielded conflicting findings. In women with primary HCMV infection during pregnancy, delayed appearance of T cell response was associated with increased risk of congenital infection\(^{25}\).

In contrast, a recent study reported that the presence of a strong HCMV-specific T cell response in the mother was associated with increased risk of intrauterine transmission\(^{26}\). In infants with congenital HCMV infection, the development of HCMV-specific T cell immunity over time was associated with an age-related decline in HCMV shedding in urine\(^{27}\). There is very limited data on HCMV-specific T cell immunity in BrM or the mammary gland. In what is probably the first report of HCMV-specific T cells in human colostrum, proliferation to HCMV was higher in the colostrum samples compared to the paired PBMC, but associations with VL or transmission were not examined\(^{28}\). A more recent study did not demonstrate a relationship between the magnitude of BrM HCMV-specific T cells using an IFN-\(\gamma\) ELISpot and the VL in BrM from mothers of preterm infants. In addition, the magnitude of the HCMV-specific T cell response in mother’s BrM between transmitters and non-transmitters was not different\(^{29}\). We have recently shown that higher BrM VL was associated with the number of mononuclear cells as well as the frequency of HCMV-specific CD8+ T cells in BrM suggesting that local HCMV replication drives the establishment of HCMV-specific T cell response in the breast\(^{30}\). Although these findings suggest that persistent antigenic stimulation is required for the recruitment HCMV-specific T cells into the breast, CD8+ T cell responses including subsets involved in the response have not been characterized.

It has been recognized that memory T cells residing in tissue (TrM) play a more important role in the control of local infections\(^{31-36}\). In the murine model, MCMV-specific TrM have been described in the salivary gland and demonstrated that the presence of antigen is critical to their recruitment and establishment\(^{37, 38}\). As TrM are not present in PB, BrM provides a unique mucosal compartment to study the role of these cells especially in the context of natural HCMV transmission. In fact, limited comparative studies between memory T cell subsets both within tissues (Tem vs TrM in the same tissue) and between blood (Tem) and tissues (TrM) and their protective capacity have been carried out. Understanding the development and establishment of TrM and/or Tem cells in BrM could help us determine how to enhance the longevity of resident memory T cell populations in tissues that can be exploited for rational T cell based vaccine design or for therapeutic modalities for transplantation.

B. Innovation

Vaccine development to prevent congenital HCMV infection is a high priority because of the significant disease burden\(^{39}\). However, lack of an understanding of the protective immune response is a major hurdle in the development of HCMV vaccines. Therefore, current efforts at developing prophylactic HCMV vaccines rely on empiric vaccine trials with candidate immunogens selected by a combination of limited data from clinical observations and studies in experimental animals. It is likely, that an effective HCMV vaccine will have to generate both robust neutralizing antibodies as well as effective memory T cells, utilizing a prime-boost strategy, since both humoral and cellular immune responses have been implicated in HCMV control\(^{40, 41}\).
HCMV acquisition from BrM is perhaps the only human setting where HCMV transmission occurs at the mucosal surface at a predictable rate in the presence of virus-specific responses. This model provides a unique opportunity to define immunologic characteristics associated with protection from HCMV transmission at the mucosal surface. Although BrM HCMV transmission does not result in serious disease except in preterm infants, a better understanding of the immune response and underlying processes that prevent HCMV transmission at the mucosal surface is crucial for the development of preventative vaccines. TrM cells have been shown to provide immediate protective immunity in tissues, however their function in BrM has yet to be studied, despite active investigation of HCMV infection in BrM. The relatively easy access of BrM as a mucosal sample allows us to further explore the function of TrM cells in humans and compare the role of different memory T cell populations in HCMV control and their unique functional and phenotypic characteristics. The proposed studies will enable us to document HCMV-specific immune responses that prevent virus transmission and to potentially define the parameters of the threshold of such a protective response. Lastly, RNA sequencing of memory T cell subsets in BrM samples will allow us to discern distinguishable patterns between the subsets and the differential gene expression between non-transmitting and transmitting mothers. Furthermore, we will investigate the impact of said differential gene expression on surface marker expression and immunological responses, opening the door for elucidation of underlying mechanisms and potential intervention points.

C. Approach

Preliminary Studies:

**BrM HCMV transmission study:** In an ongoing study, 568 women who delivered at the UAB Hospital were screened for HCMV IgG in the immediate postpartum period and their infants were tested to rule out congenital HCMV infection. A subset of these HCMV seropositive (N=114) mothers and infants completed 6 months of follow-up. Of those who completed 6 months of follow-up, 53 (46%) infants acquired HCMV. At each monthly visit, BrM samples were collected to determine HCMV VL and infant saliva samples were tested to monitor the acquisition of HCMV infection. From a subset (N=30) in the cohort, an aliquot of BrM was analyzed for virus-specific T cell responses. In addition, PB was collected from 16 mothers who consented, allowing for comparisons between T cell responses in BrM and PBMC.

**Higher HCMV load in BrM was associated with transmission:** Serial BrM specimens were processed and supernatants analyzed for HCMV DNA using a real-time PCR assay[42]. The virologic characteristics including the peak VL and the duration of HCMV shedding in BrM were compared between transmitters and non-transmitters. A significantly higher peak BrM HCMV load was observed in transmitters compared with non-transmitters (one order of magnitude difference with medians of 1.3x10^4 vs 1.2x10^3 copies/mL; p<.0001, data not shown). In addition, the duration of BrM HCMV shedding was significantly longer in transmitting women than non-transmitters (median, 42 vs 19 days, p<0.01).

