# CHARACTERIZATION OF T LYMPHOCYTES FROM PEDIATRIC HEART TRANSPLANT PATIENTS WHO CARRY EBV LOADS IN THEIR PERIPHERAL BLOOD

by

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## ABSTRACT

**Hypothesis:** The Epstein-Barr (EBV) virus causes a harmless, lifelong infection in nearly all adults, but is found in 85% of all cases of post-transplant lymphoproliferative disorders (PTLD). In healthy adults, it is a latent infection, but in some patients who have received a transplant, it is present for months at a time in the peripheral blood. This chronic viral load carrier state is an important risk factor for developing PTLD, although the viral load itself does not predict the disease. A recent hypothesis from our lab identified an inadequate Type 1 T cell response, increased EBV replication and a shift toward T follicular-like (TFh) cells as issues that may contribute to a viral load carrier state. For this thesis, our hypothesis is that pediatric heart transplant patients with EBV loads are those whose $CXCR3^+$ (Type 1) EBV-specific $CD8^+$ T cells are decreased (no adequate anti-EBV surveillance), and whose $CXCR5^+$ (TFh) $CD4^+$ T cells are increased (help to EBV-infected memory B cells).

**Objectives:** Our objectives were to assess the frequency and phenotype of Type 1 EBV-specific $CD8^+$ T cells and of $CD4^+$ T cells in pediatric heart transplant patients who have viral loads but are otherwise asymptomatic of EBV-related disease.

**Methods:** We recruited healthy controls and patients under University of Pittsburgh-approved IRB protocols. The patient viral loads were determined with real time PCR in the clinical laboratory, and the frequency and phenotype of different T cell populations were identified using flow cytometry on whole blood.

**Results:** In $CD8^+$ T cell populations, EBV-specific T cells have a decreased ability to regulate infected cells, but others maintain a high level of activation. These activated cells up-regulate EBV-specific markers that allow them to travel to B cell areas in germinal centers, possibly to control infected B cells. Conversely, in these same patients, $CD4^+$ T cell populations have also gained the ability to migrate to B cell areas, but these cells may up-regulate IL-21 and provide help to infected B cells.

**Conclusions:** Some pediatric heart transplant patients display populations of both $CD8^+$ T cells and $CD4^+$ T cells that up-regulate CXCR5 and thus can migrate to the B cell area of the tonsils, which are a key area of EBV reactivation. While $CXCR5^{high}$ EBV-lytic specific $CD8^+$ T cells may be able to control lytic reactivation, CXCR5 $CD4^+$ T cells may encourage B cell proliferation and differentiation to plasmablasts, which could lead to the viral load carrier state and therefore increase a patient's risk for developing PTLD.

**Public Health Significance:** PTLD is the leading cause of tumors in pediatric patients who have received a transplant, and while PTLD mortality has varied greatly as treatments have improved, recent research has found mortality rates still approach 50%. This research seeks to provide a better understanding of the relationship between EBV loads, EBV reactivation and T cell control. These studies may provide assistance to research that seeks to identify early markers of risk for dangerous EBV loads, as early treatment often results in better outcomes.

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## ABBREVIATIONS

APC: Professional antigen-presenting cell

CD: Cluster of differentiation molecule

EBNA: Epstein-Barr virus nuclear antigen

EBV: Epstein-Barr Virus

FoxP3: Forkhead box P3 protein

HLA: Human leukocyte antigen, also known as MHC

IL: Interleukin

INF: Interferon

LCL: Lymphoblastic cell line

LMP: Latent membrane protein

MHC: Major histocompatibility complex, also known as HLA

PD: Programmed death

PTLD: Post-Transplant Lymphoproliferative Disorder/Disease

Th1: T helper lymphocyte type 1

Th2: T helper lymphocyte type 2

TGF-β: Transforming growth factor-β

Treg: Regulatory T cell

# 1.0 INTRODUCTION

## 1.1 DESCRIPTION OF THE PROBLEM

The term “post-transplant lymphoproliferative disorder” (PTLD) refers to a diverse group of diseases involving abnormal lymphocyte growth that occurs after a transplant. While incidence is low, mortality is high, and diseases discovered and treated early generally have better outcomes. The majority (~85%) of PTLDs arise from B cells and involve the Epstein-Barr virus (EBV),[1] a gammaherpesvirus first discovered in 1964 as the causal agent of Burkitt’s Lymphoma;[2] it is now associated with a variety of human malignancies and infectious mononucleosis. It infects 90% or more of the worldwide adult population and between 40 and 80% of children in developed countries.[3] After primary infection, it preferentially infects B lymphocytes and establishes a lifelong latent infection in healthy hosts.[4]

PTLD includes milder disorders such as hyperplasia as well as more aggressive forms such as Burkitt’s or Hodgkin’s Lymphoma.[1] While they most often occur in the allograft, they can occur anywhere in the body or can be systemic.[1] They are treated by reducing immunosuppression or through radiation, chemotherapy or new immunologic approaches such as depleting B cell monoclonal antibodies or adoptive immunotherapy with EBV-specific T cells.[4]

The risk factors for PTLD include the patient’s age, EBV mismatched donations (EBV-positive donor and an EBV-negative recipient), as well as certain types of allografts, particular

HLA profiles, high levels of immunosuppression and EBV status pre-transplant.[5] Another important risk factor is a chronic high EBV viral load in the blood.[6] Patients infected with EBV may either effectively control the infection and display undetectable EBV loads in their blood, or they maintain a chronic infection with low or high EBV loads. High viral load status patients have a 45% chance of developing late-onset monoclonal PTLD,[6] a form of the disease that is more aggressive and less responsive to treatment. Recent research indicates that chronic EBV load carriers after thoracic transplantation display high numbers of EBV lytic-specific $CD8^+$ T cells compared to patients who do not carry an EBV load, suggesting increased EBV lytic replication in these patients.[7] The research here seeks to provide a further understanding of the T cell factors that may allow viral load accumulation and which characteristics of high viral load patients lead them to develop PTLD.

## 1.2 PUBLIC HEALTH SIGNIFICANCE

Post-transplant lymphoproliferative disorders affect thousands of children each year who have already gone through the duress of receiving an organ transplant. PTLD can cause severe disease, organ rejection and even death for these young patients. This research project provides new data on the peripheral blood T cell phenotypes from chronic EBV load carriers before developing this dangerous disorder. This data may help with the early identification of risk markers for developing complications (PTLD), which would allow clinicians to treat pre-emptively and limit the infection, an important aspect of achieving a positive outcome.

## 1.3 POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS

"PTLD" is a term encompassing a varied group of disorders of B cell proliferation, ranging from hyperplasia to Hodgkin-like lymphomas, which occur after an allogeneic hematopoietic stem cell or solid organ transplant. The incidence of PTLD in solid organ transplant patients is between 1 and 20 percent,[8] but it is the second leading cause of post-transplant tumors for adults and the primary cause of these tumors in children, and recent studies have indicated that mortality rates still approach 50%.[9,10] While many factors can ultimately lead to a B cell disorder, 85 percent of all PTLD tumors arise from EBV-infected cells, and it is hypothesized that the diseases most often arise from virus-infected cells in the virus's Latency III program, a form of infection in which all of the virus's latent proteins are expressed.[1,10] With all of its latent proteins active, the virus can protect the infected B cells from being destroyed through normal processes and programs them to divide indefinitely. This type of replication, which can result in B cell mutations, is normally halted by T cell surveillance.[1]

PTLD can be divided into four categories: hyperplastic, polymorphic, monomorphic and other. Mononucleosis-like PTLD and irregular growths of normal cells fall into the hyperplastic category; the second and third categories encompass more classical lymphomas where irregular lymphocytes divide, and Hodgkin-like lymphoma, a more severe form of the disease, falls into the fourth category.[1] PTLD occurring earlier during a patient's recovery is generally less aggressive than late-onset PTLD.[6]

Different types of transplants carry different risks for PTLD. Kidney transplants have some of the lowest rates of risk for PTLD, while small bowel and thoracic transplants carry the highest risk.[8] The risk for a pediatric patient to develop PTLD after a heart transplant is around 10%, and transplants of the lungs or lungs and heart carry a risk closer to 20%.[11] This may be

due to the amount of immunosuppression needed to prevent allograft loss or to the amount of lymph tissue transplanted.[5]

## 1.4 EPSTEIN-BARR VIRUS

The Epstein-Barr virus is a gammaherpesvirus comprised of double-stranded DNA that infects over 90% of the adult population worldwide.[2,4] For most people, this occurs without symptoms or as a mild illness, while for those who are first infected as adolescents or young adults, there is a 50% chance the primary infection will cause infectious mononucleosis.[12] In a typical infection, EBV enters the body through the mouth via infected saliva where it can travel to the epithelium of the tonsil tissue and cause a lytic infection within the epithelial cells. While the virus will, in the vast majority of circumstances, only establish a latent infection in B cells, the epithelial cells are in important part of its life cycle. Viruses made in epithelial cells are up to two orders of magnitude more infectious for B cells. Conversely, viruses created by B cells are less infectious for other B cells, but slightly more infectious for epithelial cells.[13]

Upon exiting epithelial cells, the virus binds to the CD21 and HLA Class II molecules of naive B cells and infects them. The viral latent proteins drive the B cell to enter the cell cycle and become a centroblast, then a plasma cell or a resting memory cell.[4] Recently, it has been proposed that EBV-infected centroblasts cannot divide faster than they die within a germinal center, which may be due to a lack of survival signals or due to T cell surveillance.[14] However, the infection is successful enough in producing infected memory cells, as EBV may be present in up to half of the total memory pool.[15]

Like all herpesviruses, EBV is capable of switching into a latent phase in which it expresses different proteins that aim to not kill the cell, but manipulate it to divide or live quietly in the memory pool. It is during latency that the virus controls B cells, and it can also prevent them from being destroyed by regulatory processes. To achieve B cell proliferation, it must express most of its latent genes, and these are prime targets for cytotoxic T lymphocytes.[4] EBV can infect memory B cells in vitro; the resulting lymphoblastoid cell lines (LCLs), divide indefinitely and provide insight into the viral life cycle in the laboratory setting.[12] An illustration of EBV's two-phase life cycle can be seen in Figure 1.

