THE CHARACTERIZATION OF EPSTEIN-BARR VIRUS INFECTED B CELLS IN THE PERIPHERAL BLOOD OF PEDIATRIC SOLID ORGAN TRANSPLANT RECIPIENTS WITH ELEVATED VIRAL LOADS

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Epstein-Barr virus (EBV) has infected 95% of the adult population. Yet, EBV stays as a harmless passenger in infected B-cells of nearly every host. EBV depends on a careful balance between the immune system and the virus that becomes evident when the host is immunocompromised. In such individuals, EBV can manifest as one of many associated malignancies. In children who have undergone solid organ transplantation, EBV-driven post-transplant lymphoproliferative disease (PTLD) can cause significant morbidity and mortality. We examined EBV-infected cells in non-diseased pediatric transplant recipients with elevated viral loads in their peripheral blood. Examination of high EBV genome copy cells in high load patients with a combined fluorescent in situ hybridization and immunofluorescence procedure demonstrated that the majority of high copy cells had no discernible expression of immunoglobulin on the surface (Ig-null cells). Such cells are lacking the crucial survival signal provided by an intact BCR and should not survive in the circulation. By flow cytometry, high load patients were shown to have the highest percentage of Ig-null cells in their peripheral blood; those with low viral loads and non-detectable viral loads had lower percentages. The phenotype of Ig-null cells was shown to differ from the resting memory B2 phenotype of normal latently infected B cells, with variable expression of CD20, CD40, and HLA Class I and II. Sorting Ig-null cells from the peripheral blood of high load carriers further demonstrated that in all patients

examined, a large portion of the viral load was carried in the Ig-null compartment. Virus was also detected in the Ig-null, CD20- and HLA Class I- compartment, with a variable enrichment of the viral load in these compartments from patient to patient. Ig-null cells have been reported in the tumors of other EBV-associated malignancies, including PTLD, but never in the peripheral blood or in a non-disease state. This study has public health relevance because PTLD carries significant morbidity and mortality to transplant recipients; the presence in the blood of aberrant Ig-null cells which should have followed a program of apoptosis might be a risk factor for the development of PTLD or another EBV-associated disease.

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1. INTRODUCTION

1.1. BIOLOGY OF EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) is the prototype of the gamma subfamily of herpesviruses. This group contains the potentially oncogenic herpesviruses, and is composed of the genera *lymphocryptovirus (LCV)* and *rhadinovirus (RDV)* (123); EBV is also known as human herpesvirus 4 (HHV4). Members of the LCV genus are restricted to primates and are likely to have evolved from early primate RDV. EBV is the only LCV found in humans, while LCVs are endemic in both New and Old World primates (14, 37, 46, 61, 82, 85, 120).

EBV related disease was first observed in the 1940s, but the causative agent was not officially discovered until 1964 (34). In the initial studies, Denis Burkitt observed characteristic extranodal lymphomas in children in regions of Central Africa with holoendemic malaria. Because of the epidemiological and clinical characteristics of the lymphomas, an infectious etiology was suspected (19). Subsequently, in a collaboration with Tony Epstein, and his graduate student Yvonne Barr the direct observation of EB viral particles was made in cells cultured from the lymphoma biopsies sent to London, England by Burkitt(34). EBV was identified as the first human oncogenic virus. Indeed, a distinguishing characteristic of the gamma herpesvirus subfamily is their potential for human malignancy, in combination with limited in vitro host range, and establishment of latent infections in lymphocytes (123). Further, LCVs and RSVs have proven to have a close nucleotide and protein sequence relationship, which has strengthened the initial classification (10, 30).

In the virion, EBV has a linear double-stranded DNA of 184 kbp (kilobase pairs) (10, 28, 48, 50). The genome circularizes into an episome upon infection, locking into a circle a variable number of terminal repeats. This variation depends on the number of tandom repeats in the parental genome and can be used, along with other repeated regions in the DNA, to track epidemics and group the virus into strains (119). Based upon its serological responses and genomic sequence, EBV has been classified into two types, Type 1 and Type 2 (6, 29, 36, 138). While Type 1 is far more common in the western world, Type 2 is prevalent in New Guinea and equatorial Africa (57, 75, 99, 168). HIV-infected individuals and other immunocompromised persons often are infected with Type 2, in addition to Type 1 (135, 164, 165). The only major difference between Type 1 and Type 2 is in the genomic sequence encoding for EBV nuclear antigens (EBNA) LP, 2, 3A, 3B, and 3C (129). A schematic of the viral genome, both in its linear and circular form is shown in Figure 1 (163).

In vitro, EBV has a host cell restriction to B lymphocytes, presumably because the required viral receptor, CD 21, is only expressed on such cells (40). CD21 is a member of the C3d component of the complement system (81, 162), and also binds to the EBV glycoprotein (gp) 350/220 outer envelope protein (109, 149, 150). After binding, CD21 aggregates, and EBV is internalized into cytoplasmic vesicles. Gp85 is required for envelope fusion to B lymphocytes (23, 108). One of EBV's defining characteristics is its ability to transform B cells into latently infected B-lymphoblastoid cell lines (LCLs), in vitro (62, 123, 145). In some circumstances, EBV can also infect epithelial cells. In vitro infection can be established in epithelial cells, albeit at a very low level and inefficiently (112, 136, 137). However, in vivo, epithelial cells are fully permissive for a lytic EB viral infection (112, 137).

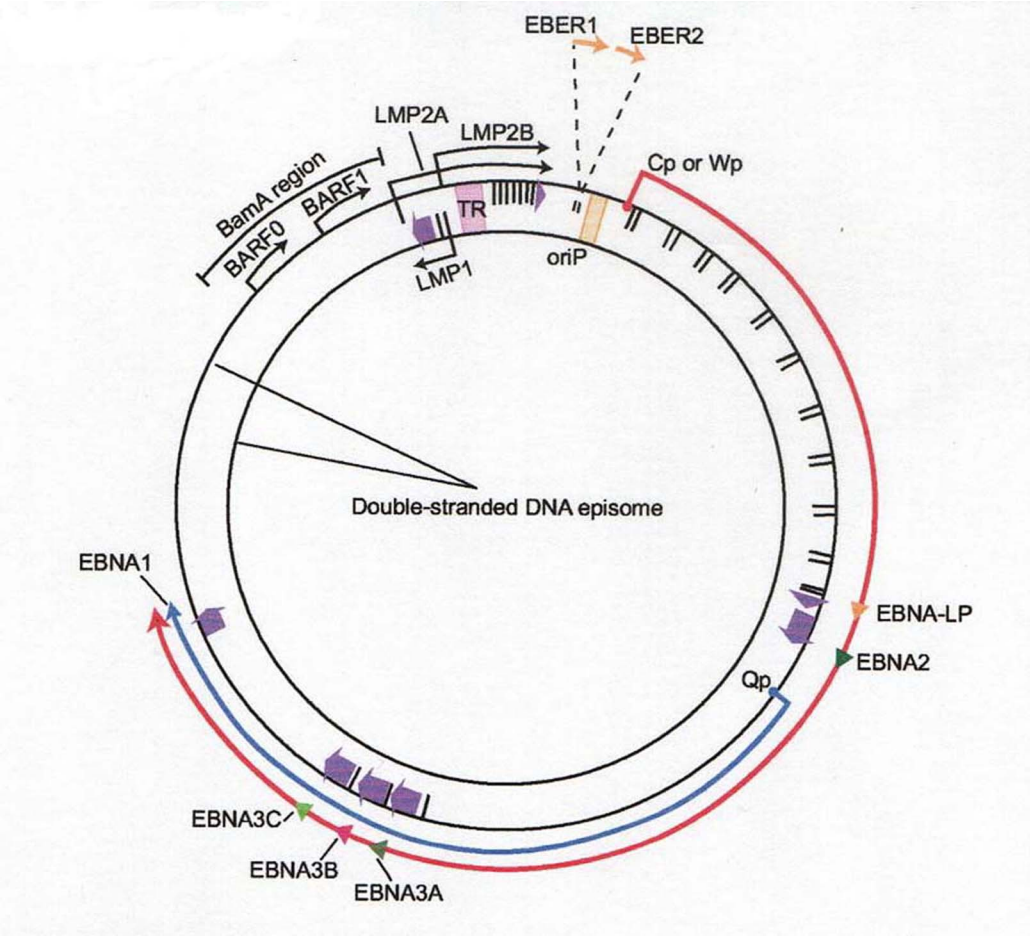

Figure 1. **The circularized EBV genome (163).**

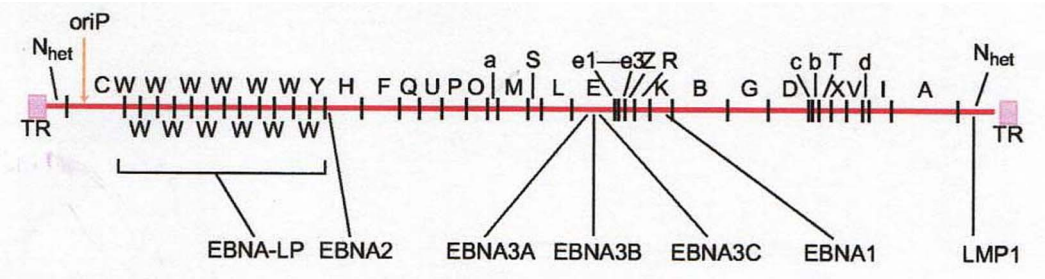

Figure 2. **The linearized EBV genome (163).**

1.2. EPSTEIN-BARR VIRAL LATENCY AND ASSOCIATED PROTEINS

Nearly all types of human primary B cells, with the exception of plasma cells, can be infected with EBV. The outcome of EBV infection in this scenario is a continuously proliferating lymphoblastoid cell line, with minimal or nondetectable virus replication and a characteristic pattern of viral gene expression, termed Latency III (66, 118). Latency III includes the expression of two Epstein-Barr Encoded RNAs (EBERS), BAMHI A RNAs, six viral nuclear antigen transcripts (EBNA 1, 2, 3A, 3B, 3C, and LP), and 3 latent membrane protein (LMP) transcripts, LMP1, LMP2A, and LMP2B (123). A brief description of form and function for each of these products is detailed below.

EBERS are small, nonpolyadenylated RNAs encoded by the virus. EBER transcripts are made by RNA polymerase III and constitute the most common viral transcript in an infected cell (7, 70). Thousands of EBERS localize to the cell nucleus, where they interact with specific cellular proteins (69). While function(s) for the EBERS have been proposed, none have been confirmed. It has been hypothesized that the EBERs have an anti-interferon effect (147) and/or are involved in RNA splicing (52), but neither hypothesis has been confirmed. Further testing must be conducted to deduce their precise role.

EBNA1 is a sequence specific DNA binding protein that binds to the origin of plasmid replication (oriP) and is the virus genome maintenance protein in EBV immortalized lymphocytes (84). It is a very stable protein and is composed of 641 amino acids with a size of 76 kilodaltons (kd) (123). EBNA1 is thought to be expressed in all EBV infected cells, although it is not always detectable. OriP is composed of two elements, the family of repeats (FR) and the dyad symmetry element (DS) (60, 121, 122). FR facilitates transcription and replication of the

EBV epsiome, and further terminates the reaction. DS is the origin from which replication moves in a bi-directional manner from the DS (43). Each molecule of EBV DNA can only replicate once per each cellular S phase (1, 166) and the newly replicated episomes are distributed randomly to the newly formed cells (144).

EBNA2 is an upregulator of virus and cellular gene expression, and the expression of the other viral proteins found in immortalized cells is dependent on this function. Its RNA is transcribed upon initiation of an EBV infection in a primary B lymphocyte. Thus, without EBNA2, transformation of B cells into LCLs does not occur (123). Cellular genes that are upregulated as a result of expression of EBNA2 include CD23 (159), CD21 (3, 25, 161), and *c-myc* (72). Between EBV Types 1 and 2, EBNA2 differs the most of any other latency associated protein, resulting in a much more rapidly growing LCL for EBV Type 2 than Type 1 (123).

EBNA3A, B, and C are tandemly located on the EBV genome, and their mRNAs are expressed at very low levels in the latently infected cell (123). These three proteins range in size from 925-1069 amino acids and are very stable proteins (132). There is a weak homology in the N terminus of these three proteins. EBNA 3A and C have shown to be essential for the growth transformation of B lymphocytes (155) and their subsequent survival, but an essential function for EBNA3B has not be deduced yet. Because of the highly immunogenic nature of EBNA 3B, it is believed to be important for recognition by the immune system. As a group, the EBNA3 proteins function as transcriptional transactivators and contribute to upregulation of cellular gene expression. Further, peptides derived from the EBNA 3's are the primary targets for the recognition of immortalized cells by cytotoxic T lymphocytes.

EBNA-LP, like EBNA2, is transcribed at the beginning of EBV infection and at very high levels. Because the N terminus of EBNA-LP is derived from the W1 and W2 repeat regions

(repeating 22/44 aa segments) of the EBV genome, this protein differs in size in cells infected with different EBV isolates (117). The carboxy terminus is 45 amino acids long. This protein is thought to cooperate with EBNA2 in the upregulation of cellular and viral gene products essential for the establishment of LCLs and viral latency (58, 113). Further, EBNA-LP has been suggested to interact with RNA transcripts and be important for RNA processing (83).

LMP1 is the latent protein responsible for many of the changes associated with EBV infection of a primary B lymphocyte. These include cell clumping, increased expression of various cell surface markers and cell adhesion molecules, increased vimentin production (with which LMP1 associates), protection from apoptosis, and increased villous projections (55, 63, 128, 160). As such, LMP1 is necessary for the growth transformation of B lymphocytes (73). LMP1 is 63 Kd with 6 transmembrane domains, a short N-terminal cytoplasmic end, and a long C-terminal cytoplasmic tail. LMP1 is homologous to the tumor necrosis factor receptor CD40, both structurally and functionally (105). LMP1 is very unstable, with a half-life of only 1.5-7 hours, depending on the cell type. LMP1 is believed to be ubiquinated and rapidly degraded by the proteosome pathway in order to avoid toxic levels of the protein in the cell (88, 89, 130). However, LMP1 transcripts are much more abundant than the EBNA transcripts in latency (39, 131).

LMP2 is an integral membrane protein with two isoforms (42, 90). LMP2A and LMP2B differ in their first exon; 2A has a 119 aa amino-terminal cytoplasmic domain, while 2B lacks this domain (130). The 2nd exon forward encodes for a transmembrane region consisting of 12 integral membrane sequences, followed by a short carboxy-terminal tail (130). Because the LMP2 transcript is encoded across the fused terminal repeat region, LM2 is only expressed in cells after circularization of the EBV genome. LMP2A associates with src family tyrosine

kinases of B lymphocytes (91), but neither 2A nor 2B is essential for the transformation of B cells into LCLs or their subsequent survival (77, 92, 93, 94, 124, 140). Rather, LMP2A is thought to block the activating signals that would occur when CD19 or sIg are cross-linked on the cell surface (100, 101). Such activation would induce the initiation of lytic replication. Further, LMP2A is thought to provide survival and anti-differentiation signals to some B cells, especially those lacking immunoglobulin on their cell surface (21). A definitive role for LMP2B has not been established.

1.3. EPSTEIN-BARR VIRAL PRIMARY AND PERSISTENT INFECTION

Latent infections by EBV have been categorized according to the patterns of viral gene expression detected and are related to the cell types involved and whether they are studied in vitro or in vivo. Latency 0 is observed in peripheral blood lymphocytes (PBLs) of healthy carriers and is defined as having no viral products expressed, with the possible exception of LMP2A. Latency 1, found in Burkitt's lymphoma cells, has the expression of only EBNA1, while Latency 2, seen in Hodgkin's and other virus-associated malignancies is defined as having the expression of both LMP1 and LMP2, in addition to EBNA1 (123). Latency III is characteristic of cells immortalized in vitro but is also detected in vivo in reactive lymph nodes of patients with active EBV infections. These latencies will be discussed in more detail in future sections. For now, a summary of their distribution is shown in Table 1.

Table 1. Programs of EBV Latency

Latency Program	0	I	II	III
LMP1	No	No	Yes	Yes
LMP2	Yes	Yes	Yes	Yes
EBNA1	No	Yes	Yes	Yes
EBNA2	No	No	No	Yes
EBNA3's	No	No	No	Yes
EBNA-LP	No	No	No	Yes
EBERS	Yes	Yes	Yes	Yes
Expression site	Resting, memory B cell	Burkitt's Lymphoma	Hodgkin's Lymphoma	PTLD, IM

Prevalence of EBV infection is near 100% in the developing world, and 95% in the industrialized nations (123). It is transmitted primarily via saliva, but can also be passed through the blood, or along with the donor organ in solid organ transplantation. Normal parent to child contact in the first years of life is responsible for most primary infections in children (54). Such infections in early childhood are nearly always asymptomatic; but primary infections in adolescence or early adulthood can be symptomatic and manifested as infectious mononucleosis (IM) (35). Why some cases of EBV infection post childhood are symptomatic, while others are not is still unknown.

IM has been described as a self-limiting lymphoproliferative disease in which up to 25% of peripheral blood memory B cells are infected with the virus (151). It is characterized by the presence of atypical mononuclear cells stemming from mostly CD8+ T lymphocytes, as well as CD4+ T lymphocytes and activated natural killer cells (NK) (123). Extensive proliferation of infected B cells and cytotoxic T cells destroy the normal histological compartments of tonsillar tissue, and a massive immune response is generated that clears the blood of most infected B cells in a few weeks. Primary CD8+ T cell epitopes are antigenically specific for mainly the EBNA 3 proteins as well as several immediate early (BZLF1 & BRLF1) and early proteins (BMLF1, BMRF1, and BALF2) (140). By molecular analysis of immunoglobulin variable region genes, it has been suggested that EBV-infected B cells do not need to pass through a germinal center reaction in order to establish a persistent latent infection (80). Typical symptoms of IM include fever, lymphadenopathy, pharyngitis, and general malaise (123). Such symptoms can last for several weeks, and are primarily due to the overwhelming proinflammatory reaction of the immune system (4, 111).

Following resolution of primary infection with EBV, a persistent carrier state is established. In a healthy individual this state is characterized by low numbers of latently infected B cells in the range of 1 to 50 per one million B cells (74, 104), secretion of low levels of infectious virus at mucosal sites with a B cell infiltrate (33, 138), and an antiviral serological response characterized by IgG antibodies to EBNA1 and VCA (viral capsid antigen), and often a low level anti-gp350 neutralizing antibody (64). Latently infected cells in the peripheral blood of healthy carriers have consistently been characterized as resting memory B2 cells (9, 78, 103, 104). These infected cells have a Latency 0 phenotype, with only the LMP2A transcript expressed. The majority of the T cells involved in the massive response specific for EBV epitopes, especially to the immediate early and early proteins, die or revert to a resting phenotype (22). However, T cells specific for epitopes, in the EBNA3's and LMP2 seem to persist and be maintained in a stable manner for long duration immunosurveillance (76, 107). Some CTL memory is maintained for the lytic epitopes, at a relatively high level (5, 22, 141, 147), as a result of continuous viral shedding at mucosal sites. The persistent and diverse immune response to EBV is probably a result of the latent and persistent nature of the virus, with episodic virus reactivations, active replication and virus shedding observed at mucosal sites in the healthy infected carrier.

Thus, EBV is extremely efficient in its persistence and achieving the balance between survival and survival of the host. By downregulating the Latency II growth transformation profile, with its strong immunodominant epitopes, to a Latency 0 profile, the virus in the circulating pool of B cells becomes undetectable by immune system. When the proliferation program is expressed, the immunogenicity of a number of essential viral proteins ensures that run-away lymphoproliferations are not a mortal threat to the host. In a healthy

immunocompetent individual, EBV is in essence a silent passenger that is easily kept in check by immunosurveillance.

1.4. ASSOCIATED MALIGNANCIES OF EPSTEIN-BARR VIRUS

In immunocompromised individuals, the balance between virus reactivation and host surveillance is often disrupted. Reduced CTL precursor frequencies are observed, in conjunction with increased viral shedding in the oropharynx and an increase in the viral load in the peripheral blood (26, 166). Studies on immunocompromised individuals have usually focused on HIV+ individuals, both children and adult, and organ transplant recipients, both solid organ and bone marrow. In adults, immunosuppression is usually imposed on an already EBV infected individual, while children who receive organ transplants or become infected with HIV are often EBV negative and must experience a primary infection in the context of immunosuppression. Congenitally immunodeficient individuals can also be at risk for diseases associated with EBV.