**BrM contains Tissue Resident Memory T-cells much like conventional tissue:** Given that BrM is mucosally derived and contains ample numbers of antigen specific T cells[80, 43, 44] and also T cells expressing the mucosal retention receptor αEβ7 (CD103)[44], we examined whether TrM (CD103+CD69+CCR7-) T cells would be also present. Phenotypic characterization of BrM T-cells (BMC) from the HCMV seropositive women described above was carried out using the gating strategy for detection depicted in Fig. 1. The data demonstrates that TrM T cells identified as expressing αEβ7 (CD103) and CD69 with a concomitant decrease in the expression of the LN homing receptor CCR7 are present in BrM. This phenotype is also seen in healthy tissue from the female genital tract (FGT, vaginal and endometrial samples from remnant tissue)[45] but absent or greatly reduced in paired PBMC (Fig. 2) obtained from a small subset of the same cohort.

**Figure 1. Gating Strategy for TrM cells in breast milk.** Sample gating strategy for the percent CD3+CD8+ Tissue Resident Memory T cells (CD103+CCR7-CD69+) obtained from unstimulated BrM from an HCMV seropositive woman. The percent gated population is shown above the gate.

**Figure 2. TrM cells are present in breast milk.** TrM CD8+ T cells are present in human BrM just as in tissue from the FGT. A subset of PBMC were obtained from the same women who donated BrM. Tissue is from unrelated women who donated remnant tissue. Horizontal line represents median value. Statistical significant differences (p<0.05) were obtained using the Wilcoxon Rank test for paired samples and for unpaired, we used the Mann Whitney.

**Figure 3. CD8+ TrM cells are inversely associated with a decreased HCMV VL in BrM.** Correlation between the %CD8+ TrM in BrM and HCMV BrM VL.
Tissue Resident memory cells are associated with a reduction in HCMV viral load: We next analyzed the frequency of CD8+ TrM cells in BrM and the HCMV virus load. We observed an inverse association between the frequency of CD8+ TrM cells and VL (Fig. 3) suggesting TrM cells may have a role in viral control. In addition, CD8+ TrM (negative association with VL, Fig. 3) and total CD8+ T cells (positive association with VL,\(^{(30)}\)) differentially associate with the presence of virus, suggesting that the properties of CD8+ TrM cells are different than other CD8+ T cell subsets.

Detection of HCMV-specific T cells in BrM from HCMV infected mothers: Although IFN-γ ELISpot assay has been used to identify antigen-specific T cells in BrM\(^{(20, 43, 44)}\), using the flow cytometry based intracellular cytokine assay (ICCS) allows us to combine phenotypic profiles with function as assessed by stimulating BrM with HCMV pp65 peptide pools (15-mers overlapping by 10). A representative plot from these experiments is depicted in Fig. 4. As can be observed, we can easily detect IFN-γ and dual IFN-γ/Granzyme B (GrB) producing HCMV-specific T cells in BrM.

Detection of HCMV-specific TrM cells in BrM from HCMV infected mothers: To determine the presence of HCMV-specific TrM CD8+ T cells in BrM, we stimulated BrM with HCMV pp65 peptide pools as shown in Fig. 5, but gated on either CD103+ or CD103+CD69+ CD8+ T cells. As shown in Fig. 5, we detected HCMV-specific TrM cells from BrM using flow cytometry.

In summary our preliminary data demonstrate that we can detect HCMV-specific TrM T cells in BrM. Given this information, we can now address the questions posed in our specific aim.

Specific Aim: Define the differences in the memory phenotypes and functional profiles of HCMV-specific T cells in BrM of HCMV infected women.

Hypothesis: HCMV will induce antigen-specific Tem and TrM CD8 T cells in BrM, but only TrM will be associated with reduced HCMV VL.

Rationale: The development of T-cell immunity in BrM is a desirable defense mechanism against transmission of HCMV to the infant. In addition, the hallmark of an effective immune response is the ability to generate long-term protection against infection. Memory T cells are long-lived antigen-specific cells that are generated after an effective immune response and can control reactivation and future infections. Despite significant HCMV transmission to the infant after birth via BrM, this transmission has not been shown to portend a negative outcome for the child, unless it is preterm. Yet, this research allows the study of transmission events in a mucosal compartment were various memory T cell subsets are present in order to unravel the role each subset plays in viral control. These studies will not necessarily have an immediate impact on mother to child transmission of HCMV, but have the potential to shed light on the different T cell memory subsets and their function with respect to HCMV control in tissues. This information should impact adoptive transfer therapies during transplants and will inform vaccine design for products aimed at inducing T cells. The phenotypes of T-cells present in BrM and whether these play a role in limiting HCMV transmission to the infant via BrM has not been characterized. Our preliminary data demonstrates that there are ample TrM CD8 T cells in BrM. Given that HCMV has been shown to generate predominately Tem T cells in the PB (reviewed in\(^{(46)}\)), and from our previous work in BrM from HIV-infected mothers\(^{(44)}\), we expect that BrM will be skewed towards the presence of HCMV-specific Tem CD8 T cells. Moreover, given our preliminary data and the role of TrM in the containment of mucosal infections in humans\(^{(36, 47, 48)}\) and in murine studies\(^{(33-35, 49)}\), we also expect to find a significant proportion of HCMV-specific T cells expressing markers of TrM T cells. In order to fully understand the function of T cells in BrM, we first need to define T cell subsets and their functions. As demonstrated in our preliminary results, the transmission of HCMV to the infant from BrM is impacted by the amount of BrM VL so we will use VL as a surrogate for transmission given the limited numbers of samples that will be analyzed. Lastly,
these studies will be important in delineating differences in Tem and TrM in the same mucosal compartment in humans, knowledge that would help establish which memory cell populations exhibit better protective capacities in mucosal tissues.