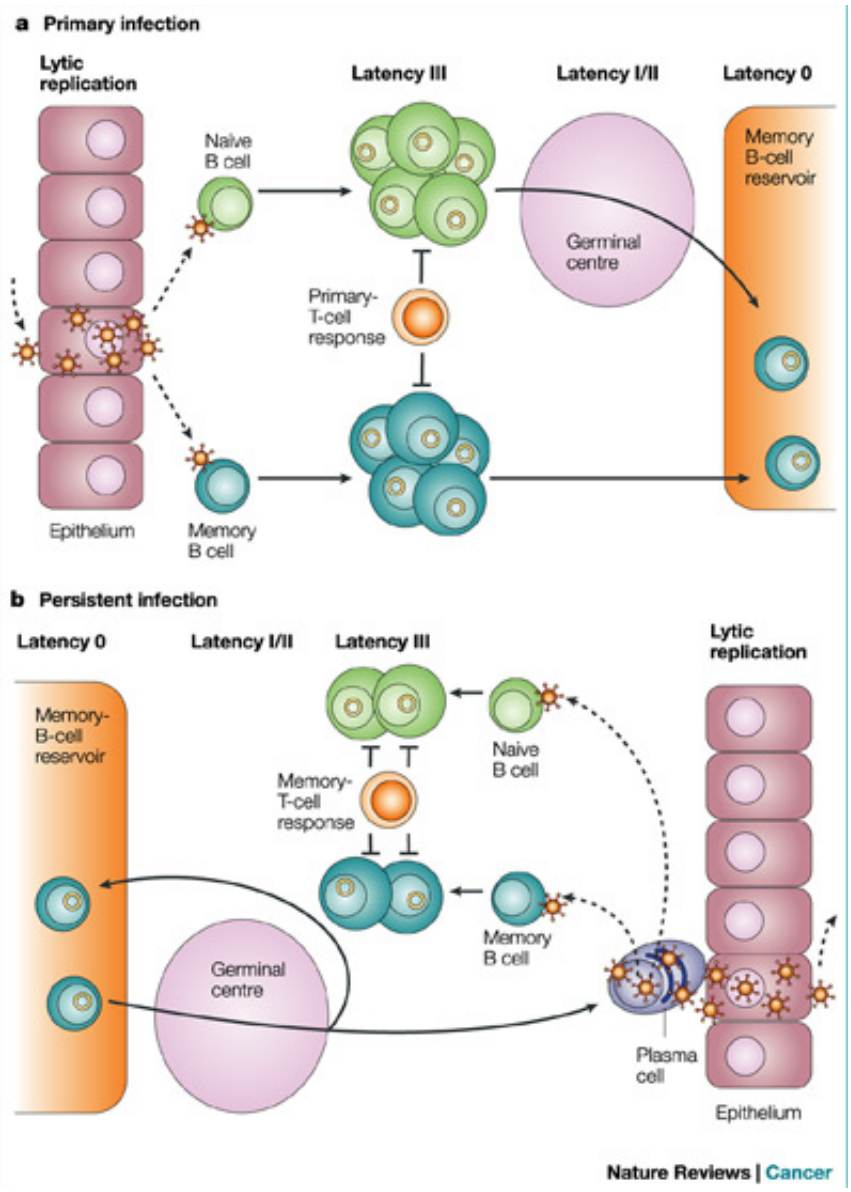


**Figure 1: The EBV Life Cycle.**[4]

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The viral genome can encode about 80 proteins, but only uses 9 during latent infections. EBV is capable of expressing four latency programs. In Latency II and III, it can cause naïve cells to migrate to germinal centers and become plasmablast cells that clonally expand, creating new cells infected with EBV.[16] In Latency I, it expresses only the small, non-coding RNAs (EBERs) and EBNA1, which are poor targets for T cells but can do little more than maintain the

genome in the cell. In Latency 0, EBV does not express any genes and easily evades the immune system. Different diseases can arise from the different latencies.[2,12]

B cells that have been "rescued" from germinal centers or were otherwise meant to be destroyed can have genetic defects, and when they are allowed to replicate out of control, this may cause the abnormal cells that are precursors to PTLD.[4] In individuals with low T cell function, such as immune-compromised transplant patients, chronic lytic infections can cause reinfection of more B cells.

**Table 1: EBV Latent Gene Expression, Cell Types and Related Disorders Genes**

| **Expressed** | **Latency 0** | **Latency I** | **Latency II** | **Latency III** |
|---|---|---|---|---|
| EBER 1 and 2 | + (probably) | + | + | + |
| EBNA1 | | + | + | + |
| LMP1 | | | + | + |
| LMP2 | | | + | + |
| EBNA2 | | | | + |
| EBNA-LP | | | | + |
| EBNA3A, B and C | | | | + |
| **Associated Disorders** | | Burkitt's Lymphoma | Hodgkin Lymphoma, Nasopharyngeal Carcinoma, T Cell Lymphoma, Gastric Adenocarcinoma | PTLD, Immunoblastic Lymphoma |
| **Cell Type** | Resting Memory B Cell, $IgD^-$, $CD10^-$ | Dividing Memory B Cell, $IgD^-$, $CD10^-$ | Germinal Center B Cell, $IgD^-$, $CD10^+$ | B Lymphoblast; Naïve B Cell, $IgD^+$ |

Composed using data from Vetsika and Callan[12] and Young, Arrand and Murray[2]

Most healthy people carry EBV in their resting memory B cells in Latency 0 or 1, with occasional asymptomatic reactivation of the lytic cycle achieved by B cell differentiation into plasma cells and reinfection of the epithelium of the oropharynx. However, disruption of immune surveillance by disease or immunosuppressants can allow EBV-infected lymphoblasts to divide out of control, resulting in a variety of malignancies.[4]

EBV was the first virus to be identified as a causative agent of human malignancy, and it was after studying an endemic childhood cancer in Africa called Burkitt's Lymphoma that the virus was discovered. Today, it is strongly associated with mononucleosis, Hodgkin's Lymphoma, post-transplant lymphoproliferative disorders, NK and T cell lymphomas, nasopharyngeal carcinomas and gastric carcinomas in addition to Burkitt's Lymphoma. It is also thought to have a role in a variety of cancers and autoimmune disorders, but the evidence for the virus causing such disorders is sparse.

## 1.5 EBV INFECTIONS AFTER TRANSPLANTATION

Transplantation of solid organs is an effective treatment for organ failure diseases, with over 100,000 solid organ transplants performed annually.[17] In order to reduce the risk for organ rejection, patients are placed on immunosuppressive therapies that decrease the activity of a patient's T cells, and thus protect them from allograft rejection. Whether a patient is EBV negative or positive before a transplant, it is common for viral status to change after the transplantation. In asymptomatic pediatric patients (who do not have PTLD or EBV disease symptoms), about 34% will not have detectable blood viral loads, 50% have low viral loads and 16% have high viral loads.[18]

Patients with a chronic high viral load (lasting longer than six months) have a 45% risk of developing late-onset PTLD, a more aggressive and usually monomorphic version of the disease.[6] While reducing immunosuppression has been shown to eliminate early-onset PTLD, which is often polymorphic and less aggressive, this is not effective for late-onset PTLD.