Diseases associated with a congenital immunodeficiency include X-Linked lymphoproliferative syndrome (XLP), fatal infectious mononucleosis, and virus-associated hemaphagocytic syndrome (VAHS). A common theme of such diseases is an inability to control the massive T-cell proliferation associated with a primary EBV infection, thereby leading to hemophagocytosis and many times, death (123). In rare instances, EBV is linked to malignancies even in the immunocompetent host.

1.4.1. Burkitt's Lymphoma

Burkitt's Lymphoma (BL), is named for Denis Burkitt who first observed these lymphomas in equatorial Africa (19). The tumors were observed in childhood/early adolescent patients, at a yearly incidence of 5-10 cases/100,000 children (18, 96). Lymphomas present at extranodal sites, commonly along the jawline, orbit, central nervous system (CNS), or as an abdominal mass. Morphologically, malignant cells are similar to germinal center B cells, but with macrophages interspersed in the tumor, giving it a "starry sky" appearance (123). The tumors are monoclonal, with usually one of three reciprocal translocations. Besides this endemic form, BL has a sporadic and an AIDS-associated form. Sporadic BL has the same characteristics as the endemic form, but occur spontaneously in Europe and the United States, albeit at a much lower rate than the endemic form and with less than 100% association with Epstein-Barr virus. Similarly, AIDS-associated BL shares a similar morphology to the other forms, both cytologically and histologically (44).

EBV has a varied association with the different forms of BL. It is highly associated (some reports say 100%) with EBV in the endemic form, while only 20-30% are associated in both the sporadic and AIDS-associated form (71, 87, 96). In EBV-positive BL's, a latency I profile is always expressed, with EBNA1 being the only latent protein detected. BL cells have the phenotype BCL6+, CD10+, CD77+, CD38+, and sIg+, with mutated V genes and often ongoing somatic hypermutation (SHM) (80). Thus, the cellular origin of BL cells has been pinpointed to germinal center B cells.

1.4.2. Hodgkin's Disease

Hodgkin's Disease (HD) is characterized by a distinct, although small in number, population of cells termed Hodgkin and Reed-Sternberg Cells (HRS). While these cells only make up 1-2 %

of a Hodgkin lymphoma, they are large, atypical, malignant cells. The remaining tumor mass is usually made up of non-malignant T and B cells, eosinophils, or other cells (59). There are 4 histologic subtypes based on the bulk of cells making up the tumor mass. The first three types, nodular sclerosing, mixed cellularity, and lymphocyte depleted, have been classified together as "classical" HD, while the fourth, lymphocyte predominant, is rare and termed "non-classical" (59). HD occurs worldwide, and overall, around 40% of the lymphomas are EBV-positive (51). However, in some areas of the world, namely Africa, Asia, and Latin America, nearly 100% of childhood HD is associated with EBV. In immunocompromised individuals, nearly 100% of HD are EBV associated (8, 45, 153).

The infected HRS cells of EBV positive HD have a Latency II profile, with the expression of LMP1, LMP2, and EBNA1. Classical HD is monoclonal, with the cellular derivation of HRS cells being B cells, specifically pre-apoptotic germinal center B cells. Such cells show a loss of B-cell phenotype, and often carry either heavily mutated Ig genes or crippled B cells with nonsense mutations or deleterious deletions. Such mutations stem from the somatic hypermutation process occurring in germinal centers, but usually do not lead to a viable B cell. Without the proper survival signals normally received in a germinal center reaction, B cells are eliminated by apoptosis. In this case, it is suspected that EBV is delivering the survival signals, in the form of LMP2A (surface Ig-mediated signal), and LMP1 (activation of the CD40 pathway) (80). Exactly how and why EBV contributes to tumor development is unclear.

1.4.3. Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a tumor of epithelial tissue arising from the fossa of Rosenmuller (an anatomical site) (123). Two classifications exist: keratinizing and non-

keratinizing, with the main difference being level of differentiation. Different incidence rates exist for different geological locations. In the western world, NPC is very rare (0.5/100,000/year) and has an equal distribution of keratinizing and non-keratinizing types. In Southeast Asian populations, as well as in the Inuit, NPC is much more common, with an incidence of 25/100,000/year, and mainly of the non-keratinizing type. This suggests that genetic factors may work with environmental factors to increase the risk of NPC in certain ethnic populations (123).

Nearly all cases of both keratinizing and non-keratinizing NPC are EBV positive (110, 114). Elevated serum antiviral antibody titers are observed in NPC patients, with a distinctive detectable level of IgA antibody responses and elevated IgG levels to VCA and EA (65). Monitoring for these antibody levels in an at-risk population has been used as a diagnostic screening tool. Another marker for NPC is EBV DNA in the serum. At a basic level, NPCs have been described as having a Latency II pattern of EBV gene expression, similar to HD (LMP1 & 2, EBNA1) (17, 20). Recently, a more accurate description of Latency I/II has been conducted for NPCs and the level of both LMP1 and 2 appears to be very low and at times undetectable (42, 86). NPC tumor cells express HLA Class I and II, CD40, CD54, CD70, CD80, CD86, and CD95, and have a cellular phenotype typical of epithelial:lymphoid interactions (2, 133).

1.4.4. AIDS-associated Lymphomas

As mentioned above, HIV+ persons are at an elevated risk for the development of EBV-associated malignancies. Up to 10% of this population will develop a B-cell lymphoma (12). The diversity of the B-cell lymphomas is wide, with estimates of EBV positivity hovering

around 50% (44). The first class of AIDS lymphomas occurs when HIV+ individuals are profoundly immunocompromised and experiencing full-fledged AIDS. The cells of these lymphomas contain and express EBV, and resemble post-transplant lymphoproliferative disease (see below) in many aspects (95). The expression of EBV genes is varied, but is usually EBNA2 and LMP1 and the lymphoma cells appear to arise from mature post-germinal center progenitors (31). Such lymphomas typically present as polymorphic B-cell hyperplasia or monomorphic immunoblastic lymphomas (95). Other categories of AIDS-associated lymphomas include Burkitt's Lymphoma (as described previously), primary effusion lymphomas, and classical Hodgkin's Disease. In HIV+ children, the second most common tumor is leiomyosarcoma (a smooth muscle tumor). Nearly all such tumors in immunocompromised children are EBV associated, although neither the mechanism by which EBV infects smooth muscle, nor the latent state of EBV is known (97).

1.4.5. Post-Transplant Lymphomas

Post-transplant lymphoproliferative disease (PTLD) is an important factor in both solid organ transplantation (SOT) and hematopoietic stem cell transplant (HSCT). This potentially fatal disease has many diverse presentations, ranging from reactive polyclonal hyperplasias to non-Hodgkin's lymphomas (53). Ninety percent of PTLDs are EBV positive and are closely linked to the immunosuppression associated with transplantation (115, 116). Depletion of cellular immunity in such patients decreases the chances of rejection and graft versus host disease, but also disrupts the balance between EBV and its control by the EBV-specific cellular immune surveillance. Patients are at the highest risk of PTLD in the first year following transplant (53), but may also occur in patients years after the transplant. Incidence rates of PTLD have a broad

range from 1-2% after HSCT (13, 27), to 1-10% following kidney, lung, heart, and liver transplant, and up to 20% following small bowel transplant (16, 32, 41). Differences in the incidence rates are most likely a consequence of many factors, including type, intensity and duration of immunosuppression, amount of lymphoid tissue present in the transplanted organ, EBV serostatus pretransplant, and, in the case of HSCT, depletion of T-cell donor cells from the graft (11).

The clinical presentation and pathology of PTLD is quite varied. Symptoms include lympadenopathy, malaise, fever and sweats, and can resemble a primary infection of EBV. The disease can present as a tumor localized to a specific area, often involving major organs, or can be a diffuse polyclonal lymphoproliferation. While many histological classifications have been developed over the years, none has been widely accepted, and the disease continues to be described in various manners. PTLDs can stem from a variety of EBV-infected B cells, including naïve, memory, and germinal center (153). Most often, a Latency III type profile is expressed, but Latency I & II have also been documented. In some PTLDs, atypical crippled germinal center cells with no surface expression of immunoglobulin have been detected, as in Hodgkin's Disease. Such cells have crippling or destructive mutations in the Ig locus (80).

Because of the wide range of nonspecific symptoms, and because early detection is vitally important for survival, specific methods to detect early signs of PTLD are imperative. One of the most commonly used is viral load monitoring. PTLD is nearly always associated with a sharp rise in EBV viral load in the peripheral blood, which can easily be detected by polymerase chain reaction (PCR) (126, 143, 156, 157). A caveat to this method is that nearly half of transplant recipients carry a stable elevated viral load in their peripheral blood, even without looming PTLD (see below). A schedule of regular testing for changes in viral load is

therefore important to distinguish between a chronically high load and one that is rising in advance of symptomatic PTLD (127). Further, assays to enumerate the CTLs specific for EBV in the peripheral blood of transplant recipients is useful in determining the risk of PTLD (24, 98).

Treatment protocols for PTLD are diverse and nonstandardized. A summary of various strategies for both solid organ and hematopoietic stem cell transplantation is shown in Table 2 (53). Boosting of the immune response to restore the balance between virus and immune control has been the main focus of disease treatment protocols. This principle is outlined in Figure 3 (53). Withdrawal or reduction of immune suppression has been the first step in solid organ transplant recipients. However, with the reduction of immunosuppression, the risk of allograft rejection rises, and careful monitoring must be conducted. For HSCT recipients, treatment with interferon-alpha and adoptive immunotherapy with donor T cells and/or EBV-specific CTLs derived from the donor has been the regimen. Another approach in both types of transplants, is to eliminate CD20+ B cells with the chimeric mouse/human monoclonal antibody, rituximab (38, 79, 102). While this treatment is effective in decreasing viral load by eliminating the viral reservoir, CD20 negative PTLDs have been reported, and the possibility of recurrence is high, once the B-cell pool is regenerated (158). Chemotherapy has also been utilized, but has been associated with high mortality rates. If the lymphoma is localized, radiation or surgery to remove the tumor is a possibility. Usually such localized approaches are in conjunction with systemic treatments, so the efficacy of localized approaches alone has not been proven.

Perhaps the treatment approach that holds the most promise is cellular immunotherapy. For HSCT recipients, antigen-specific CTL lines have been generated from the seropositive donors, using LCLs derived from donor B cells as targets (15, 67, 125). This technique has several advantages, stemming mainly from the fact that the PTLDs are mainly of donor origin

and express the highly immunogenic EBV antigens. In clinical trials, the disease has readily responded to donor-derived EBV-specific CTL infusions. In solid organ transplant recipients, the donor and recipient are typically not HLA-matched and the tumor cells are typically of recipient origin, so donor-derived CTL lines are not a therapeutic option. Autologous CTLs are often not available because many recipients are EBV negative at the time of transplant, and ongoing immunosuppression quickly eliminates the EBV-specific CTLs. In addition, time is a factor and, the generation of such CTLs is time consuming. A possibility for future treatments lies in the development of a bank of allogeneic CTL specific for EBV, which would be immediately available after cross-matching with patients' B cells (133, 146). A reduction of immunosuppression is still necessary. Despite the advances in treatment regimens, EBV-associated malignancies still remain life-threatening. The experience of immunosuppressed patients with EBV infections exposes the necessity for constant surveillance by a healthy immune system in order to maintain the precarious balance between virus and host.

Table 2. Strategies of PTLD treatment.

Transplant Type	Treatment
Solid Organ Transplantation	Reduction of immunosuppression
	Interferon-alpha
	CD20 antibody (Rituximab)
	Surgery/radiation/chemotherapy
	IL-6
	Antiviral agents
	EBV-specific CTL
Hematopoietic Stem Cell Transplant	CD20 antibody (Rituximab)
	Donor T-cell infusion
	EBV-specific CTL

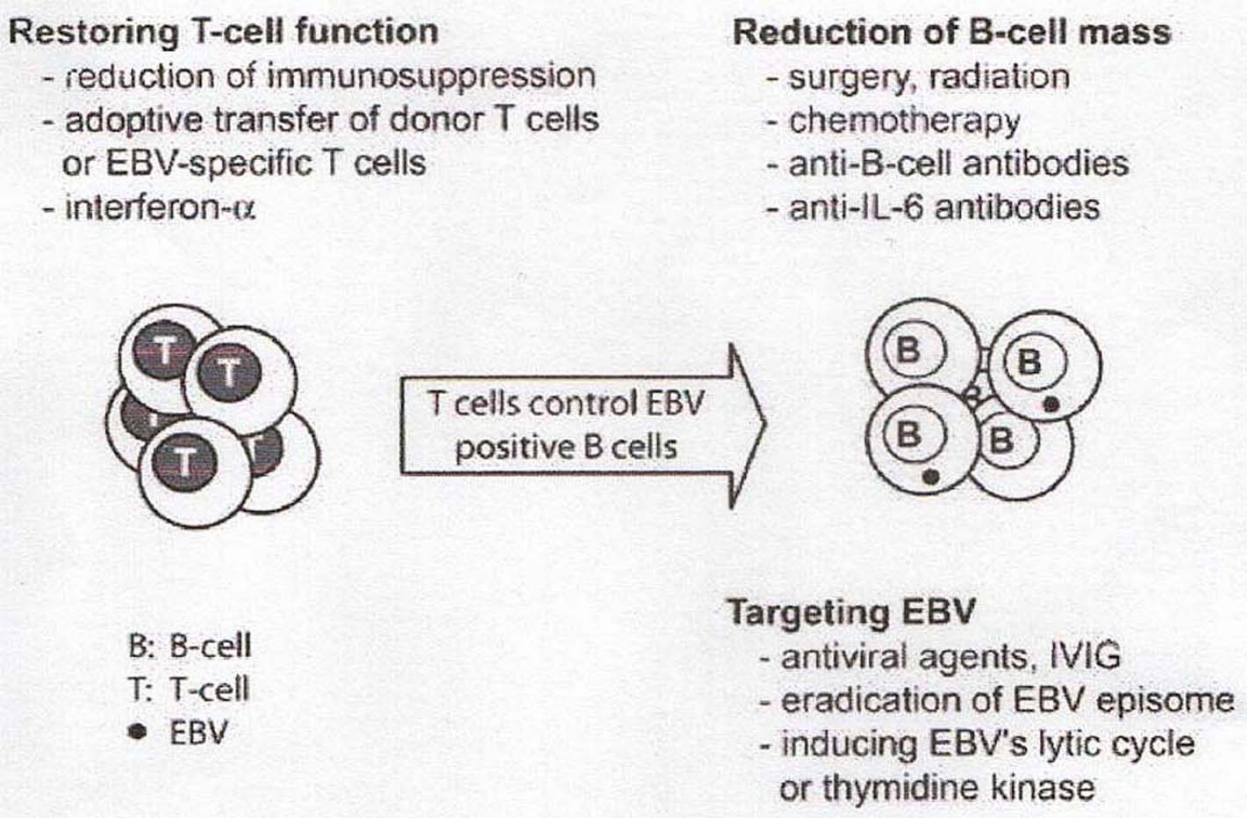

Figure 3. The principle driving treatment for PTLDs (53).

1.5. VACCINE STRATEGIES

While in the vast majority of individuals EBV is a harmless passenger, easily controlled by the body's immune system, in some individuals EBV drives a broad range of diseases than can cause significant morbidity and mortality. Therefore, a prophylactic vaccine that would not prevent primary infection, but prevent the acute disease associated with persistent infection would be most beneficial (123). Using the humoral immune approach, vaccines expressing the major viral envelope protein, gp350 have been developed with mixed results (56, 68, 150). Most recently, a live recombinant vaccinia vaccine expressing gp350 provided protection in two-thirds of vaccinated infants. Other attempts at therapeutic vaccines have been developed based on direct peptide immunization approaches. Immunodominant HLA Class I and II epitopes from LMP2, LMP1, and EBNA1 have been used in an effort to create a strong and sustained T cell response (106). Such efforts are ongoing, but have seen some success, primarily in reactivating CD4+ and CD8+ cells in vitro. Perhaps the best approach would be to combine both to elicit both neutralizing antibodies and cellular immunity.

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2. SPECIFIC AIMS

Although 90% of the world's population is infected with EBV, the typical immunocompetent host has no symptoms of the infection and carries the virus benignly for life. However, in immunosuppressed individuals, EBV has greater pathogenic potential and has been implicated in a number of disease states. One group at risk for the development of EBV-induced lymphoproliferative disease is pediatric solid organ transplant recipients who are undergoing an immunosuppressive drug regimen. Individuals with posttransplant lymphoproliferative disease (PTLD) have been shown to harbor levels of virus in their peripheral blood at orders of magnitude 3-4 times that of a normal immunocompetent individual. Furthermore, a large percentage of posttransplant pediatric solid organ recipients carry persistent abnormally high levels of viral load, in the absence of overt disease. Our lab has previously identified two groups of such individuals, and characterized them as low load carriers (LLC) and high load carriers (HLC) (1).

Previously in our lab, the virus positive cells in patient blood samples were initially characterized by the direct visualization using fluorescent in situ nucleic acid hybridization (FISH). Rose (2) began to characterize the cells of low load and high load carriers as being either low copy number cells (1-2 EBV genome copies/virus infected cell) or high copy number cells (10 or greater EBV copies/virus infected cell) with this technique. Low load patients had a strong predominance of low copy number cells; high load patients also had low copy number cells, but carried the bulk of their increased viral load in a distinct population of high copy number cells. We believe these high copy cells to stem from a different developmental process than the low copy cells, and will demonstrate different viral and cellular characteristics. The hypothesis of this proposal is that *high load patients carry the bulk of their elevated EBV load in*

a distinct population of high copy cells that have a phenotype previously thought to be incompatible with survival in vivo.

While high load carriers do not show overt symptoms of PTLD, their risk for the development of this potentially fatal disease could be increased by the presence of aberrant, EBV+ B cells in their peripheral blood. With an extensive characterization of these cells, we have further determined the compartmentalization of the viral load and to determine if the typical high copy cell has a unique phenotype that has previously been undescribed. We also determined the extent of the viral burden in such aberrant cells. To this end, the following specific aims were proposed and completed:

Specific Aims:

1. **Characterize the surface immunoglobulin (sIg) status of virus infected B cells from high and low load carriers.** Since high and low load carriers have been shown to carry their viral burden in different manners, the infected cells were further characterized. To achieve this, the surface immunoglobulin status of infected B cells from the peripheral blood of high and low load carriers was examined. It was determined if the high copy cells share a common isotype and if this differs from the low copy cells. CD19+ cells were examined by a conjunctive FISH/immunofluorescense procedure for the presence of both the viral genome and surface IgA, IgG, and IgM.

2. **Determine the frequency of Ig-null cells in high and low load carriers, as well as individuals with no detectable viral load.** To survive a germinal center reaction and

circulate in the peripheral blood, B cells must typically receive two survival signals. One of these is provided by the somatically mutated Ig molecules, with productive rearrangements that enable high affinity binding with antigen. The previous aim demonstrated the presence in the peripheral blood of CD19+, Ig-null cells. This is an aberrant type B cell, and the frequency in which these cells were present in the peripheral blood of transplant recipients was determined . To achieve this, peripheral blood mononuclear cells (PBMCs) were stained with CD19 and a pan sIg marker, and analyzed by flow cytometry.

3. **Identify cellular phenotype and viral compartmentalization of Ig-null cells.** To further identify the phenotype of high copy cells, surface markers of typical B-cell identity were examined by four-color flow cytometry in the high load patient population. These markers were HLA Class I, HLA Class II, Ki-67, CD20, CD27, CD5, CD40, CD10, CD38, CD23, and CD69. Further, Ig-null cells were isolated from PBMCs of high load carriers by both a MoFLo and a FACS Aria and the viral burden in the Ig positive versus Ig-null compartment was determined by quantitative-competitive PCR. The Ig-null compartment was further broken down into CD20 positive and negative, and HLA Class I positive and negative to see if there was further compartmentalization of the viral load.

In collaboration with Children's Hospital of Pittsburgh, we have access to a unique study population. Transplant recipients without PTLD, but yet with chronic elevated viral loads in their peripheral blood, have proven to be a valuable resource for the characterization of EBV-

infected B cells. Collectively, the studies presented here show that in this population, a large proportion of the EBV load is carried in an aberrant B cell that was previously believed to not be compatible with survival in vivo. While much progress has been made concerning the pathogenesis of EBV, this study shows that in immunosuppressed individuals, the story may be much more complicated.