**Study Design**

**Volunteer Populations:** HCMV seropositive mothers (N=30) will be enrolled in the immediate postpartum period and monitored prospectively for HCMV shedding (HCMV VL 1-4 months) and HCMV-specific T-cell responses (1,2,4 months) in BrM collected monthly for 4 visits, since our data has shown that 81% of infants are infected within 4 months. All mothers are tested for HIV-1 as part of prenatal visits and any mother testing positive will be excluded from this study. Saliva samples will be collected at each visit from infants of participating mothers to monitor for acquisition of HCMV via BrM. PB samples from mothers will be collected at the same time points as BrM. We will also enroll 10 HCMV seronegative women as controls and collect their BrM and PB at 1 month postpartum. In infants whose saliva samples test positive for HCMV by real-time PCR, urine samples will be collected at all subsequent visits.

**Cell isolation: Peripheral blood** will be collected by venipuncture at the same time points that BrM is collected. PBMC will be isolated as previously described. Breast Milk will be collected at 1, 2 and 4 months post-partum by manual expression or breast pump and isolated as previously described (30, 43, 44) for immunological studies and at 3 months for sorting CD8+ T cells (CD3+CD8+) cells for TrM (CD103+) and Tem (CD45R0+CCR7-CD45RA-) into TRIZol reagent and frozen at -80 °C for RNaseq analysis. We will sort at least 1 x 10^6 cells.

**Phenotypic Analysis:** Cell suspensions from PB and BrM will be incubated with monoclonal antibodies specific for T cell specific surface markers: CD3-APCFlour780 (eBioscience, San Diego, CA), CD4-Qdot655 (Invitrogen, Grand Island, NY), and CD8-V500; activation marker: CD69-APC; memory markers: CD45RA-FITC and CD197-PercpCy5.5 (CCR7); and homing marker: CD103-PE (αEβ7). The leukocyte marker, CD45-PeCy7 will be used in conjunction with SSC to gate BrM cells (Fig. 1), instead of using SSC vs FSC as done for PBMC. Live cells are gated by the exclusion of a dead cell dye. For reproducibility, commercially available reagents will be used. Unless otherwise specified, antibodies are obtained from BD Biosciences (San Jose, CA, USA). Samples will be stained using an Ab panel containing at a minimum 12 different fluorochromes in order to maximize information gathered while minimizing the total number of cells needed. Stained cells will be acquired using a BD FACSymphony flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FlowJo Version 9.9/10.5 software (TreeStar, San Carlos, CA). We plan to run at least 10^5 gated lymphocytes for each stained specimen. Fluorescence minus one (FMO) will be used to set gates for markers where gating is not clearly evident. Unbiased data analysis using SPADE and t-SNE will also be performed (see LOS from Dr. Kutsch).

**ELISpot assay:** We will use the IFN-γ ELISpot assay as previously described, to determine the immunogenicity of 19 HCMV proteins using peptide pools (https://shop.jpt.com/images/upload/pdf/PM-C-HCMV-2.pdf, JPT, Innovative Peptide Solutions, Berlin, Germany), in order to select the top 2 with the highest frequency for use in the flow cytometric studies. In order to determine the HCMV immunogenic pools we will use PBMC and BMC obtained at 1-month post-partum.

**Detection of virus-specific T cell responses:** Virus-specific T-cell subsets (using IFN-γ and IFN-γ+GrB+ dual positive cells) including central memory, Tcm (CCR7+CD45RA-), effector memory, Tem (CCR7-CD45RA-), terminally differentiated, Temra (CCR7-CD45RA+) and naive cells (CCR7+CD45RA+), as well as the novel TrM (CCR7-CD103+CD69+) memory subset will be defined and analyzed using polychromatic flow cytometry as previously described on cells isolated from BrM and PB. Responses to HCMV will be measured using the top 2 immunogenic peptide pools from the 19 tested in an IFN-γ ELISpot (15-mers overlapping by 11, JPT, Innovative Peptide Solutions). PBu/ionomycin will be used as a positive control. For rigor, unstimulated cells will be used as the negative control and to position effector molecule (IFN-γ-APC-Alexa700 and Granzyme B-V450) production gates, as depicted in Figures 4 & 5. For Ag-specific cell detection, we will stimulate cells for 6 hours in vitro at 37°C and 5% CO₂ (standard intracellular cytokine assay). Data will be analyzed and the presence of the various memory subsets will be compared between compartments (i.e. PB and BrM) and within the same sample (i.e. TrM vs Tem in BrM). We will also determine whether TrM cells isolated from BrM are HCMV-specific and capable of elaborating IFN-γ and Granzyme B as surrogate markers of cytotoxic T-cells. The frequency of the T-cell responses from BrM will be compared to responses from paired PBMC obtained at the same time. This data will be analyzed to determine whether TrM play a role in controlling HCMV replication in BrM (as measured by BrM VL) and HCMV VL in the infant. We will determine whether TrM T cells within BrM are associated with a reduction in VL in saliva and urine samples from infants who acquire HCMV infection during the study.