Late/monomorphic PTLD must often be treated with chemotherapy and radiation, placing extra strain on transplant patients.[19]

## 1.6 EBV-INFECTED B CELLS

By replicating B cell life cycle events, the virus effectively evades immune surveillance and establishes persistence. Naïve B cells encounter their specific antigen, become activated, divide into short-lived and potent effectors or long-lived and resting memory cells.[20] The normal B cell life cycle can be seen in Figure 2.[21]

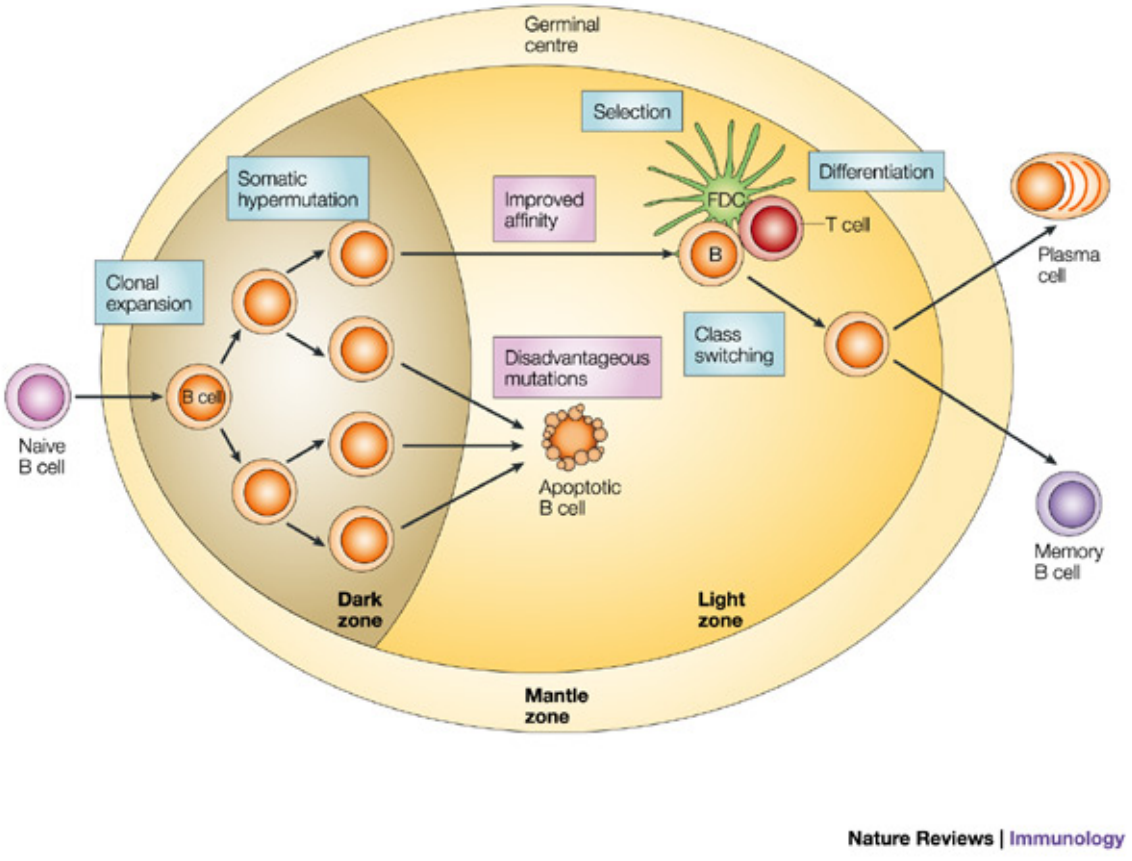


**Figure 2: B Cell Life Cycle[22]**

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As shown in the figure, B cells are first selected for survival if they display a functional B cell receptor (BCR). After this, they will quickly die off unless they have mutations that allow them to interact with follicular dendritic cells and T follicular helper cells. Both of these cells provide critical survival signals to these activated B cells. B cells that survive both of these selective processes may switch their class of immunoglobulins, differentiate into memory B cells or plasma cells, and finally are able to leave the germinal center.[22]

B lymphocytes infected with EBV are unique because the virus manipulates them to carry out normal life cycle events such as proliferation and exiting the cell cycle. For example, EBV encodes BHRF1 and BALF1, both of which are homologues of Bcl-2 and work together to help infected cells avoid apoptosis.[2] Bcl-2 is also structurally similar to CD40, a protein that provides growth signals to B cells.[4] Some research has suggested that EBV infects naïve B cells or post-germinal center B cells,[4] but the virus preferentially infects resting memory B cells.[1] In fact, Hochberg and Souza found that during a lytic infection when up to 50% of memory cells were infected, but less than one in $10^4$ naïve B cells carried the EBV genome.[15]

## 1.7 THE ROLE OF VIRAL REACTIVATION AFTER TRANSPLANTTAION

So far, EBV has been shown to result in benign latent infections or to drive malignancies through mutations occurring during its Latency III phase. However, an important risk factor for developing PTLD is a high viral load after a transplant. In healthy humans, EBV can reactivate from latently infected memory B cells and plasmablasts during times of stress or during peak seasons of allergen exposure and minor respiratory infections.[23] During lytic reactivation, EBV-lytic specific $CD8^+$ T cells from healthy subjects are promptly activated into effectors and

eliminate lytically infected cells. Because of this swift and effective response, healthy individuals do not experience chronic EBV infection or accumulation of EBV loads. Immunocompromised patients such as transplant patients display impaired T cell function, and in many patients, Epstein-Barr viral loads are present in the blood.[24]

Chronic immunosuppression may render T lymphocytes anergic or exhausted, making them much less capable of identifying and killing EBV infected cells. Anergy and exhaustion are intrinsic cellular controls that are vital counterbalances to T cell growth and proliferation, and these mechanisms, along with dominant mechanisms such as dedicated suppressor cells, ensure the immune response does not overwhelm the host.[25] IL-2, IL-4, INF-γ, IL-7, IL-15 and IL-21 are a few cytokines that stimulate T cell growth stimulate their function. IFN-γ and IL-21 in particular have been shown to assist $CD8^+$ T cells with maintaining cytotoxic activities, and were analyzed in this study.[26] Normally, T cell regulation either prevents the death of all clones involved in an infection or promotes self-tolerance,[27] but in PTLD, this regulatory response may be overwhelming, preventing T cells from controlling lesions. Recent research has found that pediatric patients who had progressed to PTLD had high viral loads and low cytotoxicity, indicated by low IFN-γ, in EBV-specific T cells, but patients with higher functionality had better outcomes.[24,28]

## 1.8 RELEVANT VARIETIES OF T CELLS

T cells begin life much in the same was as B cells. Unlike B cells, naïve T cells leave the bone marrow and travel to the thymus to mature. They initially express both CD4 and CD8 molecules, then it is postulated that TGF-β assists in the process that differentiates them into cells expressing either CD4 or CD8 molecules;[29] afterward, they leave the thymus and enter circulation, where they pass through the blood and lymph nodes in 12 to 24 hour cycles. They mature and proliferate only if they encounter MHC loaded with antigen-derived peptides. CD4 binds to MHC class II, found only on professional antigen-presenting cells (APCs), and CD8 binds to MHC class I, found on all nucleated cells. T cells require multiple signals to be activated, and these signals determine their function. The helper CD4+ T cells most relevant to this thesis are Th1 and TFh cells.[20]

### Type 1 CD8+ and CD4+ T cells

The Type 1 response is critical for the control of intracellular[20] pathogens such as viruses and is characterized by inflammation and the activation of cytotoxic T cells and macrophages. It is characterized by T cell production of IL-2, IFN-γ □□□ TNF-α. □□s□□□□□ of □□□CD4 T cells can secrete IL-21, and they may help memory B cells to differentiate into plasmablasts and produce high affinity IgG antibodies.[20] In this study, we divided Type 1 helpers into conventional and T follicular-like T cells based on the expression of (i) CXCR3/CCR5 (Th1) chemokine receptors that drive Th1 cells to migrate to the site of antigenic challenge and to promote inflammation and of (ii) CXCR5 (TFh), a chemokine receptor that allows cells to migrate into B cell areas of lymphoid follicles.[28]

### 1.8.1 Th2

The Th2 response is critical for controlling extracellular pathogens such as bacteria and parasites, and also mediates allergic reactions. These cells favor B cells and antibody production and are characterized by the production of IL-4, IL-5, IL-13 and also IL-21.[20] Skewing Th1 toward Th2 responses during EBV surveillance after a transplant results in a lack of EBV control.

### 1.8.2 Th17

Another T helper cell subset is the Th17 $CD4^+$ cell subset, which is distinguishable from the other T helper cells by the its expression of IL-17A, IL-17F, IL-22 and IL-21.[29]

Th17 cells are critical for the control of fungi, while IL-17-mediated immunopathology is linked to autoimmune diseases. Similar to Th2, this type of response is not effective for the control of a viral infection.

### 1.8.3 T Follicular Helper Cells

A last $CD4^+$ helper cell category of note is the T follicular helper cell (TFh). These cells can be found in peripheral blood, although their primary function is to provide help to B cells within the germinal centers. These cells have been shown to participate to the germinal center (GC) formation in the B cell follicles of the secondary lymphatic organs.[30] TFh cells also provide critical help to naïve B lymphocytes for the generation of memory B lymphocytes and are involved in the generation of plasmablasts that secrete high-affinity, protective T cell-dependent antibody responses to bacterial and viral infections, as well as to prophylactic vaccination.[16,31,32]

They are characterized by (i) the expression of CXCR5, a chemokine receptor that drives them toward CXCL13 in the B cell follicles, (ii) the co-expression of co-stimulatory molecules ICOS and CD40L, and (iii) production of TFh-like cytokines. Tfh-like $CD4^+$ T cells can be found in peripheral blood, and are considered to be the counterparts of GC TFh cells. In fact, it is now believed that circulating TFh are the best surrogate cell subsets to monitor for GC activity.[33] TFh cells can be divided into subsets, distinguished by cell surface chemokine receptors as follows: $CXCR5^+CXCR3^-CCR6^+$ indicates TFh Th17 cells; $CXCR5^+CXCR3^+CCR6^-$ indicates TFh Th1, and $CXCR5^+CXCR3^-CCR6^-$ indicates TFh Th2 cells.[33] Each subcategory of TFh shares common functions with the larger group of cells. For instance, TFh Th17 cells behave much like Th17 cells. Up to this point, no information on their role during EBV surveillance and viral reactivation is known.