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3. RESULTS

3.1. CHAPTER 1: SURFACE IMMUNOGLOBULIN-DEFICIENT EPSTEIN-BARR VIRUS-INFECTED B CELLS IN THE PERIPHERAL BLOOD OF PEDIATRIC SOLID-ORGAN TRANSPLANT RECIPIENTS

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3.1.1. Preface

To further characterize Epstein-Barr viral infected cells in the peripheral blood of pediatric solid organ transplant recipients, we endeavored to determine the surface immunoglobulin status of the infected cells of both high and low load carriers. The results were both novel and unexpected. After peer-review, our manuscript was accepted and published in an American Society of Microbiology journal.

3.1.2. Abstract

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, normally causes an asymptomatic latent infection with very low levels of circulating virus in the peripheral blood of infected individuals. However, EBV does have pathogenic potential, and has been linked to several diseases, including posttransplant lymphoproliferative disease (PTLD), which involves very high circulating viral loads. As a consequence of immunosuppression associated with transplantation, children in particular are at risk for PTLD. Even in the absence of symptoms of PTLD, very high viral loads are often observed in these patients. EBV-infected B cells in the circulation of 16 asymptomatic pediatric solid organ transplant recipients from Children's Hospital of Pittsburgh were simultaneously characterized for surface immunoglobulin (sIg) isotype and EBV genome copy number. Patients were characterized as high load and low load based on their stable level of circulating virus. High load patients had both high and low copy cells. Cells with a high numbers of viral episomes (>20/cell) were predominantly Ig-null and cells with low numbers of episomes were predominantly sIgM positive. Low load patients carried the vast majority of their viral load in low copy cells, which were predominantly IgM positive. The very rare high copy cells detected in low load carriers were also predominantly Ig-null cells. This suggests that two distinct types of B lineage cells contribute to viral load in transplant recipients, with cells bearing high genome copy numbers having an aberrant Ig-null cellular phenotype.

3.1.3. Introduction

Epstein-Barr virus (EBV) is a member of the *gammaherpesviridae* family. Its genes are encoded in a 172 kb dsDNA molecule, and like other gammaherpesviruses, it has a very narrow host range and produces persistent infections in the lymphoid cells of the host. EBV has a tropism for human B cells. The virus is primarily spread via intimate contact, but it is also transmissible by bone marrow engraftment, blood transfusion, or along with a donor organ during solid organ transplantation. Because of the efficient transmission of the virus via person-to-person contact, more than 90% of the world's population is infected with EBV by adulthood (25).

Despite the high prevalence of EBV infection, only very rarely does EBV cause overt disease. Most primary infections occur in childhood and are asymptomatic or exhibit only minor clinical symptoms. In adolescence and adulthood, primary EBV infection can manifest as infectious mononucleosis (11). In an immunocompetent host, a primary infection with EBV is readily resolved by cytotoxic T cell-mediated immunity, but the host remains asymptomatically infected with the virus for life. The virus has pathogenic potential in certain settings and associated disease states include oral hairy leukoplakia, nasopharyngeal carcinoma, EBV-associated Burkitt's lymphoma, Hodgkin's lymphoma, and lymphoproliferative disease in immunocompromised patients. EBV-associated posttransplant lymphoproliferative disease (PTLD) in solid organ transplant recipients is a particular concern. Following organ transplantation, patients typically undergo an immunosuppressive drug regimen to prevent rejection of the donor organ. As a result of this immunosuppression, immune responses that control EBV-driven B cell proliferation are reduced. PTLD can have a varied clinical presentation, ranging from posttransplant infectious mononucleosis to malignancies containing chromosomal abnormalities (22), and can range from localized lesions to disseminated

involvement of the whole lymphoid system (24). General risk factors for the development of PTLD include a primary EBV infection posttransplant, cytomegalovirus (CMV) status mismatch of the donor and recipient, CMV disease, type and intensity of immunsuppression (22), and the type of solid organ transplanted (19).

EBV-driven PTLD is an important cause of morbidity and mortality in the solid organ transplant recipient, especially in pediatric patients. Estimates of the incidence of disease range from 0.7% (19) to 32% (7) in various allograft recipients, depending on a number of factors. From a large number of separate studies, it has been shown that the diagnosis of PTLD is also associated with a very high load of EBV in the peripheral blood (>1000 copies/ 10^5 peripheral blood mononuclear cells [PBMCs]) (6, 8, 9, 10, 15, 17, 18, 23, 26, 29, 31, 32, 35). While normal immunocompetent adults harbor an average of approximately 0.01-0.1 genomes per 10^5 PBMCs (29), PTLD patients typically have viral loads several orders of magnitude greater at the time of their diagnosis. Before the symptoms of PTLD appear, there is a period of 2-6 weeks during which the EBV load in the peripheral blood is readily detected and rising. This increase is usually detected by quantitative PCR. Upon resolution of their symptomatic PTLD, patients often retain a much higher (by 2-3 orders of magnitude) circulating persistent viral load than a normal latently infected individual. In addition, persistently high viral load has been observed in a substantial percentage of pediatric solid organ transplant recipients who have never had a diagnosis of PTLD. This persistently elevated load has generally been attributed to the immunosuppressive drug regimen that is administered to prevent rejection of the transplanted organ (36). Serial monitoring with quantitative PCR reveals that many of these patients harbor persistently elevated viral loads asymptomatically for months to years (unpublished

observations, 10, 15, 28, 34, 37, 38). The potential complications arising from a very high viral load without overt disease have never been elucidated.

The chronic carrier states exhibited by the immunosuppressed pediatric solid organ transplant recipient population offer a unique opportunity to study viral latency and risks for development of PTLD. Previously it has been shown that the latent EBV load is carried in the resting memory B cell (CD 19+, IgD-, Ki67-, CD 23-, CD 80-) compartment in the peripheral blood, in both immunosuppressed and immunocompetent persons (1, 2, 27). Further studies have pinpointed latency to the CD27+, CD 5- B2 memory type of B cell in the peripheral blood of immunocompetent individuals (34). Immunosuppressed individuals also carry their chronic viral load in the memory B cell compartment of the peripheral blood (2, 27). A preliminary analysis of EBV infected peripheral blood B cells by fluorescent in situ hybridization (FISH) for EBV DNA found that the distribution of the viral genomes in infected cell was related to the level of the patient's viral load (28). In low load patients, cells had just one or two genomes per nucleus, while high load patients carried a large portion of their load in cells harboring as many as 20-50 genomes per cell (Figure 4). Increases in load were accompanied by an increase in the number of these high copy cells. Since high copy cells (greater than 10 genomes/cell) were found predominantly in high load patients, these cells were a distinctive marker for this group of patients. In terms of pathogenic processes, the high genome copy cells likely arise in a different manner from the low genome copy number cells. High copy cells warrant further analysis because they may be a key to understanding the development of at least some PTLDs. Recent evidence suggests that the existence of aberrant cell types may be a main constituent in PTLDs and Hodgkin's Disease lymphomas (14, 16, 33). The characterization of the immunoglobulin

phenotypes of EBV infected cells in high and low load asymptomatic carriers that is reported here lends support to this notion.

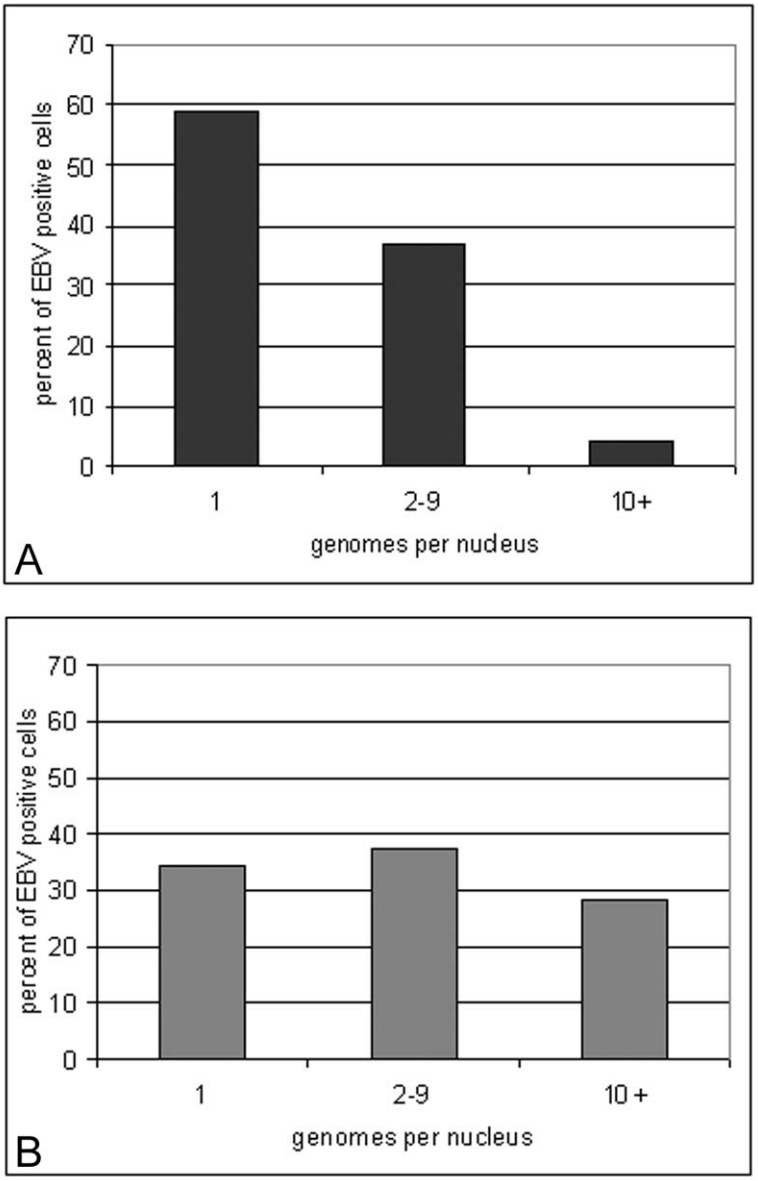

Figure 4a. EBV genome distribution per infected cell in low load patients.

Summary of the EBV copy number in infected peripheral blood B cells, expressed as a percentage of all EBV-positive cells present and determined by fluorescent in situ hybridization.

Figure 4b. EBV genome distribution per infected cell in high load patients.

Summary of the EBV copy number in infected peripheral blood B cells, expressed as a percentage of all EBV-positive cells present and determined by fluorescent in situ hybridization.

3.1.4. Materials and Methods

Study Population: Since 1995, transplant recipients attending Children's Hospital of Pittsburgh (CHP) and University of Pittsburgh Medical Center have been regularly monitored for EBV viral load using a quantitative-competitive PCR (QC-PCR) protocol developed in our laboratory (10). We have followed bowel, liver, lung, renal and heart transplant recipients for EBV-DNA copy number in peripheral blood lymphocytes. We have focused on the pediatric transplant population because within this group there are more than 190 patients for whom we have multiple prospectively collected specimens. In only 34.2% of these patients (34.2%) has there never been a detectable viral load. A chronic "low" load (defined as detectable but < 200 genome copies/10^5 lymphocytes for > two months) has been detected in 36.3% of patients and a chronic 'high' load (defined as >200 genome copies/10^5 lymphocytes for > two months) 18.4%. The remaining 11.1% of patients have had viral loads that have fluctuated. As the lower limit of the QC-PCR test is 8 genome copies per 10^5 lymphocytes (29), normal latent infection is not detected by this assay; thus, the detected loads must be at least 2-3 orders of magnitude greater than normal latency. This research has complied with all relevant federal guidelines and is covered by CHP Human rights Committee research protocol # 00-155.

Quantitative-competitive (QC)-PCR of EBV loads: Lymphocytes were prepared from whole blood samples by centrifugation onto a histopaque (Sigma-Aldrich, St. Louis, MI) cushion. The cells were washed in phosphate buffered saline (PBS) and counted. Cell pellets were stored at –20 C until ready for PCR. Plasma volume equivalent to 4 x 10^5 cells for each patient was ultracentrifuged at 14000 rpm for 90 min in order to pellet cell-free virus. To make lymphocyte and plasma lysates 20µl of PCR lysis buffer (50 mM KCL, 10 mM Tris [pH 7.6], 2.5 mM $MgCl_2$, 1% Tween 20, and 100µg of proteinase K per ml) was added for every 10^5

lymphocytes or plasma volume equivalents. The lysates were incubated at 55EC for 1 hr, boiled for 10 min to inactivate the proteinase K and chilled on ice. Primers for the PCR target sequence in the EBV genome were designed with OLIGO software (National Biosciences Inc., Plymouth, MN). TP1Q5' (AGGAACGTGAATCTAATGAAGA) and TP1Q3' (GAGTCATCCCGTGGAGAGTA) amplify a 177-bp EBV sequence (exon 1) in the *lmp2a* gene. A competitor target was made by deleting 42-bp from a 177-bp EBV amplicon derive from the viral *lmp2a* exon 1 sequence. For each sample, four tubes containing 8, 40, 200 or 1000 copies of the viral LMP2a competitor sequence along with lymphocyte or plasma lysates equivalent to 10^5 cells were subjected to 30 cycles of amplification (94EC for 1 min, 54EC for 1 min and 72 EC for 1 min). Each PCR reaction (50µl) contained 20 pmol of 5' and 3' primers, 50mM KCL, 2.5 mM $MgCl_2$, 10 mM Tris [pH 9.0], 0.1 % Triton X-100, and 0.25 mM deoxynucleotides (Pfizer, New York, New York). One unit of Amplitaq Gold DNA polymerase (Perkin Elmer, Wellesly, MA) was used in each reaction. The PCR products were analyzed on 3% agarose gels containing 0.5X Tris-borate-EDTA electrophoresis buffer and 0.5µg of ethidium bromide per ml.

The QC-PCR assay for EBV is used to quantitate viral loads in the range of 8 to 5000 copies of viral DNA in 10^5 lymphocytes. Normal latent infection (0.01 - 0.1 copies/10^5 lymphocytes) is not detected by this protocol and detectable levels of viral DNA reflect a viral genome burden at least 2 to 3 orders of magnitude above normal latency.

Cell Sorting with magnetic Beads: Lymphocytes were positively sorted for $CD19^+$ B cells by using MACS CD19 Microbeads (Miltenyi Biotech, Auburn, CA). Histopaque lymphocyte preparations from patient blood samples were mixed with 20ul of CD19 Microbeads per 10^7 total cells and incubated for 15 minutes at 4EC . The cells were washed and magnetically separated using a positive selection LS column. The retained CD19+ cells were eluted with MACS buffer,

spun onto Superfrost Plus glass slides (Fisher Scientific, Hampton, NH) with a Shandon Cytospin 3 (Thermo Electron Corporation, Waltham, MA) at 500 rpm for 5 minutes. Namalwa cells, a Burkitt Lymphoma cell line that contains two integrated copies of the EBV genome were also spun onto the same slide to be used as a control in the in situ hybridization reaction. Purity of the CD19+ population ranged from 95-80%,in a control sample of PBMC's, as confirmed by flow cytometry.

Construction of DNA Probe: The double-stranded EBV-specific DNA probe was made from the plasmid p1040, containing a cloned Bam HI WWYH fragment of the EBV strain B95-8. The 14.7 kb fragment was cloned into a holding vector and linearized with HindIII. The cut DNA was purified from the agarose gel with the MinElute Gel Extraction Kit (Qiagen Inc., Valencia, CA), and double-stranded EBV DNA probes of 250-300 bp were generated with the Prime-A-Gene Labeling System (Promega, Madison, WI) and labeled with digoxigenin-11 (DIG)-2'-dUTP (Roche, Basel, Switzerland) at room temperature overnight. To terminate the reaction, the mixture was heated to 95ºC for two minutes, followed by chilling in an ice bath. 20mM EDTA was added, followed by a standard phenol-chloroform extraction of the probe. The purified probe was stored at –20ºC until direct use in the in situ hybridization reaction.

In Situ Hybridization*:* Fluorescent *in situ* hybridization (FISH) was performed on CD19+ cells from patient peripheral blood samples. The slides were fixed in methanol:acetic acid (3:1) at room temperature for 15 minutes. After washing twice with 1X PBS, slides were aged in 2X SSC at 37ºC for 30 minutes, followed by dehydration in an increasing ethanol series. The slides were prewarmed and denatured in a 70% formamide-2X SSC solution for 2 minutes and immediately placed in cold graded series of ethanol for 5 minutes each. For each sample,50 ng of probe, 5 μg of salmon sperm DNA, and 20 μg of yeast tRNA were suspended in a solution of

50% formamide, 10% dextran sulfate, and 1X SSC. This hybridization mixture was heated to 80ºC for 10 minutes, followed by a preannealing at 37ºC for 15 minutes. After prewarming the slides at 37ºC, 30 ul of the hybridization was added to each sample for a 37ºC overnight incubation. The following day, the slides were washed in a 50% formamide-2X SSC solution for 30 minutes at 37ºC, followed by a 30 minute was in 2X SSC at 37ºC. The slides were treated with RNase H (8 U of RNase H/ml in RNase H reaction buffer) for 1 hour, also at 37ºC. The slides were then washed twice with 2X SSC.

Immunofluorescence (IF): Surface immunoglobulin expression was detected with fluorescein (FITC)-conjugated monoclonal antibodies against surface IgM, IgA, and IgG (Biosource International, Camarillo, CA). Blocked slides were incubated for 30 minutes at room temperature with their respective antibody at a 1:250 dilution. They were then washed twice with 1X PBS. A cell was either scored positive or negative based on the expression level of surface immunoglobulin.

Immunological Detection of EBV probe: To detect the bound DIG-labeled EBV probe, the blocked slides were incubated with anti-DIG-rhodamine-Fab fragments (Roche, Basel, Switzerland) for 30 minutes at room temperature. The slides were washed twice with 1X PBS, dried, mounted with Vectashield mounting media plus 4',6'-diamidono-2-phenylindole (DAPI) (Vector Laboratories, Burmingdale, CA), and sealed. A positive signal is characterized by a red dot, with a surface area of approximately 22 pixels in the DAPI-stained nucleus (blue). Each specific signal represents one EBV genome, although some genomes may be closely spatially related and are visualized as one larger signal.

Photomicroscopy: The sealed slides were examined under a Nikon E600 microscope equipped with a SPOTII charge-coupled device digital camera. Images of cells were generated and analyzed with Metamorph software (Universal Imaging, Westchester, PA).

3.1.5. Results

3.1.5.1. Surface Isotype Expression in CD19+ Cells

Immunofluorescence for surface expression of the IgM, IgA, and IgG isotypes was conducted on CD19+ cells that had been positively sorted from the PBMCs of children who have undergone solid organ transplantation at Children's Hospital of Pittsburgh. Eight high and eight low load patients (as defined in the Materials and Methods) were selected for this study (Table 3a and 3b). Detection of surface expression of IgD was not performed, since we and others have previously shown by polymerase chain reaction that persistent infection with EBV does not involve the naïve IgD+ B cells of both normal and immunosuppressed individuals (1, 2, 12, 20, 27). IgM, IgA, and IgG positive cells were observed in all patient samples (Figure 5). Similar numbers of IgG expressing cells were observed in both the high and low load patients (3.4% for high load patients vs 2.6% for low load patients). There were slightly more IgA+ cells in the high load patients (3.6% in high load patients and 2.1% in low load patients), although this average was skewed by the unusually high percent (20%) of IgA+ virus-infected cell in one high load patient (pt 2). When pt. 2 is removed, the percent of IgA+ cells in high load patients is comparable to the percent of IgA+ cells in low load patients. Low load patients had substantially more IgM+ cells than the high load patients (87.2% for low load patients *vs* 62.3% for high load patients), while the high load patients had substantially more cells with no detectable immunoglobulin (Ig)

isotype compared to the low load patients (30.8% of high load patients *vs* 9.0% of low load patients). Some of the Ig-null cells observed may be attributed to the sorting procedure, in which it is inevitable that a small percentage (5-10%) of CD19- cells will remain after the sort. In addition, in an ongoing study, high load patients were shown to have a substantially higher number of Ig-null cells, as determined by flow cytometry.