**Longitudinal studies:** We will determine the fate of the memory cell pool in BrM through time. BrM samples and PBMC will be collected at 1, 2 and 4 months post-partum and cells will be analyzed for antigen-specific
memory subset populations. The frequency of the HCMV-specific cells will be followed longitudinally and compared to the frequency detected in the PB and analyzed for associations with VL. The duration of HCMV shedding in BrM will be defined by analyzing samples collected at each visit. T cell responses will be correlated with peak HCMV VL and the duration of virus shedding in BrM to determine the relationship between T cell immunity and HCMV replication.

**In Vitro assay:** We will determine if the TrM phenotype can be recapitulated by the addition of cytokines (TGF-β, TNF-α, IL-33 and IFN-α) to PBMC, since this is a easy source of T cells to use as effectors in adoptive transfer therapies. For these studies we will take PBMC, sort out Tem and incubate these purified effector memory T cells with cytokines listed above. We expect the cytokines will modulate the expression of the activation marker, CD69 and the mucosal retention receptor, CD103 (αEβ7). These data would demonstrate that T cells not originating from tissue can be switched to TrM by the use of cytokines as was done in mouse experiments\(^{(54)}\).

**HCMV VL:** VL will be measured from BrM supernatant and plasma (mothers), and saliva and urine (infants) collected every month for 4 months. The samples will be processed for DNA extraction using commercial column kits (Qiagen, Inc., Valencia, CA). A real-time PCR protocol developed in our laboratory for testing dried blood spots and newborn saliva specimens was adapted to test DNA specimens from BrM supernatant specimens for HCMV\(^{(30, 42, 55)}\). Briefly, the performance characteristics of the real-time PCR assay were determined by analyzing BrM specimens from 10 CMV seronegative women as controls. In addition, plasmid standards incorporating target sequences within the IE-1 of HCMV in 10-fold dilutions ranging between 10\(^5\) and 10 copies are included in each PCR run to generate a standard curve. For rigor, each PCR run also includes a no-target control and DNA from CMV-negative and CMV-positive BrM as negative and positive controls, respectively. For reproducibility, each specimen will be run in duplicate using 20 μL of reaction mixture and 5 μL of test specimen. A specimen is considered positive if 1 or more copies per reaction are detected in both wells. The detection limit of our real-time PCR assay was between 50 and 100 copies/mL.

**Molecular description of memory cells guided by RNA-seq analysis.**

**Rationale:** TrM studies carried out in human tissues from transplant donors determined that TrM maintain a core signature in the expression of key transcriptional factors (TF) that are different from other memory cell populations\(^{(56)}\). Transcriptional analysis has also been performed for HCMV-specific CD8 T cells, and it was demonstrated that Blimp-1, a homolog of Hobit, identified effector CD8 T cells in humans\(^{(57)}\). This analysis suggests that maintenance of long-lived effector cells is likely regulated by Hobit. Transcriptional analyses have shed light into the understanding of the molecular pathways that program T cells, in mouse models (reviewed in\(^{(58)}\)). Understanding differences in the transcriptional profiles between TrM and Tem in transplanters and non-transmitters has the potential of expanding our knowledge of human TF regulating effective immune processes.

**RNA sequencing:** We will select frozen sorted TrM and Tem BrM from HCMV positive (transmitter and non-transmitters) and seronegative mothers. RNA extraction from frozen cell pellets stored in TRIzol and ultra-low input RNA-seq library preparation will be carried out. Total RNA will be converted into strand-specific paired-end sequencing libraries using the TruSeq stranded total RNA kit (Illumina). Samples will be DNase I treated and total RNA will be rRNA depleted. Total RNA (rRNA-depleted) will be fragmented, converted to cDNA, adapter-ligated with unique indices and PCR amplified. The final libraries will be quantified and sequenced. After sequencing, the FastQ-format files of RNA-seq reads will be generated and checked for quality. All these procedures will be carried out by GeneWiz (Plainfield, NJ) on frozen cell pellets in TRIzol.

**RNA sequencing analysis:** Once we obtain FASTQ files, The CFAR Basic Research Core will map sequence files to a Homo Sapiens reference genome (e.g. GRCh37) and will quantify expression with the accompanying annotation reference file. To identify differentially expressed genes, we will use DEseq2 and/or EdgeR (Bioconductor), depending on sequencing settings. P-values will be adjusted to correct for multiple testing. As a general guide, genes with an adjusted P value < 0.05 and an absolute log2-fold change > 1 will be considered significantly differentially expressed genes; however, we will additionally sweep over the log2-fold parameter to identify possible false negatives. We will subsequently perform automated pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) as a reference. We will additionally perform Gene Set Enrichment Analysis using the Molecular Signatures Database on the MIT/Broad Institute server, and compare results using an additional public or commercial pathway analysis software platform to quantify variations and report any visible outliers (specific comparative tools) to be selected once we analyze samples with first two methods. We will additionally perform hierarchical clustering analysis of genes that are differentially expressed in key pathways identified through pair-wise combinations of control, HCMV transmitters and HCMV non-transmitters (in both Tem and TrM BrM cells). Differential clusters will be presented as annotated heatmaps based on standardized expression values, along with clustering dendograms.
Rigor and Statistical considerations: The sample size estimation is based on enrolling and collecting complete data on 30 breast feeding women. A sample size of 30 achieves 80% power to detect difference of -11 between null hypothesis mean of 0 and the alternative hypothesis mean of 11 with an estimated standard deviation of 20 and with a significance level (alpha) of 0.05 using a two-sided Wilcoxon test assuming the distribution is normal (PASS 15 Power Analysis and Sample Size Software, 2017). In order to have complete set of samples and data from 30 HCMV seropositive women, we plan to enroll about 70 women to account for attrition from discontinuing breastfeeding and dropping out from the study. This number was calculated based on our experience in an ongoing study. The primary analyses will focus on comparing the different memory subsets and cytokine secretion from T cells and comparing the memory subsets between BrM and PB to determine the predominant population as well as the antigen-specificity. Analysis will be performed using Graph Pad Prism software 5.0 for Mac and Statistical Analysis Software version 9.4 (SAS Institute, Cary, NC). In addition, summary statistics will be provided for all the measures of immune response, e.g., %CD8+ TRM, by group, e.g. median and range will be presented. Scatter plots and Box-plot will be generated to explore the associations. Spearman correlations will be used to explore the association between immune response and VL. Phenotypes may vary overtime, thus, we will generate exploratory data. Each measurement will be modeled using a linear mixed effects approach which is well-suited for handling correlated repeated measurements, unbalanced and missing data in the study. This approach allows for comparisons and estimates of effects at any time point, and also allows comparisons and estimates across time points within compartments to evaluate the changes in the phenotypes.