## 1.9 T CELL IMMUNITY TO EBV

Primary infection elicits strong humoral (antibodies) and cellular (CD4 and CD8 T cells) immune responses that keep EBV infection under control.[12] Literature supports the pivotal role of cytotoxic $CD8^+$ T cells in controlling lytic infection and in limiting the expansion of latently infected B cells. The $CD8^+$ T cell response to EBV antigens is a classic anti-viral Type 1 (IFN-γ/TNF-α/cytotoxic) MHC class I restricted response.[17,34] Frequencies of memory EBV-specific $CD8^+$ T cells are quite high in the peripheral blood of healthy $EBV^+$ subjects and are needed for surveillance for EBV replication and reactivation from latency. However, the range of $CD8^+$ T cell specificities is narrow, with marked immunodominant $CD8^+$ T cell responses towards certain EBV-lytic (e.g. BZLF-1, BMLF-1) or latent (e.g. EBNA 3A, 3B, 3C) antigens.

Subdominant responses are also described (e.g. LMP1, LMP2), while minimal $CD8^+$ T cell responses (e.g EBNA1) occur due to EBNA1's inappropriate sequence for MHC class I processing and presentation pathway. This is compensated by vigorous EBNA-1-specific $CD4^+$ T cell responses in healthy individuals.[35,36]

# 2.0 MATERIALS AND METHODS

## 2.1 HUMAN SUBJECTS

Seventeen asymptomatic pediatric heart transplant patients and seven healthy control volunteers who consented under IRB protocols approved by the University of Pittsburgh were analyzed in a cross-sectional study of viral loads and cell surface markers. The patients included 6 females and 11 males. There were 11 patients with low viral loads (LVL) and 6 with high viral loads (HVL). All patients had an A2 HLA profile to allow analysis with EBV-specific dextramers.

EBV viral loads were determined by PCR as follows: LVL carriers had EBV loads ranging between 100 and 16,000 genomic copies/ml blood, detected in more than 80% of measurements, including the time of analysis, and HVL carriers had more than 16,000 genomic copies/ml blood on at least 50% of determinations. These levels were measured over a period of at least 6 months before the current immunologic analyses.[7]

Maintenance immunosuppression was comparable for all patients, and consisted of a tacrolimus-based immunosuppression with variable use of anti-proliferative agents and/or steroids. For the average ages, induction therapy and mean years post-transplant, please see Table 2 below.

**Table 2: Study Demographics**

| | HC (n=7) | LVL (n=11) | HVL (n=6) |
|---|---|---|---|
| **Mean Age ± SD (years)** | 43.8 ± 10.9 | 14.2 ± 5.7 | 8.9 ± 5.0 |
| **Gender** | 4M/ 3F | 9M/ 2F | 2M/ 4F |
| **Induction Therapy** | n/a | 36% | 67% |
| **EBV status pre-Tx*** | n/a | 92% seronegative | 100% seronegative |
| **Mean Years post-TX** | n/a | 12.2 | 8.2 |

All patients were EBV sero-positive at time of analysis and are on tacrolimus-based immunosuppression with variable use of anti-proliferative agents and/or steroids.

All patients were HLA-A02+

## 2.2 FLOW CYTOMETRY

T cell surface markers were detected by flow cytometry. Aliquots of 100μL of whole blood were stained with BMLF1 lytic-specific dextramers (or a negative control dextramer), as well as with a mixture of 10 fluorocrome-labeled monoclonal antibodies as indicated in Table 3. Stained blood was incubated for 20 minutes at room temperature in the dark. We then added 2mL of BD FACS Lysing Solution to lyse red blood cells, and incubated them again in the dark at room temperature for another 10 minutes. Next, the tubes were spun down in a centrifuge for 5 minutes at 1200 rpm at 4°C. Supernatants were removed, and cells were washed twice with flow wash, then stored at 4°C or immediately acquired. All events were collected on a BD Fortessa flow cytometer and analyzed with FACSDiva software (BD) and FlowJo (Tree Star). An

example of a staining protocol for a patient with an A2 profile can be seen in Table 2, with single color controls summarized in the first row.

**Table 3: Flow Cytometry Staining Protocol**

| Tube | FITC | PE | PE-Cy7 | V450 | PerCP-Cy5.5 | AF700 | APC-eFlour-780 | APC | PE-CF594 | AmCyan | eF605NC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1-12** | Single color controls | | | | | | | | | | |
| **13** | rIgG2b (1 µL) | NEG | IgG2a (1 µL) | | IgG1 (1 µL) | IgG1 (1 µL) | | IgG1 (1 µL) | CD19 (3 µL) | CD8 (1 µL) | CD3 (1 µL) |
| **14** | IgG1 (1 µL) | NEG | IgG1 (1 µL) | | IgG1 (1 µL) | rIgG2a (10 µL) | | IgG2a (10 µL) | | CD8 (1 µL) | CD3 (1 µL) |
| **15** | CXCR5 (5 µL) | BMLF (1 µL) | CD195 (3 µL) | CD45RA (3 µL) | ICOS (5 µL) | CXCR3 (3 µL) | CD62L (3 µL) | CCR6 (20 µL) | CD19 (3 µL) | CD8 (1 µL) | CD3 (1 µL) |
| **16** | PD1 (5 µL) | BMLF (1 µL) | CD25 (1 µL) | CD45RA (3 µL) | CD127 (1 µL) | TIM-3 (10 µL) | CD62L (3 µL) | IL-21R (20 µL) | | CD8 (1 µL) | CD3 (1 µL) |
| **17** | Unstained | | | | | | | | | | |

## 2.3 STATISTICAL ANALYSIS

Data was analyzed for normal distribution using the KS normality test, the D'Agostino and Pearson omnibus normality test and the Shapiro-Wilk normality test using GraphPad Prism 5 (GraphPad Software, Inc.). Using the same software, we applied one-way ANOVA or a Kruskal-Wallis Tests to determine overall differences in the data (haven't seen anywhere these tests in your graphs), then compared groups (LVL, HVL and healthy controls) using the unpaired Student's t-test or the Mann-Whitney test depending on the Gaussian distribution of the data. P values less than or equal to 0.05 were considered to be significant.

# 3.0 RESULTS

As discussed above, chronic EBV loads may be associated with an ongoing lytic EBV reactivation, detected by monitoring EBV-lytic specific $CD8^+$ T cells. High EBV load status confers a higher risk of developing late onset PTLD. It is unclear why EBV loads develop in some patients, and which failures of the immune system allow for it, so this research sought to identify phenotypic differences between healthy controls, patients with low viral loads and those with high viral loads. We analyzed cell surface phenotypes in both CD4 and CD8 T cells.

## 3.1 $CD8^+$ T CELLS

Analyses of $CD8^+$ T lymphocytes specific for the EBV lytic cycle product BMLF1 were conducted using healthy controls and patients with low and high viral loads. As shown in Figure 4, we analyzed EBV lytic-specific (BMLF1) $CD8^+$ T cells for their co-expression of CXCR3 and CCR5, which are indicators of an inflammatory or Type 1 phenotype. Results in Figure 4 demonstrate that both LVL patients and healthy controls display Type 1 phenotypes while HVL patients show a tendency of lower CXCR3/CCR5 expression, suggestive of a decline in their Type 1 function.