Table 3a. Summary of High Load Patient Population

Patient number	Sex	History of PTLD	Age (mo) at transplant	Mo. Post-transplant of specimen	EBV status pretransplant	Type of transplant	EBV load (no. of copies/10^5 PBLs)
1	F	No	10	12	Neg	Liver	5000
2	M	No	15	25	Neg	Liver	2500
3	F	Yes	8	16	Neg	Liver	2500
4	F	Yes	19	21	Neg	Heart	2000
5	M	No	44	21	Neg	Liver	500
6	F	No	6	56	Neg	Heart	500
7	M	Yes	120	80	Neg	Kidney	200
8	F	No	125	6	Neg	Double Lung	200

Table 3b. Summary of Low Load Patient Population

Patient number	Sex	History of PTLD	Age (mo) at transplant	Mo. Post-transplant of	EBV status pretransplant	Type of transplant	EBV load (no. of copies/10^5
1	F	Yes	72	70	Neg	Liver	100
2	M	No	152	3	Neg	Kidney	100
3	F	No	22	41	Neg	Liver	80
4	M	Yes	61	67	Neg	Liver	40
5	M	No	2	37	Pos	Liver	20
6	M	No	118	86	Unknown	Kidney	20
7	M	Yes	83	156	Unknown	Multi-visceral	8
8	M	No	100	28	Pos	Kidney	8

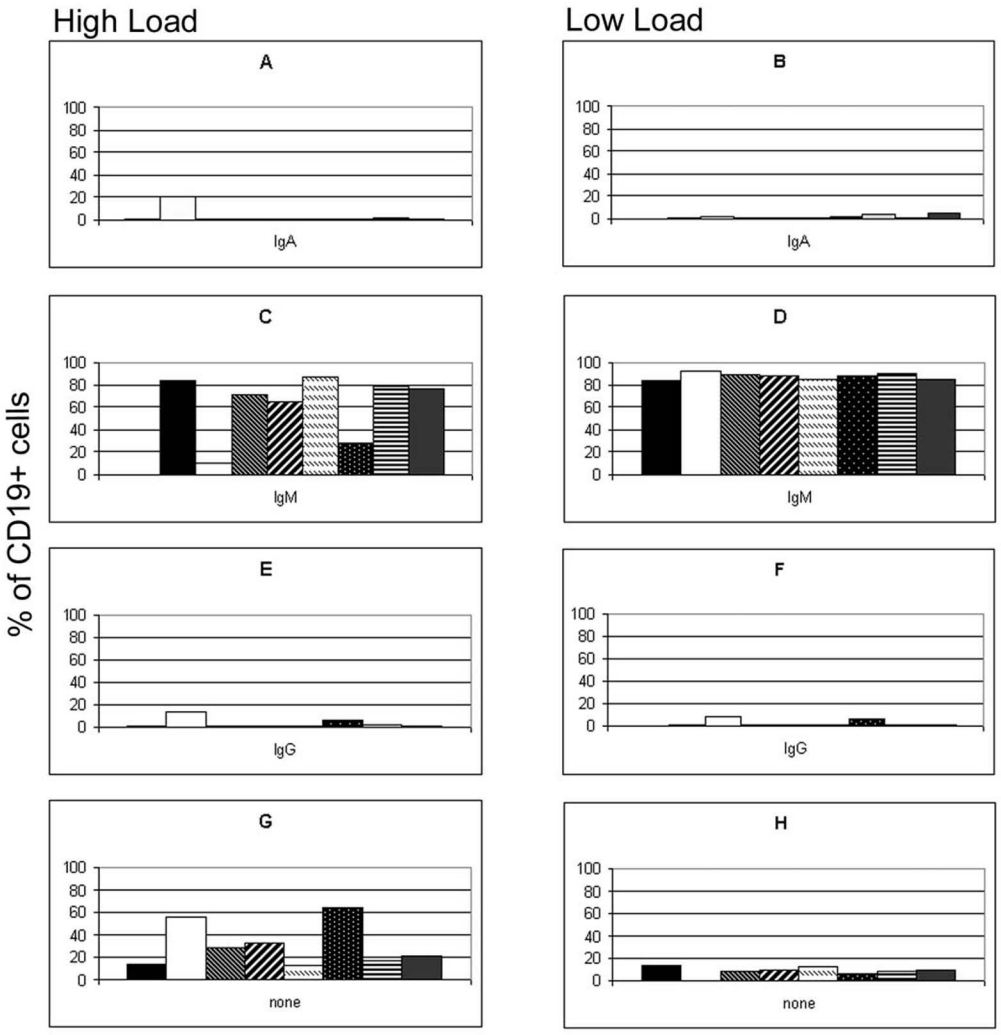

Figure 5. Distribution of isotypes in CD19+ cells of high and low load patients.

The percentage of surface IgA+ (A and B), IgM+ (C and D), and IgG+ (E and F) peripheral blood B cells was determined in chronic high (A,C, E, and G)and low (B, D, F, and H) load patients with FISH and immunofluorescence, and expressed as a percentage of all CD 19+ cells present. The percentage of cells in which no surface immunoglobulin was detected is shown in G and H. In each graph, high load patients 1-8 (left side of the figure) are shown left to right, as are the low load patients 1-8 (right side of figure).

3.1.5.2. Surface Isotype Expression of Virus-Infected Cells in Patients with Low Viral Loads

A combined FISH/IF procedure was used to generate an isotype profile for the EBV- infected cells in purified CD19+ cells from 8 low load patients (Figure 6). Examples of cells for each isotype are shown in Figure 3. Typically, low load carriers carry 1-2 viral genomes per infected B cell in the peripheral blood. Cells with a greater copy number of EBV genomes are rare or undetectable. The majority (85.5%) of the low copy cells from this patient group were IgM+. Low copy cells with IgA and IgG expression were also detected at a very low frequency (average IgA+ = 3.3%, average IgG+ = 1.0%). For low load patients, an isotype for 10.3 % of low copy virus-infected cells was not identified using our FISH/IF procedure. High copy cells in this patient population are extremely rare. Two patients had no detectable high copy cells while the remaining 6 patients had, on average, only 1 or 2 high copy cells per slide of an estimated 50,000-100,000 cells per slide. Of these high copy cells, approximately 70% did not have detectable Ig isotype expression. In addition, there were no IgG+ high copy cells identified in any of the low load patients. One low load patient (pt # 5) had a small percentage of IgA+ high copy cells. All other high copy cells detected were IgM+ (Figure 6).

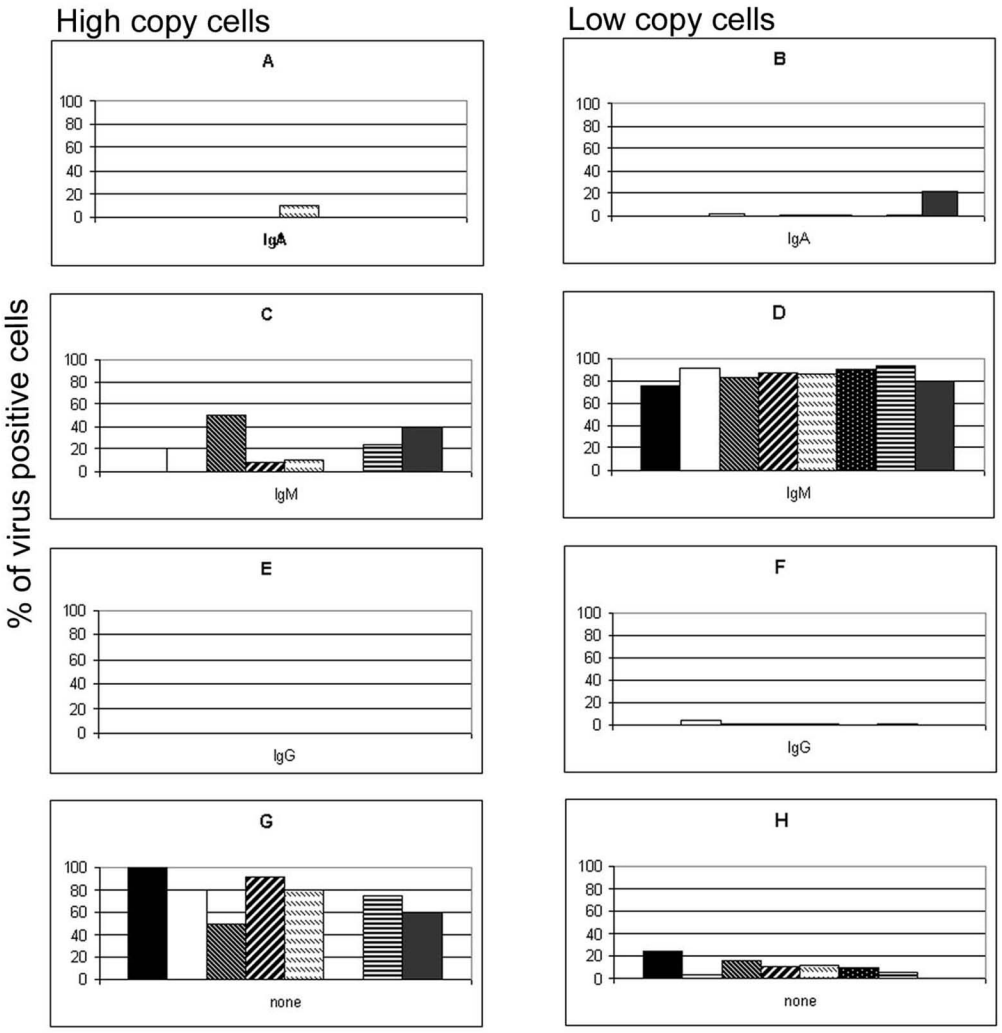

Figure 6. Distribution of isotypes in virus-infected cells in eight low load patients.

The percentage of EBV-positive, surface IgA+ (A and B), IgM+ (C and D), and IgG+ (E and F) high (A, C, E, and G) and low (B, D, F, and H) copy cells in the peripheral blood B cells of persistent low load carriers was determined with FISH and IF and expressed as a percentage of high and low copy cells detected. The percentage of infected cells in which no surface immunoglobulin was detected is shown in G and H. For each graph, low load patients 1-8 are represented left to right.

3.1.5.3. Surface Isotype Expression of Virus-Infected Cells in Patients with High Viral Loads

The FISH/IF procedure was applied to positively sorted CD 19+ cells from the peripheral blood of 8 high load patients. Examples of both positive and negative surface Ig isotype expression in virus infected cells are shown in Figure 7. Virus-infected cells with low numbers of viral genomes were present in all patients, and these cells were predominately IgM+ (67.2%) (Figure 7). IgA+ and IgG+ low copy cells were also observed at lower frequencies, in some high load patients (Figure 7). All high load patients carried high copy cells (at an average of 86 high copy cells per slide) in all high load carriers and most of these cells (68.0%) had no detectable surface Ig isotype expression. A significant difference ($p<0.05$) in the number of high copy cells per slide existed between the high and low load patients. Several of the high load patients showed no sIg expression on up to 85% of their high copy cells. Of the high copy cells with detectable surface Ig expression, 45.7% were IgM+, 31.7% were IgA+, and 22.6% were IgG+.

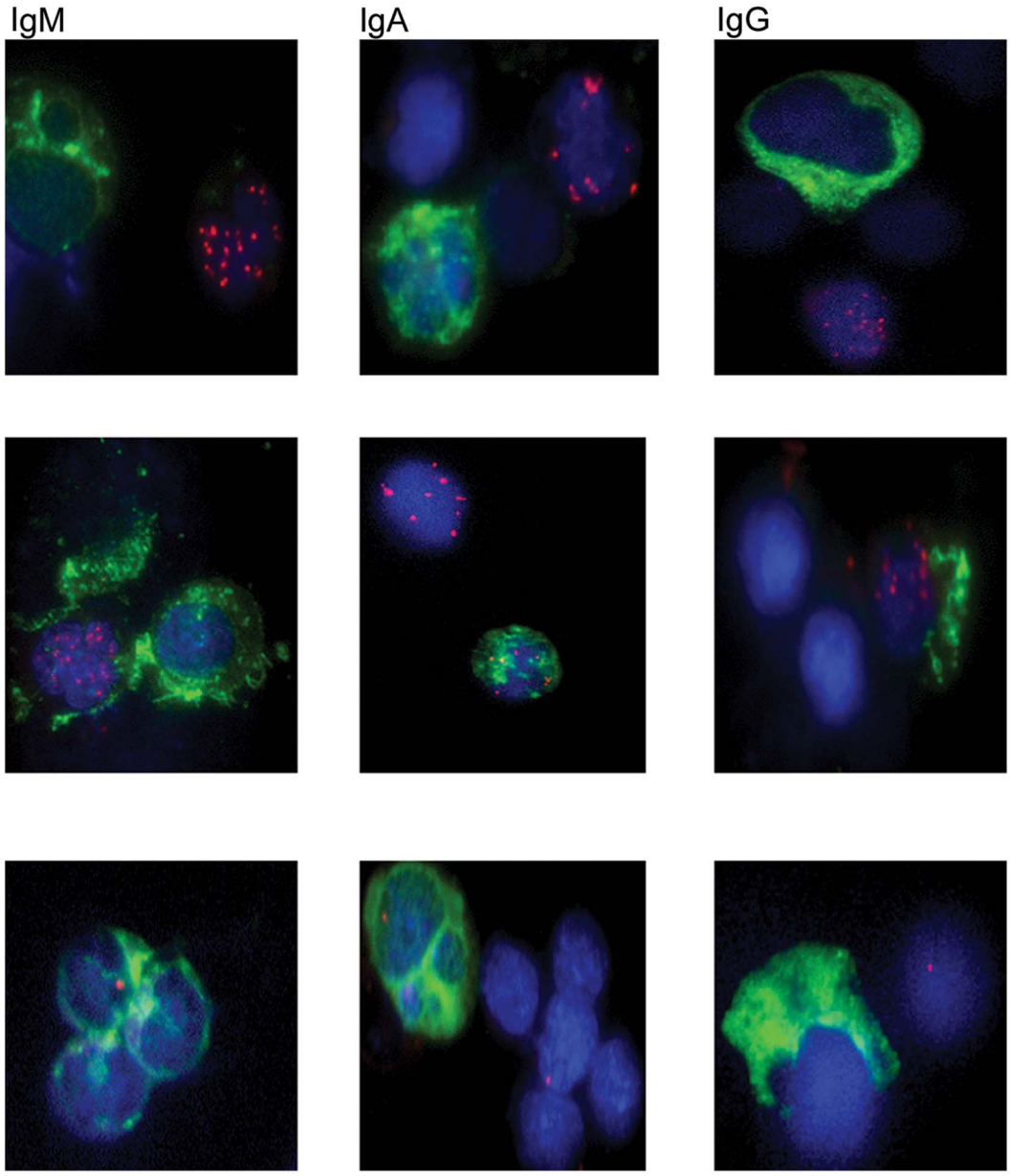

Figure 7. EBV genomes and surface immunoglobulin expression.

Representative EBV+ and surface Ig+ cells are shown, as detected by FISH and IF. The top panels represent virus positive high copy cells, without surface expression of Ig, with an IgM+ (left), IgA+ (middle), and IgG+ (right) cell nearby. The middle panels represent double positive high copy cells, with multiple copies of the virus, and surface expression of IgM (left), IgA

(middle), and IgG (right). The bottom panels represent virus positive low copy cells, with a single viral genome present in an IgM+ (left) and IgA+ (middle) cell. The bottom right panel represents a low copy cell with no surface Ig expression next to an IgG+ cell. For each panel, red is the viral genome, blue is the nucleus, and green is the surface immunoglobulin expression.

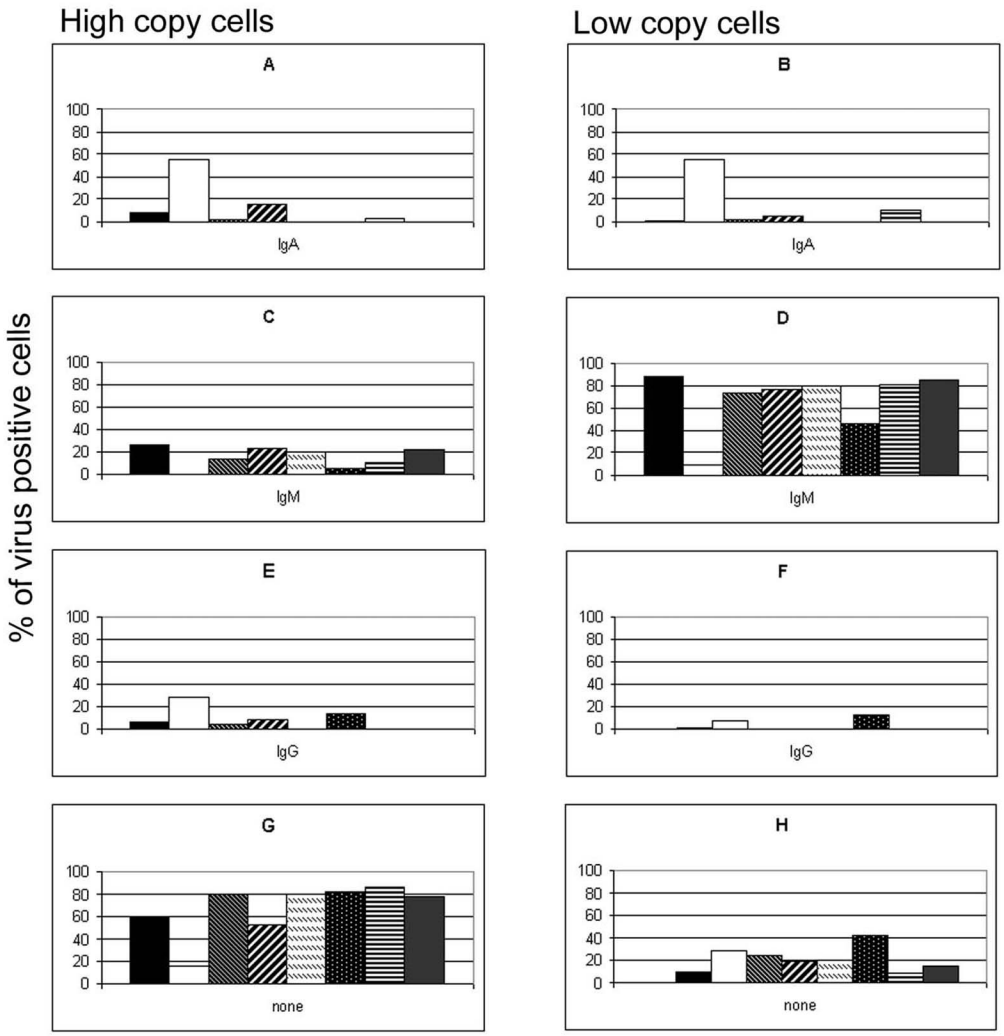

Figure 8. Distribution of isotypes in virus-infected cells of high load patients.

The percentage of EBV-positive, surface IgA+ (A and B), IgM+ (C and D), and IgG+ (E and F) high (A, C, E, and G) and low (B, D, F, and H) copy cells in the peripheral blood B cells of persistent high load carriers was determined with FISH and IF and expressed as a percentage of high and low copy cells detected. The percentage of infected cells in which no surface immunoglobulin was detected is shown in G and H. For each graph, high load patients are represented left to right.

3.1.6. Discussion

Previously, we have reported that children who undergo solid organ transplantation often carry persistently elevated viral loads of EBV in their peripheral blood. Upon direct examination of the virus infected B cells from the peripheral blood by fluorescent *in situ* hybridization, we determined that patients with loads of <200 copies/10^5 lymphocytes, infected cells had 1 to 2 copies of the viral genome per nucleus (low copy cells). High load patients (>200 copies/10^5 lymphocytes) had these low copy cells, but also had another population of virus infected cells with 20-50 genomes per nucleus (high copy cells) (28). In a larger pediatric transplant population, we have now characterized the two types of virus-infected cells for surface immunoglobulin isotype.

In our study we used fluorescent probes to directly visualize the infected cells and simultaneously determine the surface Ig phenotype and viral genome copy number of individual infected cells. For the patients in our study, the proportion of peripheral blood B cells with sIgM, sIgG or sIgA was within the normal range for children. In both low and high load patients, the EBV infected cells with low genome copy number had mainly IgM B cell receptors. Previous work had shown that for both immunocompetent and immunosupressed individuals, the EBV latent load was carried in what appeared to be the resting memory B cell compartment (CD19+, IgD-, Ki67-, CD23-, CD80-) (1, 2, 27). The current study indicates that for the pediatric transplant population, these virus-infected cells carry low number of EBV episomes and are IgM-positive. The implication therefore is that they do not appear to have undergone isotype switching after EBV infection. They do not necessarily have to have arisen from a population of cells participating in germinal center reactions within the lymph nodes.