For the analysis that will focus on determining HCMV-specific T cell responses associated with HCMV transmission via BrM we will compare virus-specific T cell responses in BrM and PBMC between transmitters and non-transmitters. For examining the relationship between T cell responses and HCMV transmission via BrM, the responses including memory T cell phenotypes will be compared between approximately 15 transmitters and a similar number of non-transmitters. As described above, power calculations will be performed for differences by using tests for paired data (Wilcoxon Signed-Rank).

Finally, comparisons will be also made between women with detectable HCMV in BrM and those with BrM samples negative for HCMV (unpaired) and between transmitter and non-transmitters. Assuming group sample sizes of 30 and 30 we can achieve 80% power to detect a difference of -22.86 between the null hypothesis that both group means are 10 and the alternative hypothesis that the mean of group 2 is 32.86 with estimated group standard deviations of 30 and 30 and with a significance level (alpha) of 0.05 using the Mann-Whitney test assuming that the actual distribution is normal.

Expected outcomes: In a recent study, we demonstrated that the frequency of HCMV-specific T cell responses was associated with VL in BrM (30), and in our preliminary data we demonstrate that the CD8+ TrM subset in BrM was inversely correlated with VL in BrM, possibly suggesting a role in controlling HCMV (Fig. 3). Given that Tem and TrM are effector cell populations shown to be involved in virus control in other tissues (33, 34, 36, 47-49, 59), we postulate that HCMV-specific memory populations in BrM will impact HCMV in the following ways:

a) A reduction in HCMV VL in BrM.

b) A reduction in transmission of HCMV to the infant.

c) HCMV-specific T cells (i.e. producing IFN-γ and Granzyme B) will be present in both memory subsets, with the majority of the response found in TrM.

d) TrM and Tem cell populations will have different transcriptional profiles.

For example, TrM cells from non-transmitters will have increased expression of transcription factors involved in the elaboration of effector molecules such as IFN-γ, GrB and TNF-α in addition, to elevated expression of activation markers. Antigen-specific cells from non-transmitters will share this profile.

Alternative approaches: We do not anticipate difficulties in detecting TrM cells and/or HCMV-specific responses in BrM as demonstrated by our preliminary and published data. Alternatively, the predominant memory T cell subset detected in BrM, could be Tem T cells, similar to PBMC. This would suggest that BrM is a unique mucosal compartment where TrM do not predominate, at least in the setting of HCMV infection. For HCMV-specific cells, it is also possible that they will be mostly contained within the Tem subset, when compared to TrM. It is also possible that all TrM are Tem (CD45R0+, CD45RA-, CCR7-, CD103+, CD69+). In which case we will limit our analysis to these cells only with respect to transmission. Differences in the expression of the various surface markers associated with memory phenotypes can also be analyzed using mean fluorescence intensity (MFI). In either case, this information will allow us to understand which types of cells are established in BrM and whether they differ from the PB. If we do not recover enough cells from the 3-month time point, will also sort cells from the 4-month time, and if needed, we will pool all both time points (3 and 4 months) in order to have enough cells for analysis. The information gained from this proposal should help inform future HCMV vaccines designed to stimulate T cell responses including local mucosal responses.
Application Number: 1 R21 AI151235-01

Principal Investigator
SABBAJ, STEFFANIE

Applicant Organization: UNIVERSITY OF ALABAMA AT BIRMINGHAM

Review Group: IHD
Immunity and Host Defense Study Section

Meeting Date: 10/24/2019
Council: JAN 2020
RFA/PA: PA19-053
PCC: M34B
Requested Start: 04/01/2020

Project Title: HCMV-specific Memory T cell Subsets and HCMV breast milk transmission

SRG Action: Impact Score:26
Next Steps: Visit https://grants.nih.gov/grants/next_steps.htm

Human Subjects: 30-Human subjects involved - Certified, no SRG concerns
Animal Subjects: 10-No live vertebrate animals involved for competing appl.
Gender: 1A-Both genders, scientifically acceptable
Minority: 1A-Minorities and non-minorities, scientifically acceptable
Age: 1A-Children, Adults, Older Adults, scientifically acceptable