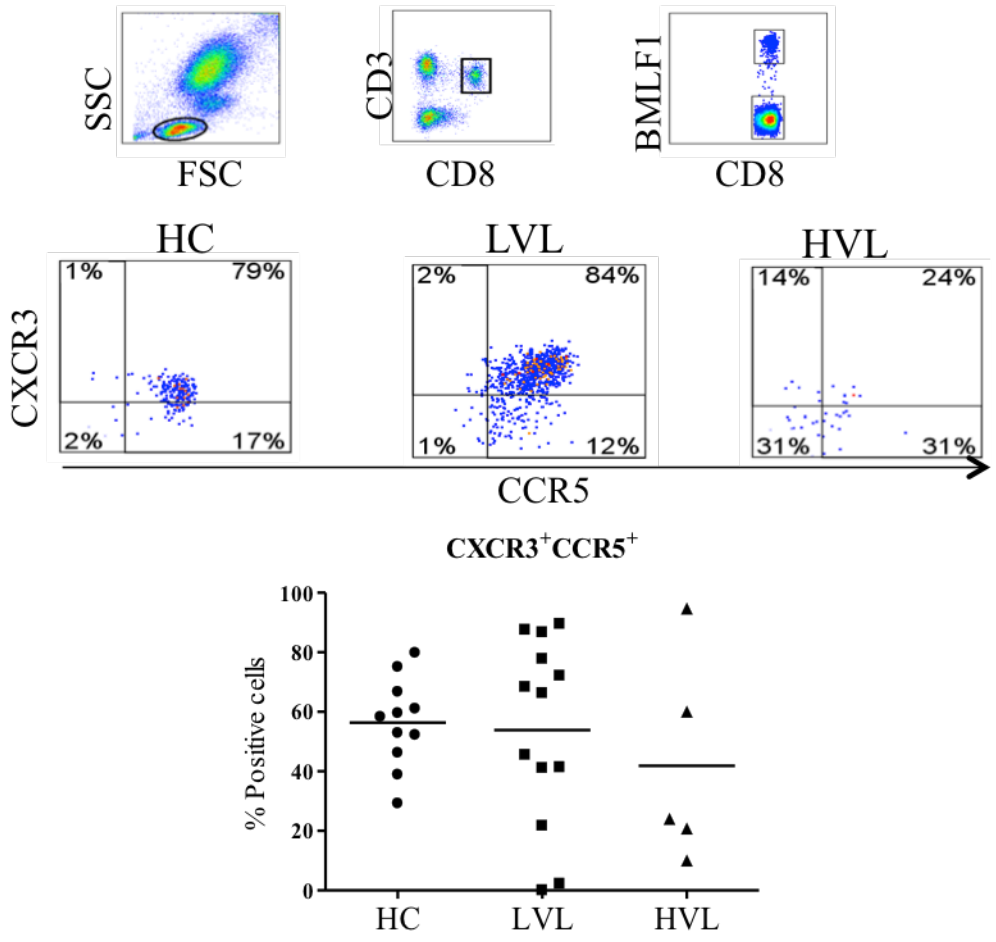


**Figure 3: Type 1 Phenotype on CD8$^+$ T Cells**

Our results further indicated that approximately half of the patients who carry EBV loads have up-regulated CXCR5 on their CXCR3$^+$ BMLF1-specific CD8+ T cells (in red), whereas healthy controls (with one exception) and rest of the patients do not express CXCR5 on their BMLF1-specific CD8$^+$ T cells. This data suggests that CXCR5 expression may allow these T cells to migrate to B cell areas of the secondary lymphatic organs and interact with B cells undergoing an EBV lytic infection (Figure 5).

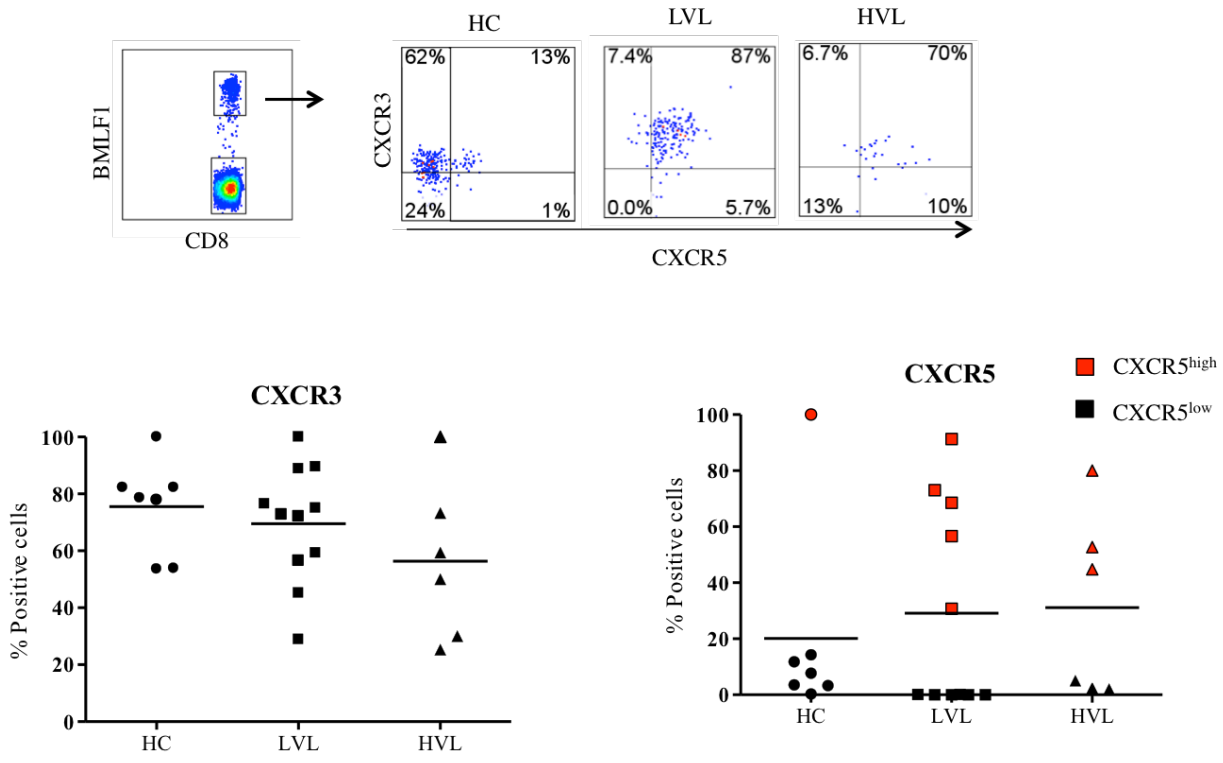


**Figure 4: Selected patients up-regulate CXCR5 on EBV-specific CD8$^+$ T cells**

To further clarify whether the Type 1 phenotype of BMLF1-specific CD8$^+$ T cells is influenced by CXCR5 expression, we compared patients who displayed high levels of CXCR5 (shown in red) to patients who lacked CXCR5 expression (shown in black). Results in Figure 6 show that while CXCR3/CCR5 levels were comparable among LVL patients regardless of CXCR5 expression, the proportion of CXCR3/CCR5 was significantly higher on CXCR5$^{high}$ than on CXCR5$^{low}$ BMLF1-specific CD8$^+$ T cells from the HVL group. These results suggest that for the HVL cohort of patients, only high levels of CXCR5 expression correlated with Type 1 EBV-lytic specific CD8$^+$ T cells, capable of an active inflammatory response in the B cell areas of the tonsils.

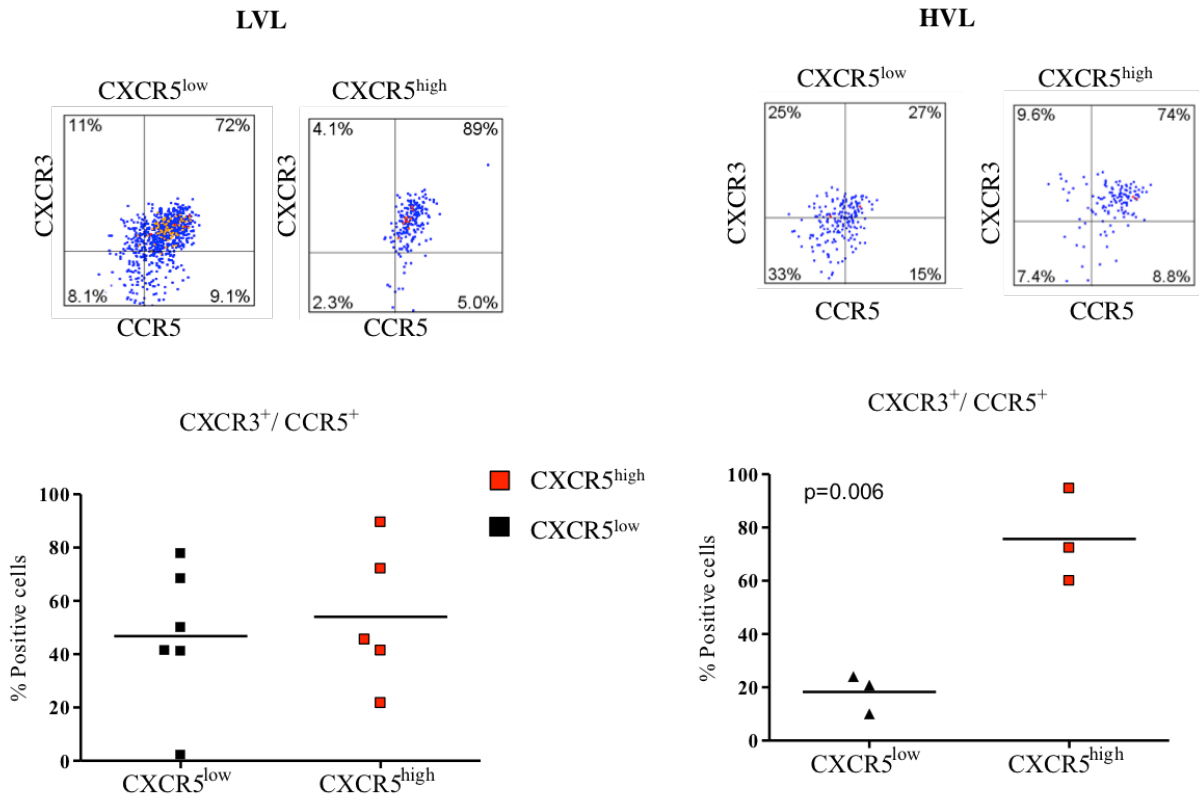


**Figure 5: CXCR5$^{high}$ EBV-specific CD8$^{+}$ T cells maintain CXCR3 expression**

We next assessed PD-1, ICOS and CD62L expression on circulating CXCR5$^{high}$ and CXCR5$^{low}$ BMLF1-specific CD8$^{+}$ T cells from LVL and HVL patients. Results in Figure 7 indicate that patients displaying CXCR5$^{high}$ expression significantly up-regulated the costimulatory molecule ICOS (critical for the interaction with B cells) and CD62L (the L-selectin that helps T cell migration to secondary lymphatic organs) in both LVL and HVL groups. Moreover, CXCR5$^{high}$ patients in both LVL and HVL cohorts showed PD-1 expression, further confirming the activated status of CXCR5$^{high}$ cells. In contrast, CXCR5$^{low}$ BMLF1-specific CD8$^{+}$ T cells do not co-express ICOS or CD62L, suggesting their inability to migrate to B cell areas of the tonsils (Fig 7).