As in our previous studies on pediatric transplant recipients, high copy cells were a distinctive feature of patients carrying high viral loads and were responsible for high circulating loads at the time of PTLD diagnoses (28). Unexpectedly, the characterization of the Ig isotype of the B cell receptors (BCRs) on these cells revealed that the majority of these cells had no detectable surface Ig expression. Internal controls demonstrated that this was not a technical problem in detection of surface Ig in these experiments. IgM+, IgA+, and IgG+ cells were observed on all slides and the frequency of the different isotypes in the circulating B cell pool was within the expected range. Some high genome copy cells did express sIg. For these, the frequency with which the virus appeared in B cell of different isotypes was not the same as the frequency of these isotypes in the circulating B cell population. The IgG isotype was underrepresented and the IgA isotype was overrepresented in the virus-infected sub-popoulation. The simplest explanation for the origin of high copy null cells would be that after infection of mature naïve B cells, the cells proliferated (increasing the copy number of viral genomes per cell) and then attempted to make an isotype switch. When an isotype switch was productive, it led more often to IgA expression. It would appear, however, that most of the switch attempts were unsuccessful, leading to the Ig-null cell phenotype.

Since B cells require a functional surface antigen receptor to receive survival signals throughout the lifetime of the cell, Ig-null cells are normally incompatible with survival *in vivo*. They would be expected to be rapidly eliminated, and not permitted to enter or persist in the circulation. The persistence in the peripheral blood of B cells with no detectable Ig expression suggests that these cells may be receiving an abnormal survival signal, most likely provided by EBV. The high copy cells are in this respect atypical B cells. It is possible that these cells have survived germinal center reactions which produced unsuccessful Ig affinity maturation and/or

isotype switching. Ig-null cells have recently been described in EBV-infected B cells in angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) (6), EBV-positive Hodgkin's Disease (14, 16), and in the B cells of EBV-positive post-transplant lymphoproliferative disease (3, 33). Lack of CD20 and surface immunoglobulin light-chain expression was also observed in various PTLD lesions by flow cytometry (13). These latter studies have led researchers to suggest that EBV interferes with the normal B cell differentiation and selection processes in PTLD (3). The Ig-null phenotype of high copy EBV infected B cells in asymptomatic children with solid organ transplants suggests that such interference is not confined to the disease state and that high load carriers, in particular, show signs of aberrant B cell development and may be at risk for further EBV-driven complications.

Acknowledgements

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3.2. CHAPTER 2: IG-NULL B CELLS IN THE PERIPHERAL BLOOD OF PEDIATRIC SOLID ORGAN TRANSPLANT RECIPIENTS WITH ELEVATED EPSTEIN-BARR VIRAL LOADS.

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3.2.1. Preface

Based on the previous results that high copy cells in pediatric solid organ transplant recipients have an aberrant Ig-null phenotype, further studies were needed. In this study, we further characterized these cells by phenotyping them and determining the compartmentalization of viral load. This manuscript is being prepared for submission to an American Society of Microbiology journal, the Journal of Clinical Microbiology for peer review.

3.2.2. Abstract

Epstein-Barr virus (EBV) causes an asymptomatic latent infection with very low levels of circulating virus in the peripheral blood of most adults. However, EBV does have pathogenic potential, and has been linked to several diseases, including posttransplant lymphoproliferative disease (PTLD), which can involve very high circulating viral loads in the peripheral blood. As a consequence of immunosuppression associated with transplantation and serostatus pre-transplant, children in particular are at risk for PTLD. Even in the absence of symptoms of PTLD, very high viral loads are often observed in these patients. Previously, we demonstrated the presence of infected B cells with high genome copies of EBV that showed no demonstrable surface immunoglobulin (Ig-null cells) in asymptomatic pediatric solid organ transplant recipients from Children's Hospital of Pittsburgh (29). In this study, we further examined Ig-null cells in the circulation of high load transplant recipients by four-color flow cytometry. We demonstrated that Ig-null cells have the surface phenotype CD19+, sIg-, CD5-, CD10-, CD27-, CD23-, CD38-, CD69-, and Ki67-, with variable surface expression of CD20 and CD40, and HLA Class I and Class II. Upon sorting of Ig-null and Ig positive cells, we saw that a significant fraction of the viral load is carried in the Ig-null compartment, with variation from patient to patient. Further sorting of the Ig-null, CD20 +/- cells, and Ig-null HLA Class I +/- cells demonstrated the presence of virus in the double negative compartment, again with variation from patient to patient. This suggests that high copy cells are an aberrant feature of EBV infection in these patients. In some patients, the bulk of the viral load is carried in Ig-null cells. Ig-null cells have been linked to other EBV-associated disease states, suggesting that these patients may be at higher risk for complications.

3.2.3. Introduction

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that establishes a persistent latent infection in the B lymphocyte compartment that is asymptomatic and lifelong in the vast majority of the human population (24). Primary infections with EBV are nearly always asymptomatic in childhood, but can be manifested as infectious mononucleosis in adolescence and adulthood (24). Despite this normally benign pathogenesis of EBV infection, it has been linked as the etiological agent to Burkitt's lymphoma, Hodgkin's Disease, and nasopharyngeal carcinoma. Furthermore, upon suppression of T-cell mediated immunosurveillance, EBV infection can opportunistically be manifested as an oral hairy leukoplakia and/or a lymphoproliferative disease (24). In transplant recipients, EBV driven post-transplant lymphoproliferative disease (PTLD) poses a significant health threat. General risk factors for this complication include type and intensity of immunosuppression, type of transplanted solid organ, EBV seronegativity pre-transplant, and presence of CMV disease (17, 21). PTLDs can have a varied clinical presentation, ranging from a clinical syndrome clinically indistinguishable from infectious mononucleosis to malignancies with chromosomal abnormalities (21). The effects range from localized lesions to disseminated involvement of the lymphoid system (23). The morbidity and mortality attributable to EBV-associated PTLDs can be very high, with estimates of the incidence of this complication ranging from 1-20%, depending mostly on the type and intensity of immunosuppressive drugs and the type of organ transplanted (8).

A very high load of EBV in the peripheral blood (>1000 copies/10^5 PBMCs) is strongly associated with a diagnosis of PTLD (5, 6, 8-11, 13, 15, 16, 22, 25, 28, 30, 31, 35). This load is at least 2-3 orders of magnitude greater than the viral load measured in normal, immunocompetent individuals, and its rise can be detected by quantitative competitive

polymerase chain reaction (QC-PCR) 2-6 weeks before patients present with physical symptoms of PTLD (32, 36). After resolution of the PTLD, accomplished by a reduction of immunosuppression or antineoplastic chemotherapeutic treatment, a viral load is present in B cells in the peripheral blood at an elevated level for an extended period of time. Longitudinal studies have revealed that many pediatric solid organ transplant recipients develop a persistently elevated viral load in their peripheral blood, even in the absence of clinical signs or symptoms of PTLD (11, 13, 27, 34, 38, 39). This load is generally attributed to the immunosuppressive drug regimen prescribed to prevent organ rejection in the patient (37). Although the level of the load varies widely from patient to patient, our experience has led us to separate and characterize patients as belonging to one of two broad categories: being either high or low load carriers. High load carriers are defined as having a persistent asymptomatic viral load of >200 copies/10^5 lymphocytes for at least two months, while low load carriers have persistent asymptomatic loads that range between 8 and <200 copies/10^5 lymphocytes for at least two months (27).

By directly examining the EBV infected cells with fluorescent *in situ* hybridization (FISH), we have shown that a distinct population of B cells carrying large numbers of EBV genomes accounts for the elevated levels of virus in high load patients (27). These High Copy Cells (HCCs) have >10 EBV genomes per cell and usually have 20 to 40 distinct FISH spots per nucleus. Up to 30% of high load patients' EBV infected cells are HCCs. The remainder of the virus infected cell population consists of Low Copy Cells (LCCs). LCCs have 1-2 viral genome copies/nucleus; these cells appear to be the predominant carriers of viral load in low load transplant patients and normal healthy carriers. Less than 5% of infected cells in low load patients have 10 or more FISH spots per nucleus (27). Further analysis of the infected cells for surface immunoglobulin expression with a combination FISH/immunofluorescence (IF)

procedure, revealed that the majority of low copy cells were IgM+ in both high and low load patients (29). Nearly 70% of HC cells in high load patients were Ig-null cells, with no discernable immunoglobulin expressed on their surface. The very rare HCCs in low load patients showed a similar phenotype (29).

Ig-null cells have also been observed in other EBV-associated diseases. 'Crippled' and 'forbidden' Ig- cells in angioimmunoblastic lymphadenopathy with dysproteinemia and in the Reed-Sternberg cells of classical Hodgkin's Disease have been observed (3, 4, 12). These latter cells have also been shown to have lost B-lineage-specific gene expression, adding to the aberrant nature of Ig-null cells (3). In EBV-positive PTLD, cells that have failed the germinal center process, and thus should have been eliminated, were shown to play an integral part in development of lymphoproliferative disease. (33) Since Ig-null cells are an aberrant type of cell that should not survive in the peripheral blood and may lead to complications in the organ recipient, we have studied them further, determining the frequency of such cells in the peripheral blood of solid organ transplant recipients and examining their surface marker phenotype and contribution to persistent viral load.

3.2.4. Materials and Methods

Patient Population: Since 1995, we have monitored children who have received solid organ transplants at Children's Hospital of Pittsburgh for viral loads of Epstein-Barr in their peripheral blood by quantitative-competitive PCR. This technique was developed in the Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh (11). We have multiple prospectively collected samples from nearly 200 patients who have had a bowel, liver, lung, renal, or heart transplant. Of these, 34.2% have never had a viral load. Slightly more than 36% have had a consistently detectable low load, defined as <200 viral genome copies /10^5 lymphocytes for >2 months. Eighteen percent of the patients have been classified as having a persistent high load, defined as having a viral load >200 viral genomes/10^5 lymphocytes for >2 months. The remaining patients have had fluctuating viral loads. The lower limit of the QC-PCR test is 8 genome copies per 10^5 lymphocytes; as such, the test does not detect a normal latent infection without purification of B cells. For a viral load to be detected, the load must be 2-3 orders of magnitude greater than the level of viral load expected to be identified in a normal immunocompetent individual.

Flow Cytometric Analysis of Ig-null cell number. Peripheral blood mononuclear cells were prepared from whole blood specimens by centrifugation onto a Histopaque (Sigma) cushion. Cells were stained for two color analysis with CD19-PC7 (Beckman Coulter) and pan goat F(ab')2 anti-human Ig's-fluorescein isothiocyanate (sIg-FITC) (Biosource International). Cells were incubated for 30 minutes at 4 degrees Celsius and fixed in 1% paraformaldehyde. Isotype controls were IgG1-PE (Pharmingen) and preimmune goat F(ab')2 IgG control-FITC (Biosource International), as well as unstained cells in some instances. The cells were analyzed on a Beckman Coulter XL flow cytometer and analysis was done with Cytomation Summit v3.1. Ten

high load patients, ten low load patients, and 11 patients with a consistently undetectable viral load were examined. The patients were picked as the first 10 high load, 10 low load, and 11 ND patients whose blood was available for study.

Flow cytometric analysis of phenotype: Peripheral blood mononuclear cells from high load patients were prepared from whole blood specimens by centrifugation onto a Histopaque (Sigma) cushion. For four color analysis, each set of cells was stained with CD19- PC7 (Beckman Coulter) and pan goat F(ab')2 anti-human Ig's-FITC (Biosource International), plus HLA Class I-PE (BD Pharmingen) and HLA Class II-PC5 (Immunotech), or CD27-PE (Immunotech) and CD20-PC5 (Immunotech), or CD38-PE (BD Pharmingen) and CD10-PC5 (BD Pharmingen), or CD23-PE (BD Pharmingen) and CD69-PC5 (BD Pharmingen). For three color analysis, each set of cells was stained with CD19- PC7 (Beckman Coulter) and pan goat F(ab')2 anti-human Ig's-FITC (Biosource International), plus CD5-PC5 (Immunotech) or CD40-PC5 (BD Pharmingen). For the nuclear proliferation marker Ki67, cells were first fixed with 1% paraformaldyhyde, washed and blocked in FACS buffer plus saponin, and then stained with Ki-67-PE (BD Pharmingen). All antibody incubations were for 30 minutes at 4 degrees Celsius. Isotype controls were IgG1-PE (BD Pharmingen), IgG2a-PC5 (Beckman Coulter), IgG1-PE (Pharmingen), and preimmune goat F(ab')2 IgG control-FITC (Biosource International). For each set of phenotypic markers, cells from 10 high load patients were analyzed on a Beckman-Coulter XL flow cytometer and analysis was done with Cytomation Summit v3.1.

Magnetic bead separations: Peripheral blood mononuclear cells were presorted before separation in the MoFlo to help purify the cell populations. Monocytes/macrophages and myeloid cells were positively sorted from the PBMC's with Dynabeads M-450 CD14 and Dynabeads CD15 (Dynal Biotech Oslo, Norway). Histopaque prepared PBMC's from patient

blood samples were mixed with Dynabeads at a concentration of 10^7 beads/ml and incubated at 4 degrees Celsius for 30 minutes, with gentle rotation. The positively selected cells were isolated with a Dynal MPC and discarded, while the negative fraction was saved for staining and further sorting.

Cell staining for cell sorting: PBMCs from high load patients that had been depleted of CD14+ and CD15+ cells were stained for two or three color sorts by incubating the cells with CD19-PE (Immunotech) and sIg-FITC (Biosource International), and in some cases, CD20-PC5 (Immunotech) or HLA Class I-APC (BD Pharmingen). The cells were incubated with the antibodies for 30 minutes at 4 degrees Celsius, then washed and resuspended in PBS.

Cell Sorting: The stained PBMC's, devoid of CD14+ and CD15+ cells, were gated on the basis of CD19 positivity and side scatter, then sorted in a BD FACS Aria or Cytomation MoFlo (Modular Flow High Performance Cell Sorter) based on Ig positivity. Cells that were at least one log less than the median of the Ig positive cloud were considered Ig negative. Both CD19+ and CD19- fractions were collected directly into PCR lysis buffer (see below for composition), and a small amount of unsorted cells were also saved for QC-PCR. For some patients, Ig-null cells were further sorted on the basis of CD20 or HLA Class I. In such cases, the Ig-null CD20 positive and negative fractions, or HLA Class I positive or negative fractions, were collected, as well as unsorted cells.

Quantitative-Competitive PCR: Peripheral blood mononuclear cells were prepared from whole blood patient samples by centrifugation onto a Histopaque (Sigma-Aldrich, St. Louis, MI) cushion. The cells were washed in phosphate buffered saline (PBS) and counted. Cell pellets were stored at –20 degrees C until ready for PCR. To make lymphocyte lysates 20µl of PCR lysis buffer (50 mM KCL, 10 mM Tris [pH 7.6], 2.5 mM $MgCl_2$, 1% Tween 20, and 100µg of

proteinase K per ml) was added for every 10^5 lymphocytes. The lysates were incubated at 55EC for 1 hr, boiled for 15 min to inactivate the proteinase K and chilled on ice. Primers for the PCR target sequence in the EBV genome were designed with OLIGO software (National Biosciences Inc., Plymouth, MN). TP1Q5' (AGGAACGTGAATCTAATGAAGA) and TP1Q3' (GAGTCATCCCGTGGAGAGTA) amplify a 177-bp EBV sequence (exon 1) in the *lmp2a* gene. A competitor target was made by deleting 42-bp from a 177-bp EBV amplicon derive from the viral *lmp2a* exon 1 sequence. For each sample, five tubes containing 8, 40, 200, 1000 or 5000 copies of the viral LMP2a competitor sequence along with lymphocyte lysates equivalent to 10^5 cells were subjected to 30 cycles of amplification (94EC for 1 min, 54EC for 1 min and 72 EC for 1 min). Each PCR reaction (50µl) contained 20 pmol of 5' and 3' primers, 50mM KCL, 2.5 mM $MgCl_2$, 10 mM Tris [pH 9.0], 0.1 % Triton X-100, and 0.25 mM deoxynucleotides (Pfizer, New York, New York). One unit of Amplitaq Gold DNA polymerase (Perkin Elmer, Wellesly, MA) was used in each reaction. The PCR products were analyzed on 2% agarose gels containing 0.5X Tris-borate-EDTA electrophoresis buffer and 0.5µg of ethidium bromide per ml.

The QC-PCR assay for EBV is used to quantitate viral loads in the range of 8 to 5000 copies of viral DNA in 10^5 lymphocytes. Normal latent infection (0.01 - 0.1 copies/10^5 lymphocytes) is not detected by this protocol and detectable levels of viral DNA reflect a viral genome burden at least 2 to 3 orders of magnitude above normal latency.

3.2.5. Results

3.2.5.1. Frequency of Ig-Null Cells in High Load, Low Load, and No Load Patients

To determine the frequency of Ig-null cells in transplant recipients and to determine if the frequency of Ig-null cells increases with viral load, we probed peripheral blood mononuclear cells from patients at Children's Hospital of Pittsburgh with pan sIg-FITC and CD19-PE and analyzed them by flow cytometry. Ten high load patients, 10 low load patients (as defined previously), and 11 transplanted patients with no detectable viral load (ND) were selected for the study. CD19+ cells were gated based on the log of their side scatter and CD19 expression (Figure 9, top panel). The gated cells were then examined for the expression of surface immunoglobulin. A CD19+ cell was defined as Ig-null when its surface expression of Ig was at least one log below the median of the surface Ig expression of the Ig+ cloud. (Figure 9, middle panel). The presence of the Ig-null cells within the CD19+ gated population was then confirmed (Figure 9, bottom panel).

Ig-null cells were present in every patient examined. The 10 high load patients examined had an average of 2.52%, with a broad range of 0.22-9.72% of CD19+ cells (Figure 10, top). Two patients in particular had a much higher frequency of Ig-null cells. Data from high load patient #9 is shown in Figure 9. The frequency of Ig-null cells for this patient was 8.86% of the peripheral blood B cells. High load patient #10 had the highest frequency of Ig-null cells at 9.72%. The 10 low load patients had lower frequency of Ig-null cells, with 0.95% of the CD19+ cells being Ig-null (Figure 10, middle). This patient group also had a broad range, varying from 0.12-4.25% of B cells. The frequency of Ig-null cells was very low in the ND patient population,

but still detectable. This group averaged only 0.20% of CD19+ cells as Ig-null, with a range of 0.01-0.92% (Figure 10, bottom).

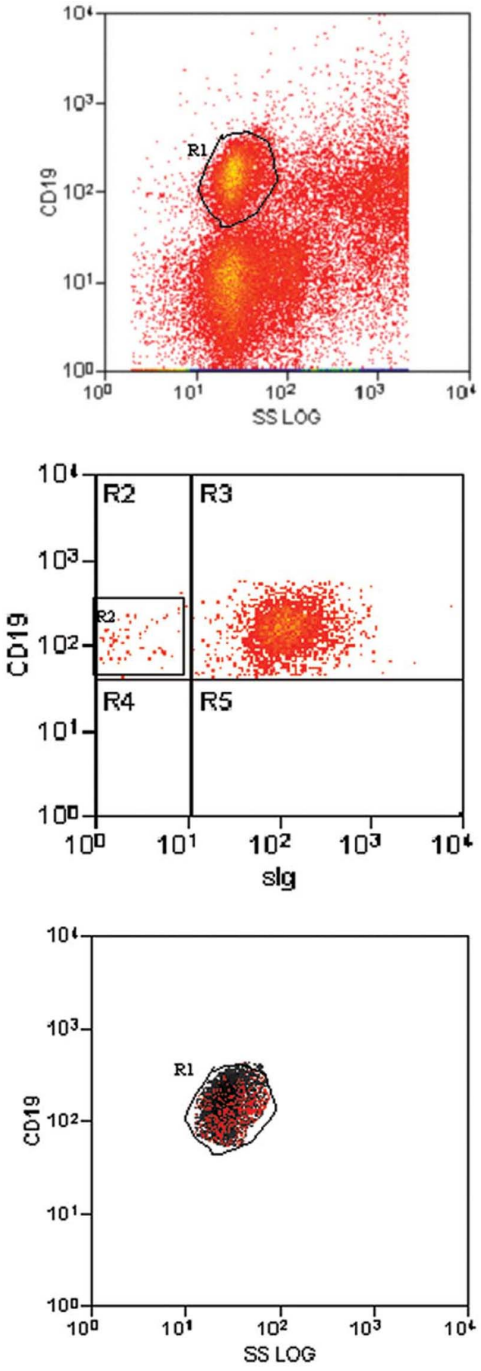

Figure 9. Procedure for the detection of Ig-null cells (high load patient #9).

The top panel shows the determination of the CD19+ cloud for gating purposes. B cells are defined based on both the surface expression of CD19 and the log of the side scatter. The middle panel shows Ig expression vs. CD19, as gated on the CD19 R1 region. An Ig-null cell (box R2) is defined as being one log less than the median of the surface Ig positive cloud. The bottom panel shows the cells highlighted from box R2 as they are dispersed in the CD19+ cloud.