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<th>Estimated Total Cost</th>
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ADMINISTRATIVE BUDGET NOTE: The budget shown is the requested budget and has not been adjusted to reflect any recommendations made by reviewers. If an award is planned, the costs will be calculated by Institute grants management staff based on the recommendations outlined below in the COMMITTEE BUDGET RECOMMENDATIONS section.
1R21AI151235-01 SABBAJ, STEFFANIE

RESUME AND SUMMARY OF DISCUSSION: This R21 application proposes to use a longitudinal cohort study of mother-infant pairs to investigate peripheral memory T cell populations present in breast milk as a model for studying tissue immunity, control of Human cytomegalovirus (HCMV) infection, and the role in transmission to the infant. Reviewers found significance in the work based on generating data on an important issue in clinical immunology, the mother-child transmission of Human Cytomegalovirus (HCMV) through breast milk and the role of tissue resident memory (TrM) CD8+T cells. The productive Investigators have extensive expertise in the role of maternal T cells in host defense and is a strength of the application. The application is based on strong preliminary data supporting both the scientific rationale and driving hypothesis. Some reviewers had questions on interpretation of the premise and supporting data concerning the contributions of CD8 T cell memory subsets. During discussion panels found the conformation of route of infection an important issue. Some reviewers found the use of data from RNA-seq library analysis was not well described. There were some questions on the significance given the predicted functional role of CD8 T cell memory cells is similar to other tissues. Overall, strengths outweighed weaknesses and there was considerable enthusiasm for the application.

DESCRIPTION (provided by applicant): Human cytomegalovirus (HCMV) is the most frequent cause of congenital infection and a leading non-genetic cause of hearing loss and neurologic disabilities in children, yet no efficacious preventative vaccine is available. About 50% of infants exposed to HCMV in breast milk will become infected by 6 months of age providing a natural HCMV acquisition model with a unique opportunity to define antiviral immune responses that prevent transmission at the mucosal surface. Studies of T cell-mediated immunity in breast milk have been limited. In humans, little is known about the types of T cells and their function in breast milk and whether they play a role in the control and the transmission of pathogens to the infant. In addition, this information will allow us to begin to understand what effector T cell populations are desirable for effective vaccines and/or immunotherapies. This knowledge is especially pertinent in the context of HCMV transmission to the infant via breast milk because this is a unique in vivo model of natural HCMV acquisition in humans. Lastly, these studies will also add new information to the study of human mucosal immunology.

PUBLIC HEALTH RELEVANCE: The hallmark of vaccine success is the generation of effective immunologic memory, which provides long-term protection against infection. An optimal vaccine needs to generate protective immune responses at the site of infection, however the study of T cell immunity in breast milk is lacking. This proposal will study memory HCMV-specific T cells and their role in control of HCMV in breast milk, in order to better understand virus transmission to infants.

CRITIQUE 1:

Significance: 4  
Investigator(s): 1  
Innovation: 4  
Approach: 5  
Environment: 1

Overall Impact: The application is designed to address a problem of high/moderate importance but weaknesses in the Significance, Innovation, and Approach criteria bring down the Overall impact score to medium. Score driving was the focus on antigen-specific CD8 T memory cell subsets. It appears
that expectations are that viral control mediated by specific subsets of memory T cells will lead to
decreased HCMV transmission to infants. This is despite the fact that antigen-specificity could/will be
shared amongst the CD8 T memory subsets (even potentially at the clonal level of the TCR). The new
knowledge will come from RNA-seq of memory CD8 T cell subsets that are hypothesized to yield
unique transcriptional signatures amongst T cells in BrM from mothers that are transmitters versus non-
transmitters of HCMV. The application has numerous strengths including the expertise and experience
of the PI and Co-Investigator in unique (non-invasive) sampling techniques, including breast milk, and
creative strategies for assessing immune and pathogen profiles in such samples. They have a
collection of a sufficient number of appropriate human specimens that have been, and are proposed to
be, evaluated. There is rich environment of mucosal immunologists UAB with diverse proficiency in
overcoming challenges unique to mucosal surfaces that have led the field for decades. It appears as
though some of the foundational questions were addressed prior to submission and the preliminary
data are shown, e.g. the relationship between virus transmission and T_{RM} in breast milk. In the end, the
prediction is that similar to other tissues various subsets of CD8 T cell memory cells will have functional
roles in breast milk to control transmission to infants. It is not clear how the information gained from
conducting the study will inform design of future HCMV vaccines and local mucosal responses,
particularly in BrM. Thus, there are concerns that there will be limited value to advancing the
expressed purpose of conducting the study.

1. Significance:
Strengths
• Project aims to address a significant problem in the field, notably breast milk transmission of
Human Cytomegalovirus (HCMV). The impact of this natural route of infection in breastfed
infants is not understood and under certain conditions of maternal immunity could be beneficial.
Weaknesses
• Although the prior experience of the study team in conducting rigorous investigation of breast
milk transmission of HIV, the rationale of the current study to dissect the contributions of CD8 T
cell memory subsets is not supported by rigorous prior research.
• It is not straight-forward to imagine the potential for the knowledge gained from conducting the
study, as is currently focused largely on T_{RM}, will address a critical barrier to progress in the
field. It is not clear that cells that express some of the hallmarks of T_{RM} are resident in the
breast tissue or exist within the breast milk, or both.