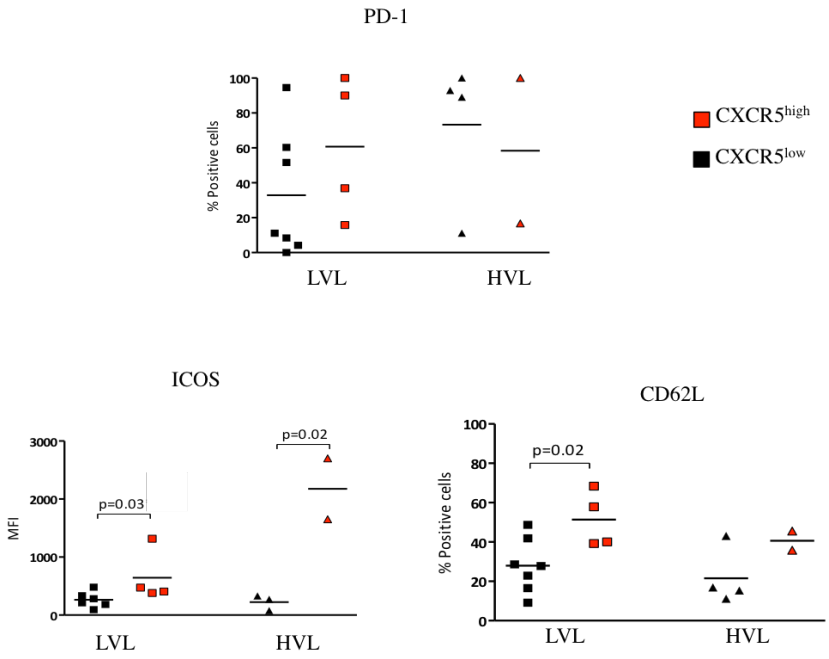


**Figure 6: CXCR5$^{high}$ EBV-specific CD8$^{+}$ T cells express PD-1 and up-regulate ICOS and CD62L**

Next, we investigated the ability of BMLF1-specific CD8$^{+}$ T cells to respond to the helper cytokines IL-2 and IL-21, which are pivotal cytokines for T cell growth and Type 1 polarization (IFN-γ production). In addition, IL-21 is critically involved in generation of TFh cells. Results in Figure 8 demonstrate that only CXCR5$^{high}$ BMLF1-specific CD8$^{+}$ T cells from both LVL and HVL patients up-regulate receptors for IL-21 and IL-2, while the CXCR5$^{low}$ cells do not, suggesting a direct role of these cytokines in rendering these cells activated (IL-2 and IL-21) and gaining a TFh like phenotype (IL-21).

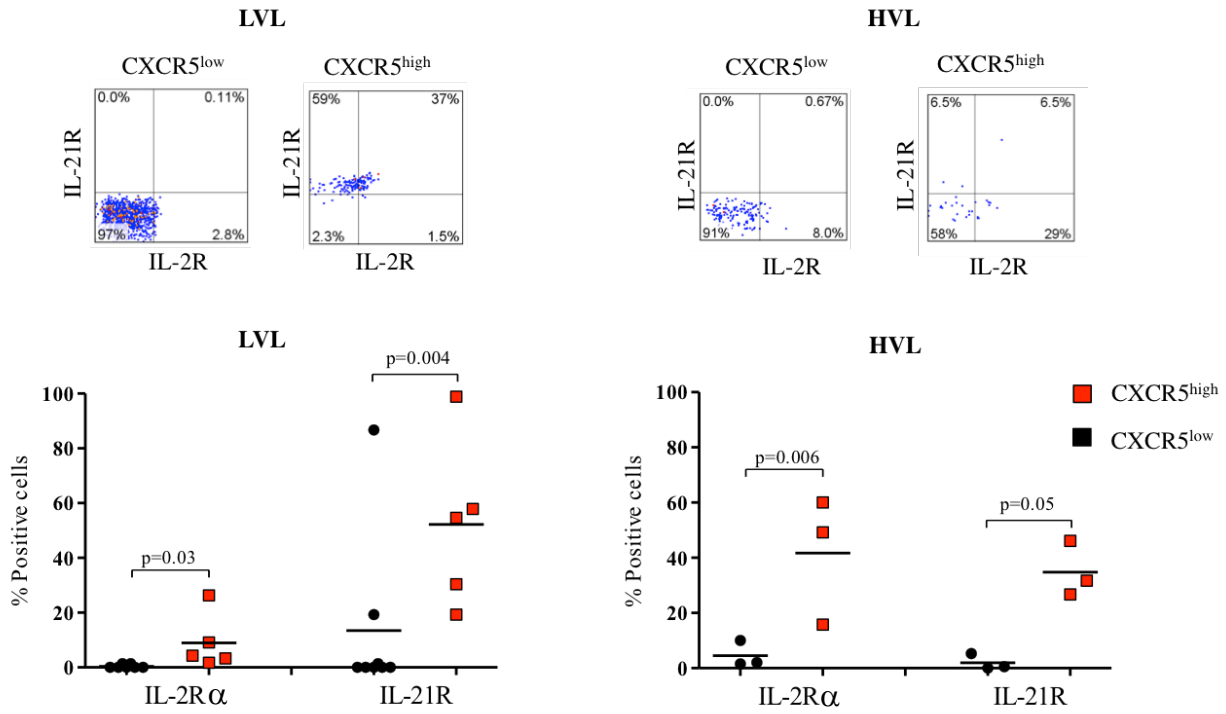


**Figure 7: CXCR5$^{high}$ EBV-specific CD8$^{+}$ T cells up-regulate IL-2Rα and IL-21R**

In summary, CD8$^{+}$ T cells that are specific for the EBV lytic protein BMLF1 selectively up-regulate CXCR5, ICOS, CD62L, are responsive to helper cytokines and display Type 1 phenotypes in approximately 50% of LVL and HVL patients. These CD8$^{+}$ T cells resemble activated TFh-like cells and may interact with B cells expressing BMLF1 antigens in the B cell areas of the tonsils to control the EBV lytic reactivation.

## 3.2 CD4$^{+}$ T CELLS

We next analyzed the phenotype of helper CD4$^{+}$ T cells in the same patients and healthy controls studied above. We assessed CD4$^{+}$ T cell polarization based on CXCR3 vs. CXCR5 distribution.

Results in Figure 9 show that while the overall proportion of CXCR3$^{+}$ CD4$^{+}$ T cells remained largely unchanged among healthy controls, LVL and HVL cohorts, pediatric heart transplant patients displayed significantly higher levels of CXCR5 as compared to healthy controls. Moreover, red data points identify subjects who showed CXCR5$^{high}$ expression on BMLF1-specific CD8$^{+}$ T cells, while the black data points identify patients with CXCR5$^{low}$ BMLF1-specific CD8$^{+}$ T cells.