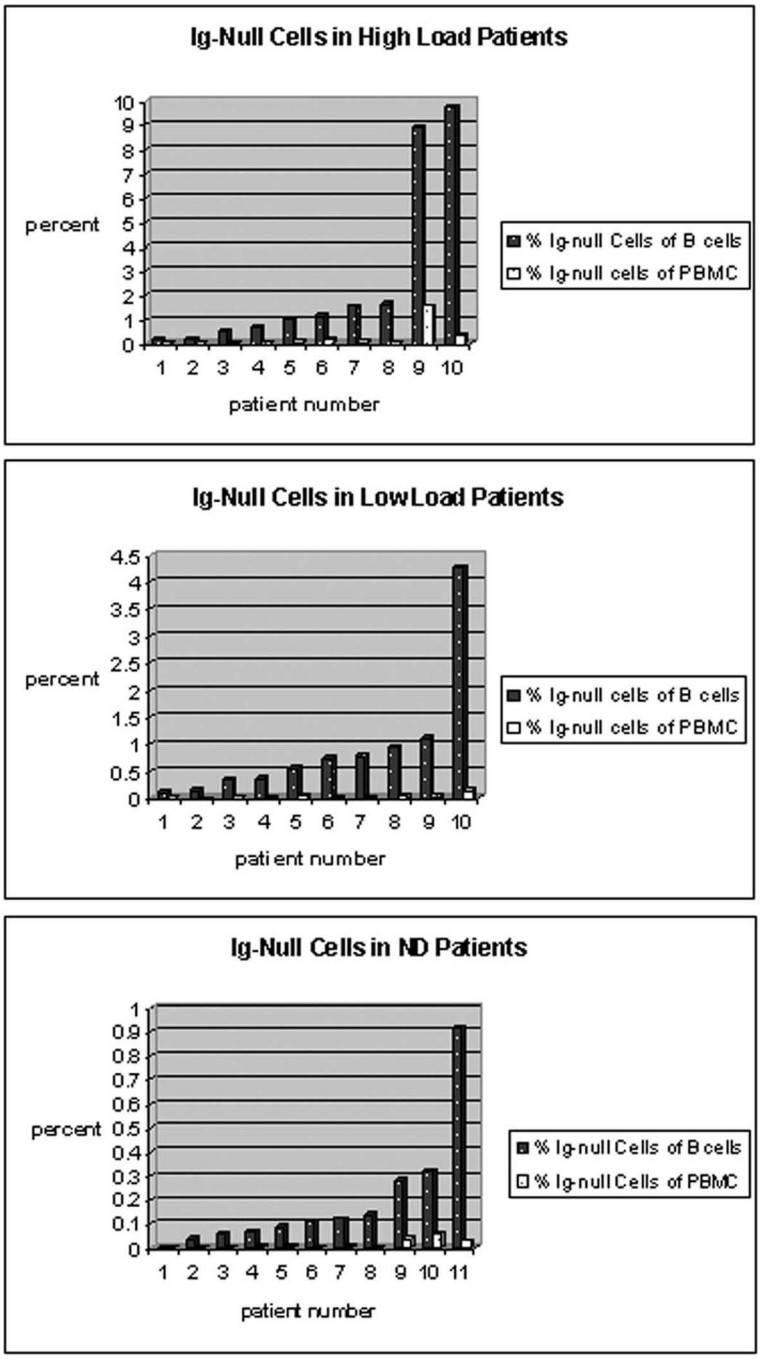

Figure 10. Percent of Ig-null cells in the peripheral blood of high load (top), low load (middle), and ND (bottom) individuals.

The percent of Ig-null cells of B cells from the peripheral blood was determined by staining PBMCs with CD19-PE and sIg-FITC and gating on the CD19+ cloud.

3.2.5.2. Distribution of Viral Load in High Load Patients

To determine what proportion of the increased viral load seen in high load patients is carried in the population of Ig-null cells, we sorted peripheral blood mononuclear cells based on the surface expression of CD19 and pan sIg. PBMCs from high load patients were presorted for initial purification with CD14 and CD15 magnetic beads to remove contaminating monocytes and granulocytes. At different times either a MoFLo (Modular Flow High Performance Cell Sorter) or a BD FACS Aria were used to sort the stained cells. CD19+, sIg- and CD19+, sIg+ cells were collected from three patients; quantitative competitive PCR was performed on each sorted cell sample to determine the number of viral genomes in each sorted fraction. QC-PCR was also conducted on a population of unsorted PBMC's from each patient for a total viral load estimate. QC-PCR results and the number of cells in each isolated cell population are shown for one representative patient. (Figure 11) For the unsorted fraction, the number of CD19+, Ig +/- cells was calculated based on the known percentage of lymphocytes, CD19+ cells, and Ig-null cells. This number was then adjusted to reflect the number of cells per 20 ul reaction. For the patient shown, a viral load of 400 was observed in the Ig-null sorted fraction (336 cells per PCR reaction). In the CD19+, Ig+ sorted fraction a viral load of 200 was measured, (3682 cells per PCR reaction). In the unsorted fraction, a viral load of 600 was observed in a fraction calculated to contain 321 Ig-null cells and 4760 CD19+, Ig+ cells. By comparing the number of Ig-null cells in both the sorted and unsorted fraction, two-thirds of the viral load is in Ig-null cells. While the distribution of viral load varied from patient to patient, in all patients examined in this manner, it was clear that the Ig-null population of cells held a substantial portion of the viral load. These results are shown in Table 4.

Sample 1	Unsorted-derived	(CD19+, sIg-)	(CD19+, sIg+)
CD19+, sIg+ (20 ul)	4760	N/A	3682
CD19+, sIg- (20 ul)	321	336	N/A
Unsorted cells (20 ul)	17, 589	N/A	N/A

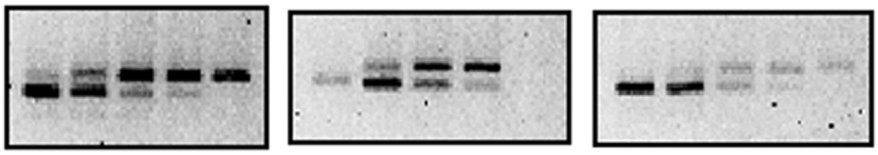

Figure 11. Distribution of viral load in one representative high load patient.

The boxed gels represent QC-PCR reactions from each fraction of cells (left to right-unsorted, Ig-null, and Ig+). For each gel, the 5 lanes represent (left to right) 5000,1000, 200, 40, and 8 copies of the competitor.

Table 4. Viral loads detected by QC-PCR in Ig positive and Ig-null cells, as well as in the unsorted fraction.

sIg	Patient 1			Patient 2			Patient 3		
Fraction	Unsorted (Ig+/Ig-null)	**Ig-null**	Ig+	Unsorted (Ig+/Ig-null)	**Ig-null**	Ig+	Unsorted (Ig+/Ig-null)	**Ig-null**	Ig+
Cells/ reaction	17,589 (4760/321)	**336**	3682	$4x10^5$ (10^4/1760)	**416**	4000	95,000 (4896/104)	**442**	9030
Viral load	600	**400**	200	5000+	**400**	2000	2000	**4000**	10,000

sIg	Patient 4			Patient 5		
Fraction	Unsorted (Ig+/Ig-null)	**Ig-null**	Ig+	Unsorted (Ig+/Ig-null)	**Ig-null**	Ig+
Cells/ reaction	13,000 (151/7)	**657**	7030	6000 (42/1)	**543**	8190
Viral load	80	**200**	100	N/A	**200**	200

N/A = not-amplifiable

3.2.5.3. Phenotype of Ig-Null Cells in High Load Patients

Since Ig-null cells by definition have an aberrant B cell phenotype and also appear to disproportionately carry the viral load in high load patients, we analyzed Ig-null cells for the expression of other B cell phenotypic markers with four color flow cytometry. CD19 and sIg were used to define the Ig-null population in each sample in conjunction with other surface markers. Because of limited cell number from each patient, different groups of high load patients (groups a-f) were examined for various surface markers. There were no significant differences between the high load patient group examined for each cell surface marker. Initially the Ig-null cells in the peripheral blood of 10 high load patients (group a) were examined for HLA Class I and Class II (Figure 12a). More than one third (36.9%) of the Ig-null cells in these patients were HLA Class I negative, with two patients in particular (patients 4 & 10) having a high percentage of the Ig-null cells HLA Class I negative, with 85.5 and 85.6 % respectively. Approximately one third (36.4%) of Ig-null cells were HLA Class II negative. Interestingly, the two patients with high numbers of Ig-null, HLA Class I^{neg} cells did not have a high number of Ig-null, Class II negative cells, although two other high load carriers (patients 6 & 9) did show high levels of HLA Class II^{neg}, Ig-null cells, with percentages of Class II ^{neg} cells at 76.5 and 95.3% of Ig-null cells, respectively.

B cell phenotypic markers were also examined on Ig-null cells from 12 high load patients (group b) (Figure 12b). B1 and B2 type cells can nominally be classified by the surface expression of CD5. Ig-null cells were predominantly CD5 negative (88.2%) suggesting a possible B2 type origin. CD20 is a B lineage marker found on all B cells and is particularly important because it is the basis of destruction of B cells with anti-CD20 monoclonal antibodies

in clinical trials treating PTLDs that fail to respond to conventional therapies. In the high load carrier population, half (49.2%) of the CD19+, sIg- cells were also $CD20^{neg}$. One patient (Patient #3) for whom 1.3% of all B cells were Ig-null cells, 79.1% of the Ig-null cells were $CD20^{neg}$. Another patient (#10), with 5.5% Ig-null cells in the B cell population showed a similar pattern with 78.6% of Ig-null cells also being $CD20^{neg}$. CD20 was broadly expressed on B cells in these patients, as 95.45% of all CD19+ cells were also CD20+ (data not shown). Memory B cells can be detected by the presence of somatically mutated Ig genes and expression of CD27. In 11 high load carriers (group c), 87.5% of the Ig-null cells were $CD27^{neg}$. Overall, 86.6% of the CD19+ cells in the circulation were $CD27^{neg}$ (data not shown). Peripheral blood B cells have been shown to express CD40. In this group of 10 high load patients (group f), 96.2% of CD19+ cells expressed this marker. However, nearly 40% (39.1%) of the Ig-null cells did not have CD40 on their surface.

Ig-null cells from high load patients were examined for the presence of surface activation (CD23 and CD69) (group d) and germinal center markers (CD10 and CD38) (group e) (Figure 12c). Flow analysis suggested the Ig-null cells were non-activated, with a very small percentage of CD23 and CD69 expressing cells (1.45% and 2.66% respectively). In the CD19+ population, percentages of 1.6% (CD23) and 1.01 % (CD69) were observed. Similarly, Ig-null cells did not carry the germinal center markers CD10 and CD38 on their surface (2.22% positive and 9.42% positive, respectively). The circulating CD19+ population as a whole had similar small percentages of these markers, with 3.4 and 2.8% expressing CD10 and CD38. Ig-null cells from 10 high load patients (group f) were permeabilized and stained for the proliferating cell marker Ki67 (Figure 12c). 2.72% of the Ig-null cells were positive. Overall, in the gated CD19+ population, only 1.2% were positive for Ki67.

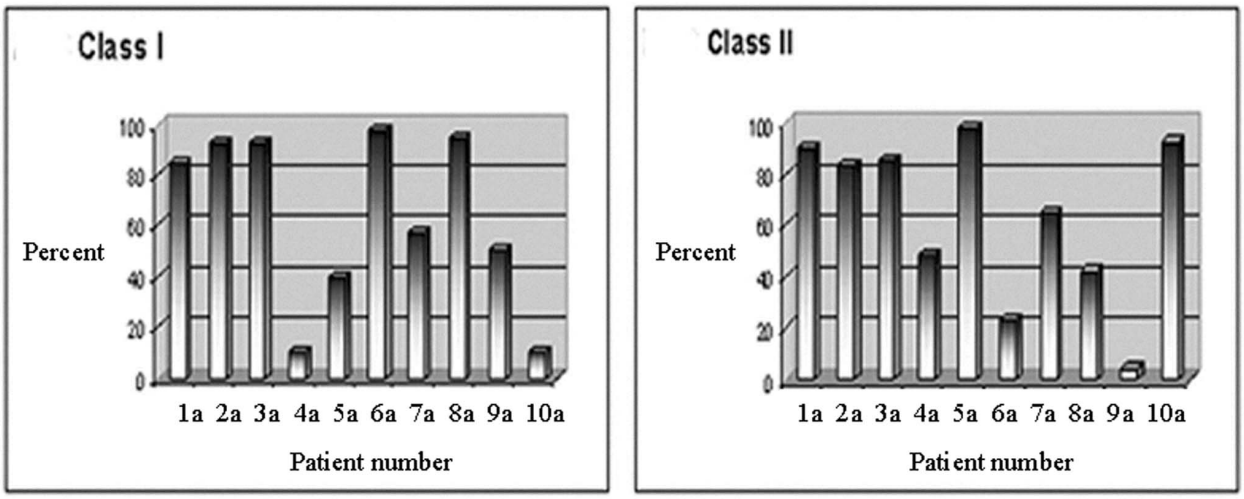

Figure 12a. Percent of Ig-null cells displaying B cell phenotypic markers.

PBMCs from 10 high load patients were stained with various surface antibodies (see materials and methods for complete list) and analyzed by flow cytometry. A shows the percent of cells expressing HLA Class I and II. For each graph, percents are expressed as percent of B cells, and the patients are ordered 1-10 according to the increasing amount of Ig-null cells in their peripheral blood.

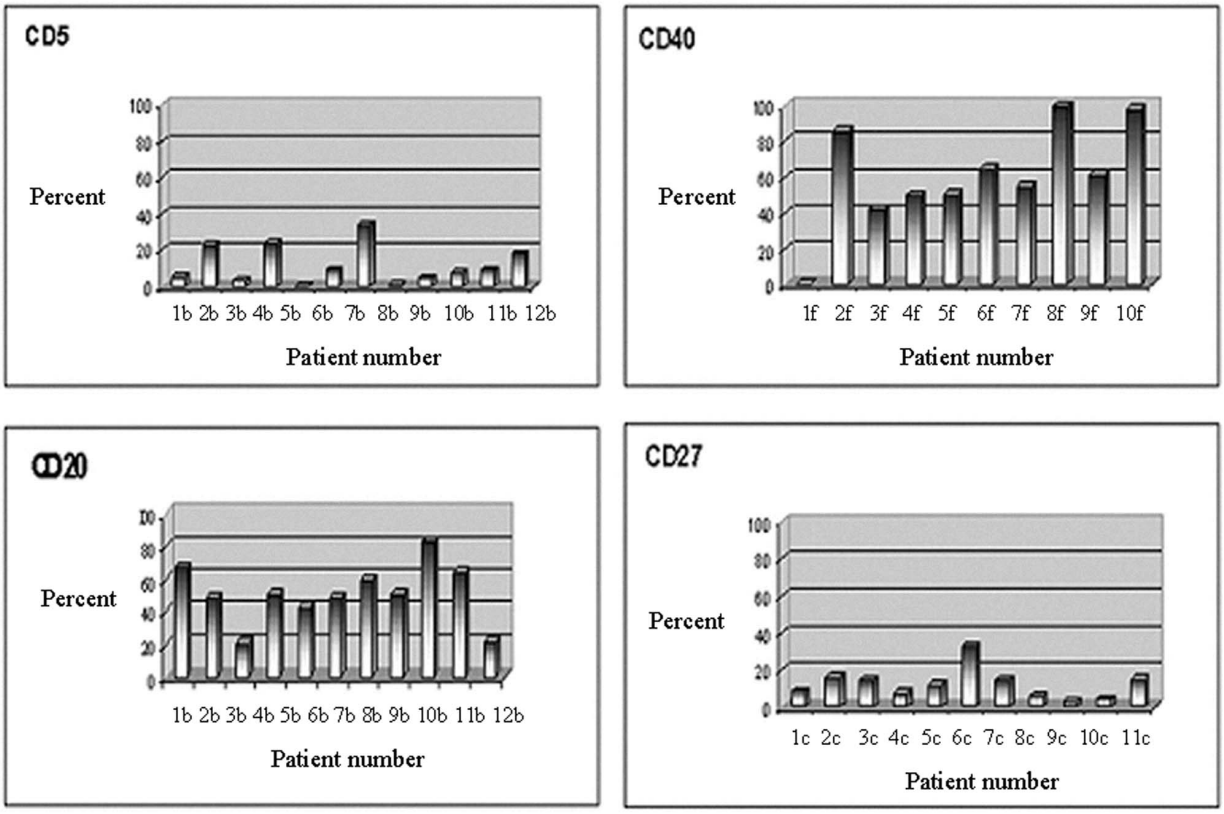

Figure 12b. Percent of Ig-null cells displaying B cell phenotypic markers.

PBMCs from 10 high load patients were stained with various surface antibodies (see materials and methods for complete list) and analyzed by flow cytometry. B shows the percent of cells expressing B cell markers CD5, CD20, CD40 and CD27. For each graph, percents are expressed as percent of B cells, and the patients are ordered 1-10 according to the increasing amount of Ig-null cells in their peripheral blood.

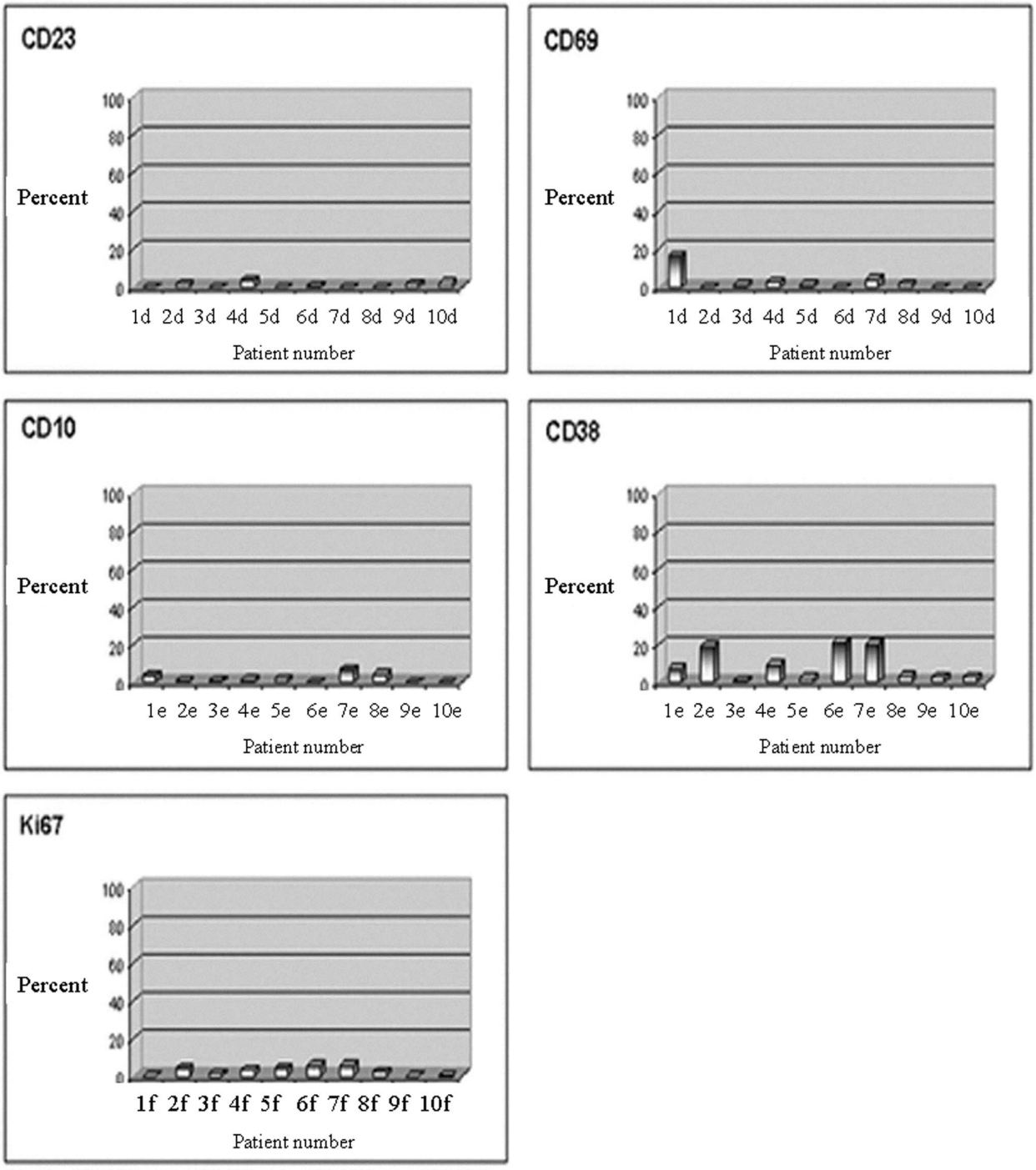

Figure 12c. Percent of Ig-null cells displaying B cell phenotypic markers.