2. Investigator(s):
Strengths
• Talented junior investigator well trained and uniquely qualified to conduct the study proposed.
(Title in biosketch not up to date)
• Team is outstanding for supporting the PI’s career development and growth into the HCMV
niche. Particularly valuable is Dr. Boppana, an outstanding expert in studying HCMV, even in
dried saliva from newborns.
Weaknesses
• None noted

3. Innovation:
Strengths
• Attempting to resolve immune factors influencing virus transmission or diminution of
transmission of HCMV in breast milk is novel.
Weaknesses

- $T_{RM}$ cells could play a role in preventing HCMV transmission in breast milk. But then again, perhaps they are a unique population related phenotypically but not functionally to those in other mucosal tissues, such as the female reproductive track. It is difficult to think how cells localized in breast milk per se, recognize and eliminate virus in the breast environment.

4. Approach:

Strengths

- They have a collection of a sufficient number of appropriate human specimens that have been, and are proposed to be, evaluated. The congenital route of transmission in the specimens for study has been eliminated using a rigorous approach.

- Unique sampling technologies well understood by the experienced study team.

Weaknesses

- Evidence presented to suggest that there is an inverse association between $T_{RM}$ and viral load in breast milk is weak.

- As presented, the Flow Cytometry data to support the distribution between memory CD8 T subsets is not as strong as it could be. Nor is the flow cytometry data regarding IFN-$\gamma$ production. The elispot assays proposed should be more rigorous.

- It is not clear how viral control by CD8 cells (with $T_{RM} >> T_{CM}$) would occur in the breast milk, or elsewhere before migrating into the mammary gland.

- Distinguishing gene signatures for different subsets of antigen specific T cells in breast milk may not identify functional differences between the cell types.

- CD4 cells were largely ignored and it is likely that they are major contributors to control of virus.

5. Environment:

Strengths

- Exceptional environment for conducting this study from scientific and academic perspectives.

Weaknesses

- None noted

Protections for Human Subjects:
Acceptable Risks and/or Adequate Protections

Vertebrate Animals:
Not Applicable (No Vertebrate Animals)

Biohazards:
Acceptable

Resource Sharing Plans:
Acceptable

Authentication of Key Biological and/or Chemical Resources:
Acceptable
CRITIQUE 2:

Significance: 1
Investigator(s): 1
Innovation: 1
Approach: 1
Environment: 2

Overall Impact: This is an outstanding application that proposes to characterize cytomegalovirus (HCMV)-specific T cells in breast milk to determine their role in control of virus replication and transmission. The investigators make a case that since only 50% of infants exposed to HCMV in maternal milk are infected, this is a good model of virus transmission to identify protective responses. The hypothesis to be tested is that breast milk HCMV transmission is due to inadequate T cell control of virus replication, with resident memory T cells being efficient in this function, as compared to effector memory T cells. This is an engaging, well prepared application that this reviewer enjoyed reading. It has a compelling hypothesis and experimental plan. It asks the right questions. The breast milk virus transmission model is indeed an excellent natural situation to define immune correlates. Well controlled samples from a longitudinal cohort will allow to accomplish the aim proposed. The idea of comparing with peripheral blood to contrast systemic vs. milk T cell responses in a kinetic, Ag-specific manner is excellent. The analysis of T cell phenotypic and transcriptional data between mothers of infants who have or have not acquired HCMV has the potential to uncover protective elements. The studies are innovative and have high translational value. They have the potential to identify T cell memory phenotypes and their ability to control HCMV infection in tissues. The PI, Dr. Sabbaj is well qualified to conduct the work proposed, and the environment at UAB are supportive. The nature of this work fits the R21 high risk high reward mechanism. If an association is found between memory resident T cells in maternal milk and infant infection, it would be an important target response to achieve through vaccination. This is a potentially impactful proposal worth supporting.

1. Significance:

Strengths
- This is a clinically relevant application to discern T cell signatures in breast milk that may be associated with protection from CMV transmission in infancy.
- The work proposed is novel and likely to generate important information of translational value.
- The experimental plan is straightforward and has a focused, well defined aim.
- Studies will be performed in the target population. The investigators have access to a unique longitudinal cohort to obtain well characterized samples and clinical data.
- Although not needed for an R21, this application has critical preliminary data supporting both the feasibility and the proposed hypothesis.
- The PI has demonstrated expertise, access to resources and a supportive environment.
- The application is well written and has high scientific quality.

Weaknesses
- None identified.

2. Investigator(s):

Strengths
Dr. Sabbaj has specialized in the study of mucosal T cell immunity against HIV and HCMV and correlates of protection against these pathogens. Her expertise is demonstrated by excellent publications.

Pertinent to this application, in a recent paper with her collaborator, Dr. Boppana, Dr. Sabbaj describes the association between HCMV in breastmilk and establishment of T cells in infected mothers.

The research team has ample expertise and is uniquely qualified to conduct the work proposed.

Weaknesses
- None identified.

3. Innovation:
Strengths
- The in-depth characterization of T cells to HCMV in breast milk and its association with virus transmission proposed is conceptually and technically innovative.
- The idea of study TrM in breast milk and their association with tangible protective anti-viral activity is also novel and unique.

Weaknesses
- None identified.

4. Approach:
Strengths
- Clear and straightforward research plan with the aim of defining differences in memory phenotype and function of HCMV-specific T cells in breast milk from infected and non-infected women and their association with virus transmission to their infants.
- Compelling preliminary data showing that HCMV-specific resident memory T cells can be detected in breast milk. A strategy for selection of reactive peptides is described.
- T cell responses measured will be specific and over time. RNAseq will provide additional gene expression data for a complete signature of cell phenotype and function.
- Side by side comparison of T cell responses in blood vs. breast milk. This is a relevant comparison that will yield information about the two compartments.
- A bonus experiment is the attempt to convert peripheral Tem into TrM by in vitro exposure to cytokines.
- Methods, clinical samples and tools are all available for this work.
- Adequate consideration of sample size and enrolment needs.