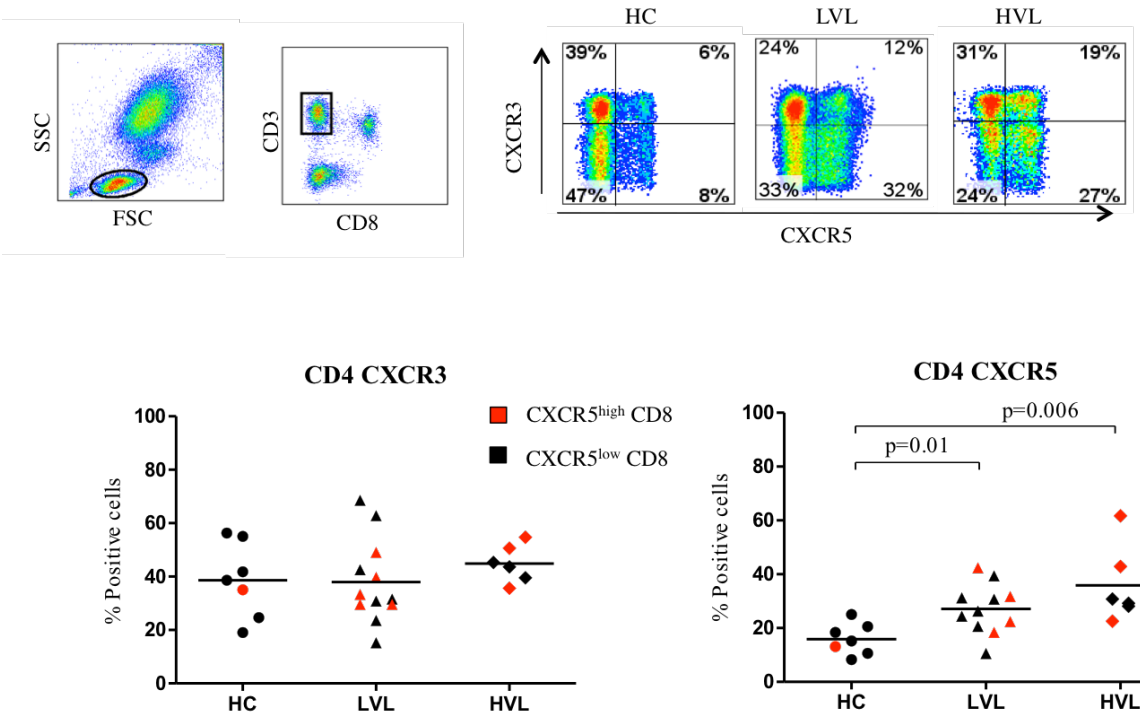


**Figure 8: Pediatric patients that carry EBV loads display high levels of CXCR5 TFh cells**

In addition, CD4$^{+}$ T cells can be classified into subsets based on expression of CXCR3 and CXCR5 as follows (Figure 10): (i) CXCR3$^{+}$CXCR5$^{-}$ (conventional Th1); CXCR3$^{-}$CXCR5$^{-}$ (conventional Th2/Th17); (iii) CXCR5$^{+}$CXCR3$^{+}$ (TFh Th1), (iv) CXCR5$^{+}$CXCR3$^{-}$ (TFh Th2/Th17). Results in Figure 10 further indicate that all TFh cells are increased in patients, with Th1 TFh cells being most significantly elevated in HVL patients.

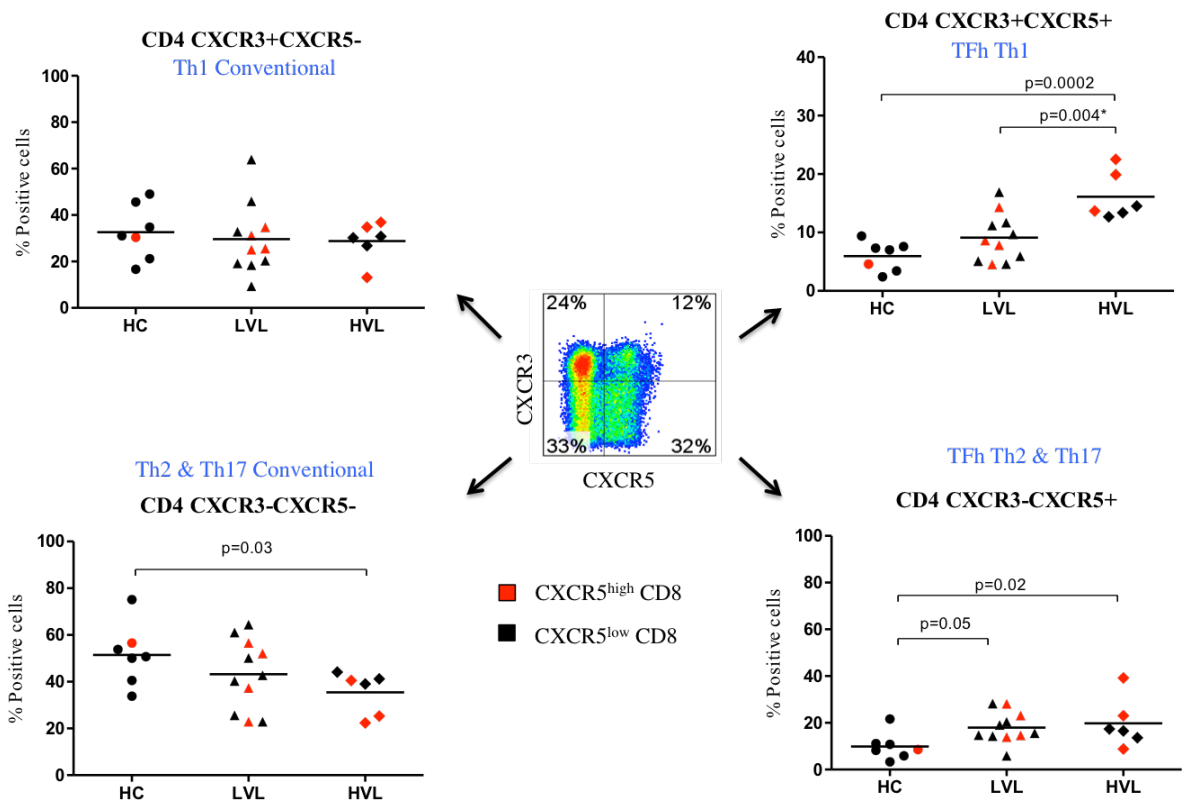


**Figure 9: HVL patients display highest levels of TFh Th1 CD4$^+$ T cells**

We found differences between patients with high and low levels of CXCR5 in their CD8$^+$ T cell populations. The CXCR5$^{low}$ groups produced some IFN-γ and IL-21. In contrast to this, CXCR5$^{high}$ groups had a significant shift toward IFN-γ and IL-21 production (Figure 8). Similar to our findings for CD8$^+$ T cells, it appears that CD4$^+$ cells that display more CXCR5 are more activated. Indeed, TFh Th1 cells directly correlate with EBV loads in patients and show increased effector memory phenotypes in HVL patients, indicative of an ongoing activation. (Figure 11).

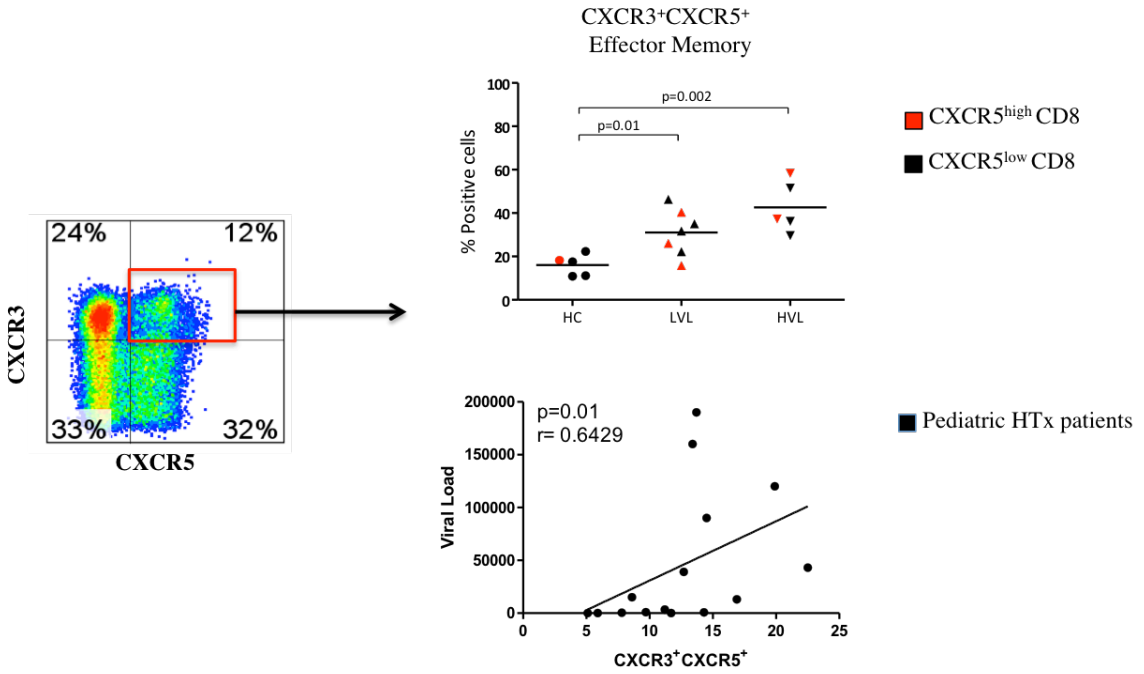


**Figure 10: TFh Th1 CD4$^+$ T cells up-regulate EM and correlate with EBV loads in patients**

Lastly, we stimulated CD4$^+$ T cells from healthy controls and pediatric heart transplant patients who expressed CXCR5$^{high}$ EBV-specific CD8$^+$ T cells to assess to assess their function. We found that conventional Th1 and TFh Th1 CD4$^+$ T cells from one HVL patient with CXCR5$^{low}$ EBV-specific CD8$^+$ T cells produced some IFN-γ and no IL-21 (Figure 12). In contrast, conventional Th1 and TFh Th1 CD4$^+$ T cells from one HVL patient with CXCR5$^{high}$ EBV-specific CD8$^+$ T cells produce significant levels of IFN-γ and IL-21, elucidating the source of IL-21 in these patients. In addition, these TFh Th1 CD4$^+$ T cells appear to interact with and help B cells hosting a lytic EBV infection.