PBMCs from 10 high load patients were stained with various surface antibodies (see materials and methods for complete list) and analyzed by flow cytometry. C shows the percent of cells expressing activation and germinal center markers, as well as a proliferation marker. For each graph, percents are expressed as percent of B cells, and the patients are ordered 1-10 according to the increasing amount of Ig-null cells in their peripheral blood.

3.2.5.4. Compartmentalization of Viral Load in High Load Patients

Approximately one half of the Ig-null cells in high load patients were CD20 negative and thus would presumably be resistant to rituximab therapy. Another third of Ig-null cells are HLA Class I negative and thus unable to present targets for antigen specific cytotoxic T cells. An increased concentration of viral load in these cell populations would have important implications for immunotherapeutic approaches to lymphoproliferative disease.

We examined the elevated viral load in these patients to determine whether it is carried in the Ig-null, $CD20^{neg}$ and/or HLA Class I^{neg} compartment. In the two patients whose Ig-null cells were sorted into HLA Class I positive and negative fractions, different distributions of viral load were observed. Patient one (Table 5) had a viral load of 400 in the unsorted fraction. From the total number of cells and the percentages of CD19+, Ig-null, and HLA Class I^{neg} cells, it can be calculated that the PCR reaction on the unsorted fraction contained approximately 35 Ig-null cells of which only 1 would be a double negative cell. In the Ig null fraction, the HLA Class I^{pos} reaction had 205.7 cells, and a viral load of 200 genome copies. The HLA Class I^{neg} reaction had just 5 cells, and a viral load of 40 genome copies. By comparing the viral load in the sorted populations to the viral load in the unsorted fraction, we determined that the viral load was not enriched in the Ig-null, HLA Class I^{neg} fraction. On the other hand, the same analysis in the other high load patient examined did have an enriched viral load in the Ig-null, HLA Class I^{neg} compartment. The unsorted fraction had a viral load of 400, with a calculated number of Ig-null, Class I positive and negative cells of 106 and 2 cells, respectively. The sorted Ig-null, HLA Class I^{pos} fraction had a viral load of 80 genome copies in 171 cells. The sorted double negative

fraction had a load of 400 genome copies indicating that in this patient there was a significant enrichment of the viral load in the Ig-null, HLA Class I^{neg} compartment (Table 5).

PBMCs from three patients were sorted for their Ig-null, $CD20^{neg}$ populations (Table 6). While virus was detected in the $CD20^{neg}$ population in 2 patients, the levels were not significantly enriched. For patient three, the 8.66 sorted Ig-null, $CD20^{neg}$ cells per PCR reaction did carry an elevated viral load of 100 genome copies, while the 81.2 Ig-null, $CD20^{pos}$ fraction had a viral load of only 80. In this patient, the Ig-null, $CD20^{neg}$ fraction disproportionately carried more of the viral load. Patient 2 had no detectable viral load in the Ig-null, $CD20^{neg}$ compartment, while Patient 1 carried a proportional viral load. These results are summarized in Table 6.

Table 5. Viral loads detected by QC-PCR in HLA Class I positive and negative Ig-null cells, as well as in the unsorted fraction.

HLA Class I	Patient one			Patient two		
Fraction	Unsorted (Ig-null)	Ig-Positive	Ig-negative	Unsorted (Ig-null)	Ig-positive	Ig-negative
Cell#/rxn	34.8	205.7	5.1	108	171	2.8
Viral load	400	200	40	400	80	400

Table 6. Viral loads detected by QC-PCR in CD20 positive and negative Ig-null cells, as well as in the unsorted fraction.

CD20	Patient one			Patient two			Patient three		
Fraction	Unsorted (Ig-null)	Ig-Positive	Ig-negative	Unsorted (Ig-null)	Ig-positive (Ig-null)	Ig-negative	Unsorted (Ig-null)	Ig-positive	Ig-negative
Cell#/ Rxn	35.5	299	130	4.1	126	1.73	25.8	81.2	8.66
Viral load	800	400	40	80	8	0	200	80	100

3.2.6. Discussion

Previously, we reported the presence of CD19+, Ig null cells in the peripheral blood of pediatric solid organ transplant patients with a high load of Epstein-Barr virus (29). By fluorescent in situ hybridization, we demonstrated that a large portion of the viral load was carried in high copy numbers with in Ig null cells, while cells with a low copy number of EBV genomes were predominately IgM+, in both high and low load carriers (29). In the current study, we present data that suggests that Ig null cells are present in all transplant recipients and that their level is highest in high load patients. The high load group had, on average, two and a half times the percentage of Ig-null cells in the peripheral blood as compared to the low load group, and more than ten times the percentage of Ig-null cells as compared to the ND group.

In an effort to determine the phenotype of Ig-null cells, we examined a number of immunologically relevant surface markers on the CD19+, Ig-null population. Normal latently infected B cells are CD27+, and have a resting B2 cell phenotype (1, 2, 19, 20, 26). While we found that Ig-null cells were non-proliferating, and lacked germinal center and activation markers, we also determined that nearly all these cells were $CD27^{neg}$ and approximately half of the cells did not have the ubiquitous CD20 B cell marker on their cell surface. Moreover, approximately one third of Ig-null cells also lacked HLA Class I and II markers. Ig-null cells were $CD5^{neg}$ consistent with a B2 cell type profile. Thus, while the Ig-null cells had variable surface expression of some markers, as a whole they did not match the recognized phenotype of normal latently infected cells (1, 2, 19, 20, 26). In addition, it is unclear how long-lived Ig-null cells are in the circulation. Many high load carriers over a period of six to 12 months tend towards reduced viral loads and eventually become, by definition, low load carriers. These converters to load status do not carry an elevated number of Ig-null cells or high genome copy

cells suggesting a direct relationship between the drop in viral load and the loss of the null cells from the circulation as time progresses (data not shown).

By sorting Ig-null cells from the peripheral blood of high load patients and performing quantitative PCR on the sorted populations, we determined that a large fraction of the elevated viral load was being carried in the Ig-null subpopulation. Importantly, it can be deduced that not all Ig-null cells are high copy EBV+ cells. This is demonstrated by both the detectable population of Ig-null cells in the ND patients, as well as the viral loads detected in the sorted CD19+, Ig-null fractions from the high load patients. While these viral loads were certainly elevated and concentrated in this fraction, the load would still be expected to be much higher if all the cells were carrying 10 or more viral genomes. This confirms the previous study, in which a percentage of Ig-null, EBV negative, CD19+ cells were observed in both high and low load patients (29). The origin of Ig-null cells seems most likely to be from germinal center reactions in which unsuccessful Ig affinity maturation and/or isotype switching has occurred. This explains the lack of expression of surface immunoglobulin. In the context of an EBV infection, EBV could provide the necessary survival signals to prevent apoptosis. However, Ig-null, EBV negative cells were detected in this study and this suggests that the process is likely not entirely EBV dependent. The immunosuppression of transplant recipients is likely to be a major factor in allowing Ig-null cells to escape apoptosis and persist. EBV may be a key player in the modification of a microenvironment within the germinal center that allows for the enhancement of protection of both uninfected as well as infected Ig-null cells. Such an environment might also contribute to the loss of B-cell differentiation and maturation markers from a large fraction of the Ig-null B cells. Kuppers et al (2003) observed a similar loss of B-cell markers on the Reed-Sternberg cells of classical Hodgkin's Disease (3). While their group examined RNA

expression, and in the present study we analyzed surface protein expression, a similar phenomenon of B-cell identity loss in the Ig-null cells of transplant patients appears to be occurring. A comprehensive gene expression profiling of the Ig-null cells would further clarify their phenotypic status.

Throughout this study we have used CD19 to define the B cell population. We have not determined the prevalence of CD19 negative null cells in our patient populations. CD20 is also often used to gate B cells from mixed populations. When PBMCs from 10 high load carriers were gated on CD20, on average approximately 0.78% of the gated cells were Ig-null and only 0.05% of the CD20+ cells were both $CD19^{neg}$ and Ig-null (data not shown). These observations show that there were very few CD20+, $CD19^{neg}$, Ig-null cells in the circulation. Thus, very few Ig-null cells were missed by gating on the CD19+ population. Since nearly half of the CD19+, Ig-null cells are CD20neg, CD19 is clearly a better B cell marker for defining the population and examining Ig-null B cells. Because Ig-null cells carry a significant viral load and half of Ig-null cells are CD20-, the possibility exists that some PTLDs cannot be effectively treated with rituximab (anti-CD20 antibody). Previous studies examining the efficacy of rituximab have shown varied responses, with overall response rates around 60-70% (7). The largest study to date involved 32 patients, with either solid organ or bone marrow transplantation. The overall response included 20 complete responses and 2 partial responses, for an overall rate of 69% (18). Various factors, including CD20 positivity and performance status, appear to be the major predictors of response to rituximab (7). Our current study shows that elevated viral loads are often carried in aberrant B cells, which would be predicted to be non-responsive to rituximab-based therapies.

Taken together, the results suggest that the persistent EBV infection of pediatric solid organ transplant recipients is complex and dynamic. Because Ig-null cells have been shown to be involved in such EBV-associated disease states, as angioimmunoblastic lymphadenopathy with dysproteinemia (AILD), EBV positive Hodgkin's Disease, and PTLD (3, 4, 12, 14, 33), their presence in the peripheral blood of asymptomatic children with high EBV loads warrants monitoring. If such cells would give rise to a PTLD or lymphoma, current therapeutic approaches may be ineffective.

3.2.7. Literature Cited

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4. DISCUSSION

While Epstein-Barr virus is normally carried as a harmless passenger in infected individuals, it has also been linked to several severe malignancies. The focus of this project was a population of individuals who are at an elevated risk for EBV-associated posttransplant lymphoproliferative disease (PTLD). Pediatric transplant patients are an elevated risk group because they are seronegative at the time of transplant. Advances in understanding the pathogenesis of EBV infection and the development of PTLD in this patient population are needed.

4.1. THE PRESENCE OF IG-NULL CELLS

The patients in this study were transplant recipients who did not have PTLD. Many of these patients did carry a characteristic elevated viral load in their peripheral blood. They were classified as either high load carriers, low load carriers, or non-detectable (ND) patients, according to previously published criteria (16). Briefly, high load carriers have a viral burden in their peripheral blood of >200 viral genomes per 10^5 lymphocytes. Low load carriers have a viral burden between 8 and 200 viral genome copies in 10^5 lymphocytes, and ND patients have <8 viral genome copies in 10^5 lymphocytes. In each case, this viral load must be carried stably for at least two months to meet the classifications. Previous work in this laboratory involved a characterization of EBV infected B cells in the peripheral blood from this patient population. The two main findings were 1) the EBV load is carried exclusively in the IgD negative B cell compartment (non-naïve), and 2) high and low load carriers harbor their elevated viral loads differently (18, 19). The difference is that high load carriers have a distinct population of infected cells that have high copy numbers of viral genomes (10+). They also have a population

of low copy number cells with just 1-2 viral genomes per nucleus. Low load carriers carry the their viral load predominately in low copy number cells. High copy cells were only detected in low load carriers in very rare instances (19). In normal individuals, the EBV genomes are carried in the peripheral blood as low copy cells, just as it is in the low load carriers.

One issue that is important to understanding the pathogenesis of disease in immunocompromised patients is how high and low copy cells arise during the course of virus infection. The presence of viral genomes in low copy cells can be readily accounted for by the Random Assortment Model for virus replication (Figure 13). This model requires three assumptions about virus infection: 1) only one virus particle infects each cell, 2), the viral episomes only replicate once per cell cycle and 3) there is EBNA1 mediated random assortment of the viral genomes to the daughter cells at the M phase of the cell cycle (19). The high copy cells observed in the high load patients cannot be accounted for by a mechanism involving random assortment alone (19). This suggests that high copy cells probably have a different pathway of infection than low copy cells. The present work was initiated to analyze the characteristics of high copy cells that could yield an explanation of their origin and fate in the context of EBV infection, immunosuppression and lymphoproliferative disease. While there are more questions to be answered (see Future Directions, below), some important conclusions were uncovered concerning the origin of high copy cells within the overall scheme for ontogeny of B cells.

It was already determined that the EBV load was carried exclusively in an IgD- population (18). Since IgD is a marker for naïve B cells, the result suggested that B cells might be further along in the B cell maturation process. Determining if the infected cells had gone through a germinal center reaction, and thus had possibly undergone isotype switching would be

useful. An examination of B cell receptor usage in 8 high load patients examined by a combination FISH/IF procedure, 68% of the infected cells with high copy numbers had no detectable surface expression of immunoglobulin (Ig-null cells). Ig-null cells were observed in every high load patient, and in some patients, up to 85% of their high copy infected cells were Ig-null. Low copy cells in both high and low load patients were predominately IgM positive. In both the high and low load carrier groups, three patients had a prior history of PTLD. The PTLDs in all 6 patients were fully resolved at the time of the current study. These 6 patients did not differ significantly from another of the other patients in the isotypes of their infected cells. The 3 low load patients that had a prior history of PTLD did have the highest percentages of Ig-null high copy cells, but these values were not dramatically different. Further, high copy cells were still very rare in these patients. High percentages of Ig-null cells were detected in low load patients that did not have a prior history of PTLD.

To appreciate the significance of Ig-null cells, an understanding of fundamental B-cell ontogeny is necessary.

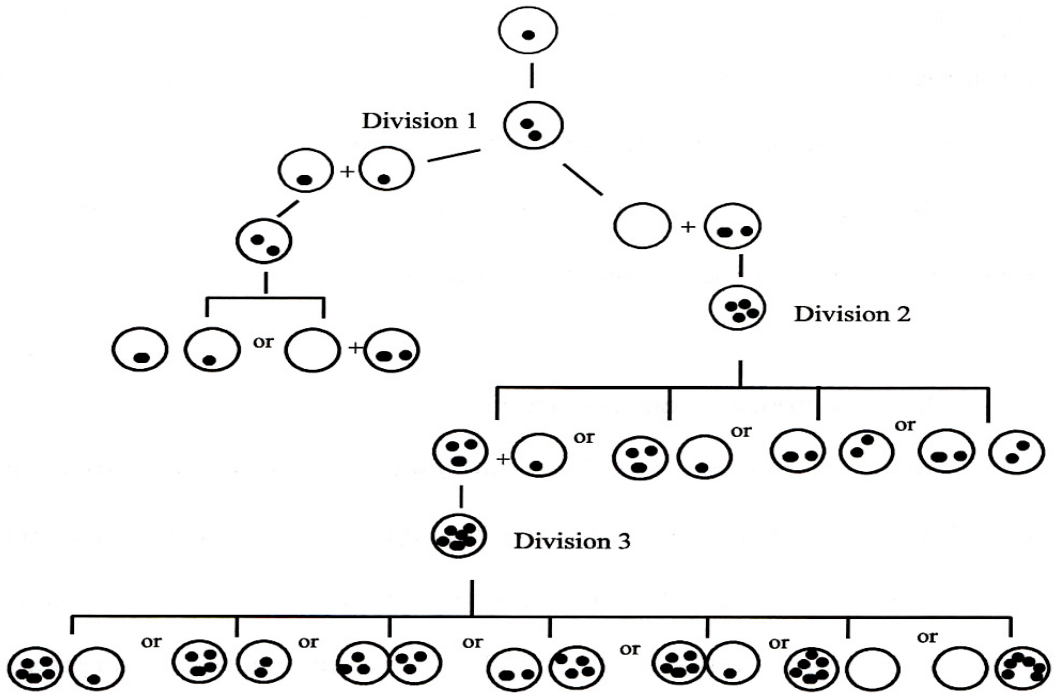

Figure 13. The Random Assortment Model (19).

4.2. B-CELL ONTOGENY

The earliest stages of B cell development occur in the bone marrow and are dependent on non-lymphoid stromal cells. B cells derive from pluripotent hemopoietic stem cells, and the earliest form of B-lineage cell is called a pro-B cell (7). In these B cells, the rearrangement of heavy chain immunoglobulin gene segments takes place. Pro-B cells differentiate into pre-B cells, which express an intact heavy chain on the cell surface as a preliminary step in the assembly of a functioning B cell receptor (BCR). Once one of the light chain genes is rearranged and a light chain is synthesized, a complete IgM molecule can be expressed on the surface. The cell is now viewed as an immature B cell (7). After passing self-tolerance tests, the immature B cell is released from the bone marrow to circulate in the peripheral blood and further differentiate into naïve B cells, expressing IgD in addition to IgM. As they recirculate through the secondary lymphoid organs, they await contact with antigen to develop into mature B cells. Throughout this initial developmental process, a large number of B cells have died because of nonproductive rearrangements owing to imprecise joining of their immunoglobulin gene segments. The heavy chain locus in particular leads to a high percentage of cell loss (11).

When antigen-naïve, self-tolerant B cells leave the bone marrow, they circulate to the secondary lymphoid organs and enter through high endothelial venules (17). If they encounter their cognate antigen, usually in the outer T-cell zone of lymphoid organs, they become activated, with the help of their interaction with CD4+ T helper cells and follicular dendritic cells. These activated B cells then migrate to B-cell follicles in the lymphoid organs, and start to proliferate and differentiate into centroblasts. These B cell follicles become germinal centers (13), where intense proliferation and somatic hypermutation of their rearranged V-region genes

takes place (10). Centroblasts differentiate into resting centrocytes, which must be selected for high affinity of their recently rearranged BCR to cognate antigen. At this point in B cell development, two survival signals must be received by the centrocyte to rescue it from a default pathway leading to apoptosis. The first signal is delivered through the somatically mutated immunoglobulin molecule as it demonstrates high affinity binding to cognate antigen (12). The second signal is delivered by a cognate interaction between CD40 on the B cell and a T-cell expressing CD40L (the CD40 counter-receptor) (12). If such signals are received, the B cell can recycle to further mutate and/or undergo the isotype switching process to become a mature memory B cell. Alternatively, without the two crucial survival signals, they are programmed to undergo apoptosis. Memory B cells express IgA, IgG, or IgM, and have lost the expression of IgD. After selection, a B cell can also terminally differentiate into a plasma cell. Plasma cells do not express CD19, nor do they express surface immunoglobulin. Both memory and plasma cells exit the germinal center and enter the peripheral circulation. See Figure 14 for a pictorial view of B cell ontogeny, as described above.

A subset of B cells in humans develops in a different manner and these cells are termed B1 cells. These cells develop in the fetus and are self-renewing. They have distinctive receptors and functions from conventional (B2) cells. These cells were first identified by their surface expression of CD5, and are IgM positive and IgD negative (7). While they are sparsely found in the lymphoid organs, they are the main B-cell population in the peritoneal and pleural cavities. B1 cells somatically hypermutate very little, if any, and tend to bind to many different ligands with low affinity, suggesting that they mediate a more primitive early immune response (7). B1 cells continually divide in peripheral sites and have been linked to the chronic lymphocytic leukemia. Some speculation exists that PTLDs may have a linkage to B1 cells.

As the CD19+ B cells in this study do not express surface immunoglobulin (functional BCR), theoretically they should have apoptosed during the germinal center reaction, as the first survival signal was not received. Without a functional BCR, B cells are pre-programmed to undergo apoptosis. The aberrant Ig-null cells detected in this study must be receiving an alternative signal to allow them to survive in the periphery without surface expression of immunoglobulin. The majority of low copy cells in this study express IgM, suggesting that they did not undergo the isotype switching process in the germinal center and have not necessarily arisen from a germinal center reaction. Because the high copy Ig-null cells have an aberrant immunoglobulin phenotype and are missing one of two crucial signals necessary for the survival of the B cell, we investigated further and determined the frequency of such cells in the peripheral blood of pediatric solid organ transplant recipients by flow cytometry.