Weaknesses
- Which is the cell yield of TrM cells in breast milk?

5. Environment:
Strengths
- Dr. Sabbaj has access to adequate lab space, resources and support.
- The research environment at UAB is excellent; it has been the home of some of the best mucosal immunologists.

Weaknesses
- None identified.

Protections for Human Subjects:
Acceptable Risks and/or Adequate Protections
- Women and infants enrolled to monitor HCMV infection and immunity
Inclusion Plans:
- Sex/Gender: Distribution justified scientifically
- Race/Ethnicity: Distribution justified scientifically
- For NIH-Defined Phase III trials, Plans for valid design and analysis:
  - Inclusion/Exclusion Based on Age: Distribution justified scientifically
- Special groups needed (lactating mothers and infants)

Vertebrate Animals:
Not Applicable (No Vertebrate Animals)

Biohazards:
Acceptable

Resource Sharing Plans:
Acceptable

Authentication of Key Biological and/or Chemical Resources:
Acceptable

Budget and Period of Support:
Recommend as Requested

CRITIQUE 3:
Significance: 2
Investigator(s): 2
Innovation: 2
Approach: 4
Environment: 2

Overall Impact: This new R21 proposal seeks to determine the memory phenotypes and functional profiles of HCMV-specific T cells in breast milk (BrM) of HCMV infected women. The central hypothesis that tissue resident memory (TrM) CD8+T cells are associated with a reduction in HCMV titer is supported by strong preliminary results, which indicates that rigor of prior research is high. Likewise, the significance is high since studies of T cell-mediated immunity in breast milk can provide new information on HCMV transmission and also the role of TrMs in controlling the infection. The PI is well trained and highly qualified to carry out the proposed studies. She has in the past successfully isolated and characterized HIV-specific TrMs from the BrM of HIV-infected women. Hypothesis for the proposal shows innovative thinking and the experimental design is well proposed including acceptable attention to rigor and statistical considerations. Facilities are likely to be sufficient for the proposed studies. A couple of problems that somewhat reduce the enthusiasm for this proposal include relatively poor attention to alternatives and somewhat vague plan for the second half of the project. It is not very clear what the PI will do if the TrMs were found to be characteristically indifferent from each other or Tem, especially between transmitters and non-transmitters. Similarly, any specific use of data from RNA-seq library analysis is not well described. The PI will possibly identify a lot of differences especially between HCMV transmitters and HCMV non-transmitters but where she will go from there is not very clear.
Protections for Human Subjects: 
Acceptable Risks and/or Adequate Protections

Vertebrate Animals: 
Not Applicable (No Vertebrate Animals)

Biohazards: 
Acceptable

Resource Sharing Plans: 
Acceptable

Authentication of Key Biological and/or Chemical Resources: 
Acceptable

Budget and Period of Support: 
Recommend as Requested

THE FOLLOWING SECTIONS WERE PREPARED BY THE SCIENTIFIC REVIEW OFFICER TO SUMMARIZE THE OUTCOME OF DISCUSSIONS OF THE REVIEW COMMITTEE, OR REVIEWERS’ WRITTEN CRITIQUES, ON THE FOLLOWING ISSUES:

PROTECTION OF HUMAN SUBJECTS: ACCEPTABLE

INCLUSION OF WOMEN PLAN: ACCEPTABLE

INCLUSION OF MINORITIES PLAN: ACCEPTABLE

INCLUSION ACROSS THE LIFESPAN PLAN: ACCEPTABLE

COMMITTEE BUDGET RECOMMENDATIONS: The budget was recommended as requested.

Footnotes for 1 R21 AI151235-01; PI Name: SABBAJ, STEFFANIE

NIH has modified its policy regarding the receipt of resubmissions (amended applications). See Guide Notice NOT-OD-14-074 at http://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-074.html. The impact/priority score is calculated after discussion of an application by averaging the overall scores (1-9) given by all voting reviewers on the committee and multiplying by 10. The criterion scores are submitted prior to the meeting by the individual reviewers assigned to an application, and are not discussed specifically at the review meeting or calculated into the overall impact score. Some applications also receive a percentile ranking. For details on the review process, see http://grants.nih.gov/grants/peer_review_process.htm#scoring.
MEETING ROSTER

Immunity and Host Defense Study Section
Immunology Integrated Review Group
CENTER FOR SCIENTIFIC REVIEW
IHD
10/24/2019 - 10/25/2019

Notice of NIH Policy to All Applicants: Meeting rosters are provided for information purposes only. Applicant investigators and institutional officials must not communicate directly with study section members about an application before or after the review. Failure to observe this policy will create a serious breach of integrity in the peer review process, and may lead to actions outlined in NOT-OD-14-073 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-14-073.html and NOT-OD-15-106 at https://grants.nih.gov/grants/guide/notice-files/NOT-OD-15-106.html, including removal of the application from immediate review.

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* Temporary Member. For grant applications, temporary members may participate in the entire meeting or may review only selected applications as needed.

Consultants are required to absent themselves from the room during the review of any application if their presence would constitute or appear to constitute a conflict of interest.