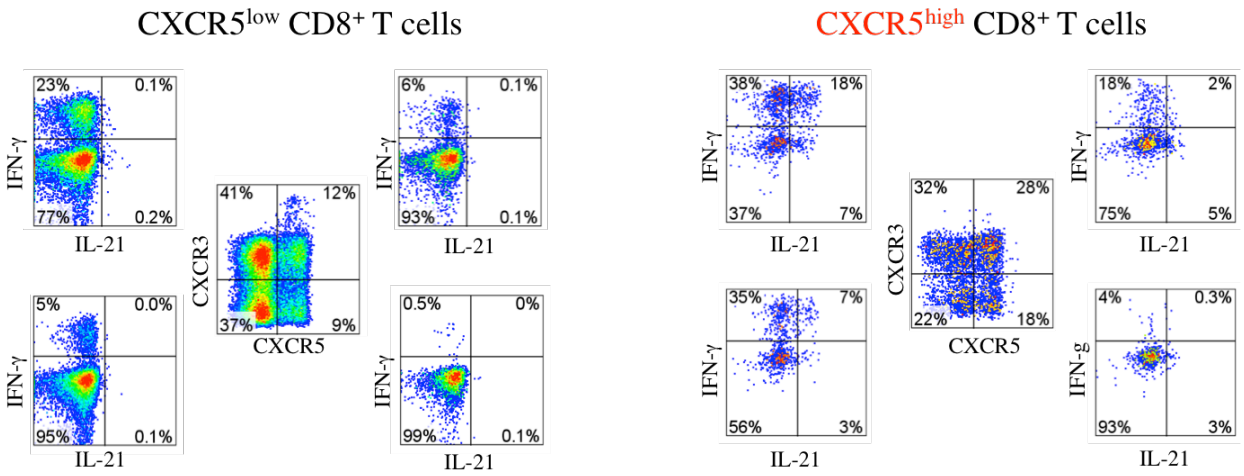


**Figure 11: CD4$^+$ T cells from patients who display CXCR5$^{high}$ EBV-specific CD8$^+$ T cells produce IFN-g and IL-21 with CD4$^+$ T cells**

To summarize the CD4$^+$ T cell findings, patients up-regulate circulating T follicular-like CD4$^+$ T cells. Patients who carry high EBV loads display the highest percentage of TFh Th1 CD4$^+$ T cells, and this population also shows a positive correlation with viral loads and a higher percentage of effector memory cells. Finally, both conventional and T follicular-like cells from patients who were previously shown to up-regulate CXCR5 on their BMLF1-specific CD8$^+$ populations produce IL-21, which may help B differentiation and switch EBV into lytic replication.

# 4.0 DISCUSSION

This research suggests that there are indeed CD8 and CD4 phenotypic differences between healthy controls and pediatric heart transplant patients and between LVL and HVL carriers. We analyzed chemokine receptors and costimulatory and activation markers that show $CD8^+$ and $CD4^+$ T cell polarization, their ability to migrate to the periphery or to B cell areas of the secondary lymphatic organs and their up-regulation of cytokine receptors that may impact how well T cells can control EBV-infected B cells.

EBV-specific $CD8^+$ T cells that are $CXCR5^{high}$ $CXCR3^+$ express ICOS and are responsive to IL-21 and IL-2 may effectively control EBV-lytic infected B cells and EBV reactivation. This Type 1 response is perhaps lacking in the similar $CXCR5^{low}$ populations, where effector cells may not be able to migrate to B cell areas in secondary lymphatic organs, but rather be effective (or not) in the periphery.

For $CD4^+$ helper T cells, our results are more complex. On one hand, conventional $CXCR5^-$ Th1 cells that lack the ability to migrate into B cell areas are most likely helpers via IFN-γ and IL-21 for BMLF-specific 1 $CD8^+$ T cells, making them potent $CXCR5^{high}$ $CD8^+$ effector T cells that may control EBV reactivation. On the other hand, $CXCR5^{high}$ $CXCR3^+$ $CD4^+$ T cells may migrate to the B cell areas of the tonsils, and due to their IL-21 production, these cells may help B cells differentiate into plasmablasts, a state that may favor EBV switch from the latent to the lytic cycle (may sustain EBV reactivation) and thus deleterious for the

EBV lytic control. Finally, TFh Th1 $CD4^+$ T cells correlated to EBV loads, suggesting their negative impact on the EBV control in this cohort (see diagram in Figure 13).

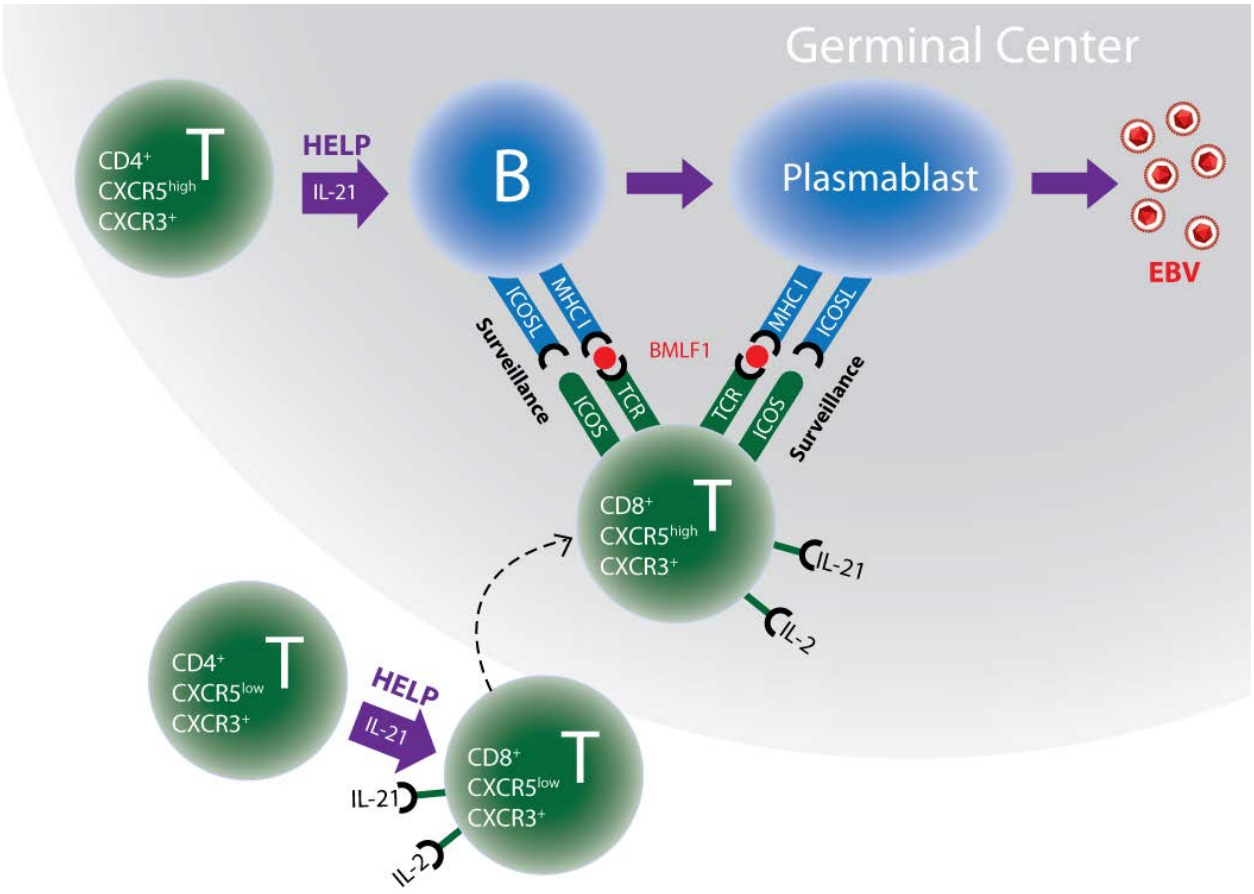


**Figure 12: Summary of Results, with some populations contributing to EBV proliferation and others contributing to EBV control**

In future research, this lab and others will need to confirm that $CXCR5^{high}$ $CXCR3^+$ EBV lytic-specific $CD8^+$ cells are truly effector cells that control EBV infections. It will also be necessary to determine whether increased $CXCR5^{high}$ $CXCR3^+$ $CD4^+$ cells promote plasmablast formation and EBV reactivation. Finally, researchers will need to monitor both of these subsets to determine if they are robust biomarkers that identify an HVL clinical state early, allowing clinicians to treat the disease early and reduce the risk for PTLD in transplant patients.

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