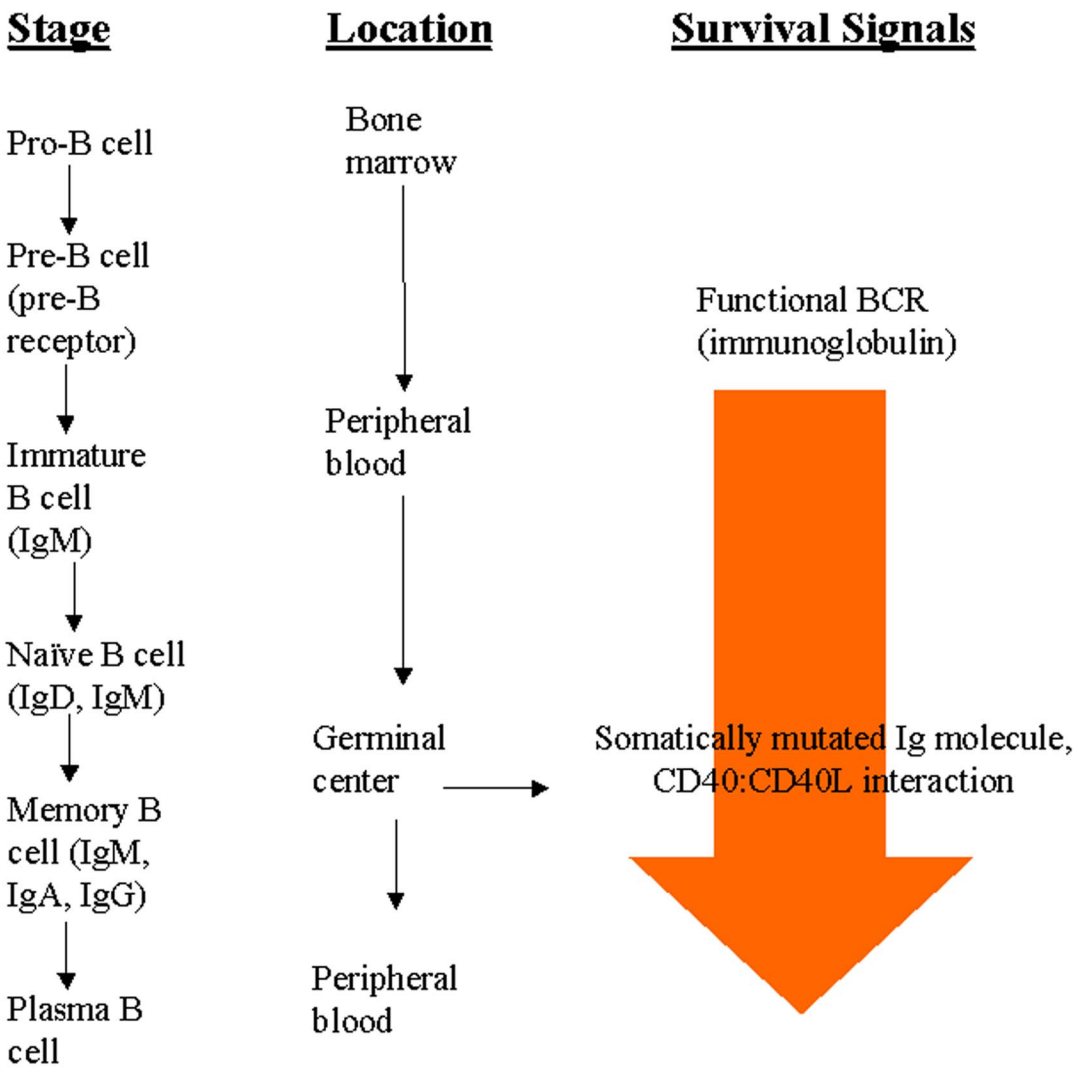

Figure 14. Normal B2 cell ontogeny.

4.3. IG-NULL CELLS IN PATIENTS AND EBV-ASSOCIATED MALIGNANCIES

Prior to determining the frequency of Ig-null cells in the peripheral blood of pediatric solid organ transplant recipients, we suspected that high load patients would have the highest frequency of Ig-null cells. These patients had the highest frequency of high copy cells. The analysis revealed that high load patients had the highest frequency of Ig-null cells, followed by low load patients and finally transplanted patients with no detectable viral load. One striking feature of the data is the broad range of Ig-null cell frequencies observed in each patient group. It would be interesting to follow the patients with the highest number of Ig-null cells in their circulation over time to see if they develop an EBV-associated complication. Additionally, it can be deduced that Ig-null cells are not extremely long-lived, at least in some patients. Low load patients that were formerly high load patients did not demonstrate the presence of high copy EBV-infected Ig-null cells at levels associated with high load patients.

Ideally, we need to examine the frequency of Ig-null cells in the peripheral blood of normal immunocompetent children who have not received an organ transplant. We would then be able to determine the extent to which such cells are a normal constituent of the B cell repertoire or if they are an aberration connected to organ transplantation and thus the degree to which they are an aberration connected to organ transplantation the immune suppression associated with it. When developing the flow cytometry protocol for this specific aim, we examined the frequency of Ig-null cells in the peripheral blood of a normal immunocompetent adult. Although the frequency was very small, Ig-null CD19+ cells were present. It would be interesting to determine if the presence of Ig-null cells is consistent across all age groups, or if they increase or decrease with age. In any case, it is clear that without surface expression of

immunoglobulin, these CD19+ cells are circumventing the normal checkpoint controls over B cell ontogeny and must be receiving a survival signal or ignoring an apoptotic one.

This is the first examination of Ig-null cells in the peripheral blood. In other EBV-associated malignancies, B cells deficient in the expression of immunoglobulin have been detected within the tumor mass. In Hodgkin's lymphoma, tumors are characterized by Hodgkin-Reed Sternberg Cells (HRS), even though they typically encompass less than 1% of the tumor. Such cells are atypical B cells that often have nonsense mutations, stop codons, or other deleterious mutations in their Ig locus, preventing the expression of immunoglobulin on the cell surface (9). These cells are "crippled" germinal center cells and are receiving a survival signal to prevent their normally efficient elimination. It has been proposed that the survival signal is provided by EBV, as HRS cells express the Latency II pattern (6). In this case, LMP2A would replace the BCR signal, and LMP1 would replace CD40 stimulation. Further, after examination of HRS cells by serial analysis of gene expression (SAGE), it was found that these cells demonstrated a loss of B-lineage specific genes and (in some cases), the protein product (20). It is possible that the loss of B cell identity exhibited by these cells might also contribute to their survival in the absence of the normally required survival signals.

Additionally, such "crippled" (Ig-null) cells have been described in angioimmunoblastic lymphadenopathy with dysproteinemia (AILD). AILD is a frequent T cell lymphoma in the Western world (3), in which large numbers of EBV-infected B cells sometimes infiltrate the tumor. Upon examination of the immunoglobulin genes in these B cells micromanipulated from cases of AILD, it was shown that the EBV infected cells represented clones of an EBV infected memory or germinal center B cell. Further, these cells showed marked continued hypermutation, in the absence of the typical germinal center environment. Often times, these clones showed

destructive mutations in their originally functional variable gene rearrangements, which resulted in both the survival and clonal expansion of Ig-null B cells. In rare instances, the crippled B cells were even shown to be EBV negative. Clearly, the microenvironment associated with AILD, interplayed with EBV infection, enables an aberrant B cell to survive and clonally expand (3).

A third scenario in which an EBV-associated malignancy shows the presence of Ig-null cells is PTLD. Recent evidence (21) demonstrated that in a proportion of cases of PTLD, tumor development is associated with post-germinal center cells that have randomly mutated or sterile (crippled, Ig-null) heavy chain genotypes. Such cells have failed the germinal center process that selects naïve B cells into memory, but still survive in the periphery and become involved in the pathogenesis of PTLD. Because of the immunosuppression associated with organ transplantation, the ongoing somatic hypermutation sometimes observed in these cells is occurring in the absence of the normal physiological environment of germinal centers (CD4+ T cells and follicular dendritic cells) (4). Further, a flow cytometric evaluation of PTLD tumors showed a distinct lack of surface immunoglobulin light chains in 36% of the cases studied (8). Overall the presence of Ig-null cells in EBV-associated PTLDs suggests that they may be involved in the pathogenesis of this disease.

Ig-null cells have not been described in disease states not associated with EBV. This is the first study showing that they are present in disease-free individuals. While the patients studied here have elevated viral loads in their peripheral blood, they showed no signs or symptoms of PTLD or any other EBV-associated malignancy. With the presence of Ig-null cells with high copy numbers of EBV in their peripheral blood, they may have an elevated risk of developing an EBV-associated malignancy. In a previous study by our lab, RNA transcripts for

both LMP2a and LMP1 were detected in high load patients; transcripts for LMP2 only were detected in low load patients (16). With the preponderance of high copy, Ig-null cells surviving in the peripheral blood of high load patients, it is proposed that LMP1 and LMP2A are providing the necessary signals for their survival. In future experiments, this will be addressed.

4.4. PHENOTYPE OF IG-NULL CELLS

Because of the aberrant nature of Ig-null cells, we were interested in determining the status of other phenotypic characteristics that could describe their differentiation and activation states. The loss of B-cell identity associated with HRS cells in Hodgkin's Disease, led us to suspect that the Ig-null cells might have similar aberrations. We examined a variety of surface phenotypic markers typically associated with B cells in an effort to clarify this issue. It has been shown that latently infected B cells in the peripheral blood of immunocompetent (non-transplanted and non-diseased) individuals have the phenotype of resting memory B2 cells (1, 2, 15); we endeavored to determine if Ig-null cells in high load patients displayed a similar phenotype.

In addition to the lack of surface immunoglobulin, Ig-null cells had significant aberrations in their B cell marker phenotype. The typical Ig-null cell in a high load patient was CD19+, sIg-, CD5-, CD10-, CD38-, CD27-, CD23-, CD69-, and Ki67-, with variable expression of HLA Class I and II molecules, CD20 and CD40. Based on the expression of this set of markers, Ig-null cells appear to be inactivated (CD23 and CD69 negative), nonproliferative (Ki67-), B2 (CD5-), nongerminal center cells (CD10- and CD38-), and in addition did not display a marker for B cell memory (CD27-). Further, one third of Ig-null cells were HLA Class I negative; and another one third were HLA Class II negative. Without HLA molecules to

display antigenic peptides on their surface for recognition by antigen specific cytotoxic T cells, these cells would not be eliminated by the immune system. Approximately 40% of the Ig-null cells were also negative for surface expression of CD40 and would be unable to receive T-cell help through CD40-CD40L interactions. These cells are missing both the survival signals (functional Ig molecule, CD40:CD40L interaction) required by B cells to avoid apoptosis. Finally, half of the Ig-null cells examined had no surface expression of CD20. In a previously mentioned study of PTLD tumors examined by flow cytometry, one fourth of the tumors analyzed showed a complete lack of CD20 expression (8). CD20- B cells could be unfortunate for therapeutic reasons. PTLDs have been treated with rituximab, a monoclonal anti-CD20 antibody to purge the patient of B cells and coincidentally of EBV infected cells. CD20- B cells would not be eliminated by this treatment, and if the virus is residing in these cells, the chance for non-responsiveness to treatment and/or relapse is greater. To date, the response rate to rituximab is around 60-70% (5, 14). Our study suggests that if a PTLD is non-responsive to therapy, the patient should be examined for the presence of CD20- Ig-null cells in the peripheral blood, and treatment adjusted accordingly.

4.5. COMPARTMENTALIZATION OF VIRAL LOAD

To determine if a significant viral load is carried in Ig-null cells, they were purified from PBMC's. By sorting out the Ig-null cells (based on CD19 and pan-Ig expression), we were able to determine that a substantial portion of the elevated viral load in high load patients was carried in the Ig-null compartment. In some patients, it seemed that the bulk of the viral load was carried in this compartment. It should be noted that this compartment of B cells often makes up

as little as 0.01% of PBMC's. To find a large portion of the virus concentrated in this tiny compartment is interesting.

Because of the low frequency of expression of CD20 and HLA Class I on the surface of high copy cells, we wanted to determine if the viral load was further compartmentalized into the Ig-null/CD20 negative and or Ig-null/HLA Class I negative compartment. As these were extremely small populations in the peripheral blood, it was difficult to sort for these cells and obtain large numbers for study. Nevertheless, we did recover sufficient cells to support some preliminary findings. CD20 had a variable enrichment of viral load in the Ig-null/CD20 negative compartment. HLA Class I negative, Ig-null cells also showed varying enrichment in two patients. As cells that do not have expression of HLA Class I on their surface are normally efficiently eliminated by natural killer cells, it is unclear how the HLA Class I negative cells are surviving in the periphery. Notably, in some patients, viral load was concentrated into less than 0.01% of the peripheral blood mononuclear cells. In patients showing no signs of clinical disease, EBV survives in an aberrant B cell. Since these cells have many characteristics of malignant B cells, these patients might benefit from close monitoring of viral load in their peripheral blood in an effort to detect possible complications as early as possible.

4.6. PROPOSED ORIGIN OF IG-NULL CELLS

While firm conclusions cannot yet be made regarding the origin of Ig-null cells, a model can be proposed to explain the possible generation and persistence of Ig-null cells in immunosuppressed individuals (Figure 15). In this model, EBV infects naïve or memory B cells in the oropharynx. Both types of cells migrate to germinal centers and undergo clonal expansion and somatic

hypermutation. The cells that will eventually become Ig-null cells undergo random somatic mutation, with nonproductive rearrangements. Further, these potential Ig-null cells express the Latency II type profile (LMP1 and LMP2a). Low copy cells may also express this profile or develop in a manner similar to normal uninfected B cells. Because EBV is providing the two crucial survival signals necessary for the prevention of the apoptotic pathway, the infected cells can circumnavigate the light zone of the germinal center. Normally in this area of the germinal center, somatically mutated B cells interact with follicular dendritic cells and T cells to receive the signals to prevent apoptosis. Because LMP2a can provide the signal normally provided by an intact BCR and LMP1 can provide the signal normally provided by CD40:CD40L interaction, the infected cells do not need to receive these signals in the microenvironment of the light zone of the germinal center. Further, LMP1 and LMP2 are not highly immunogenic proteins; immunosuppressed patients have decreased CTL and thus the host is not as vigilant in eliminating virus-infected cells. This would allow the virus infected cells to persist in the peripheral blood of pediatric solid organ transplant recipients. Low copy cells emerge from the germinal center without isotype switching, but with the loss of IgD. These cells persist in the peripheral blood as resting memory B2 cells, as in a normal immunocompetent host. High copy Ig-null cells emerge from the germinal center, as well, but could continue to undergo somatic hypermutation and continue to express the latency II profile. Downregulation of B cell identiy markers may also be occurring in the peripheral blood in a gradual process.

An alternate explanation for the generation of Ig-null cells could involve B1 cells. These are the cells that follow the alternative pathway to B-cell development. There are several observations that could be construed to support the idea that Ig-null cells arise from B1 cells. PTLDs most often arise in extranodal sites; B1 cells are very rare in lymph nodes, but are the

predominant B cell population in the peritoneal cavity. Further, PTLD's are more common in pediatric transplants than in the adult transplanted population, and as B1 cells develop early in fetal development and then self-renew in the peripheral blood, they may be more common in children. B1 cells also express high levels of IgM, and are not known to express IgD or CD23. It is well-known that the EBV viral load in infected individuals is carried in the IgD- population, and it was shown in the present study that Ig-null cells are CD23 negative. Further, while B1 cells express CD5 in the peritoneal cavity, they lose surface expression of CD5 when they migrate to the periphery. We did not observe CD5 on the Ig-null cells in the peripheral blood of this study. While the developmental process of B1 cells is not as well characterized as B2 cells, it is not believed that they participate in germinal center reactions or develop into memory B cells as do B2 cells.

Based on this information, a model that poses Ig-null cells as B1 cells, is entirely plausible (Figure 16). When a susceptible transplant recipient is initially infected (in the same manner as a normal host), B1 cells may become directly infected, or they may become infected at some later point when normal latently infected cells (resting memory B2 cells) reactivate and infect bystander cells. Because B1 cells do not obey the rules of the more characterized B2 cells, EBV may undergo a burst of replication or clonal proliferation in the peripheral blood to obtain high copy numbers of the virus. Because B1 cells are polyreactive and self-renew in the peripheral blood, controls governing the positive selection process for a functional BCR might not be as strict as those governing the survival of B2 cells. It is not known whether B1 cells must receive the same 2 survival signals as B2 cells. B cells that have no surface expression of immunoglobulin might therefore be able to survive and avoid the apoptotic pathway more easily. Most low copy cells in high and low load carriers were IgM+, IgD-. This implies that most of

the low copy cells did not undergo isotype switching in the germinal center. As B1 cells are known to be IgM+, IgD-, low copy cells could be B1 cells that have not downregulated or heavily mutated their immunoglobulin genes. Experiments to further phenotype Ig-null cells as well as to determine the mutations that are preventing the expression of the immunoglobulin protein can help confirm or deny this model.

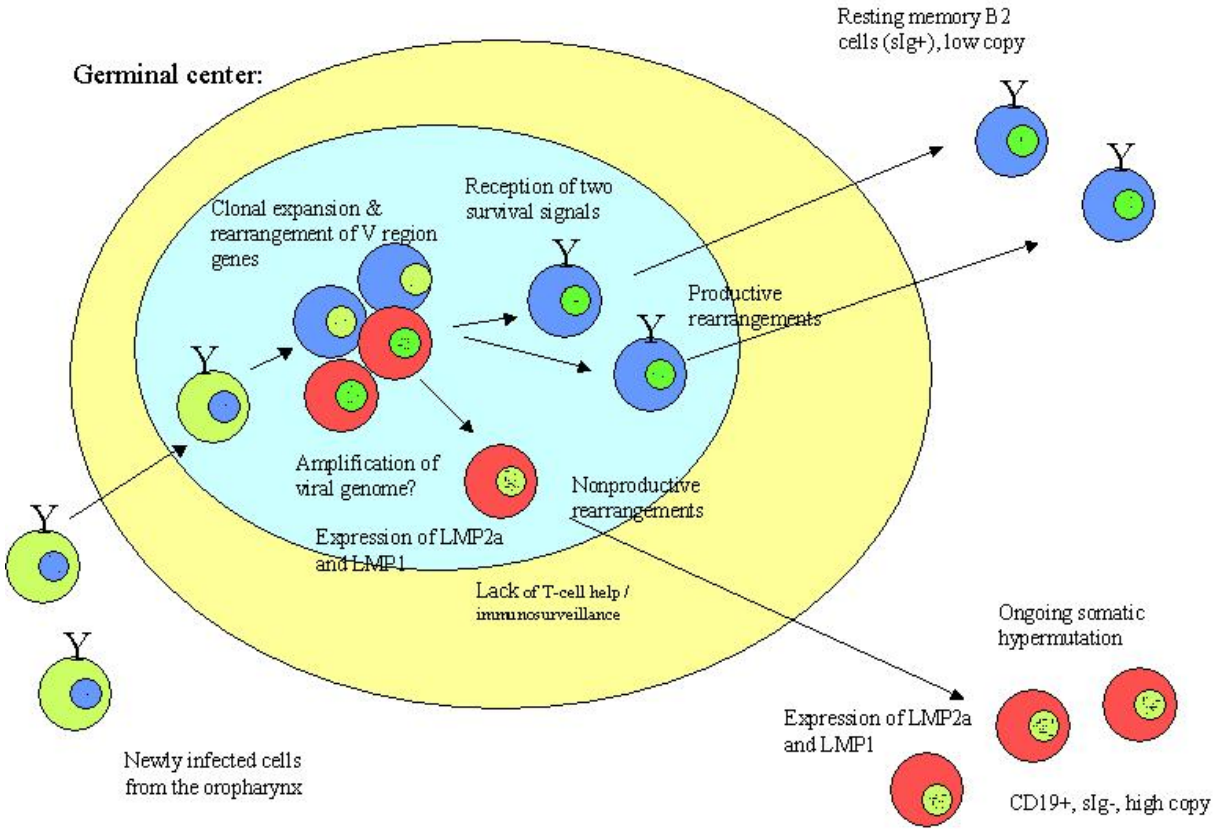

Figure 15. The proposed origin of Ig-null cells in the B2 model.

In this model, newly infected cells originating from the oropharynx enter the germinal center and begin to clonally expand and rearrange their V region genes. Some of these cells also undergo amplification of the viral genome, and have nonproductive rearrangements of their heavy chain genes. These cells are rescued by the expression of LMP2a and LMP1 and exit the germinal center to persist in the periphery as high copy cells. These cells may continue to undergo SHM, and may begin to lose the hallmarks of B-cell identity. Alternatively in the germinal center, cells may have productive arrangements of their heavy chain locus, and receive survival signals either through the accepted means (FDC and T-cell help), or by expression of LMP2a and LMP1. These cells exit the germinal center to persist as low copy cells, with the expression of IgM. In rare cases, isotype switching may occur and the low copy cells persist as IgG or IgA+ cells.

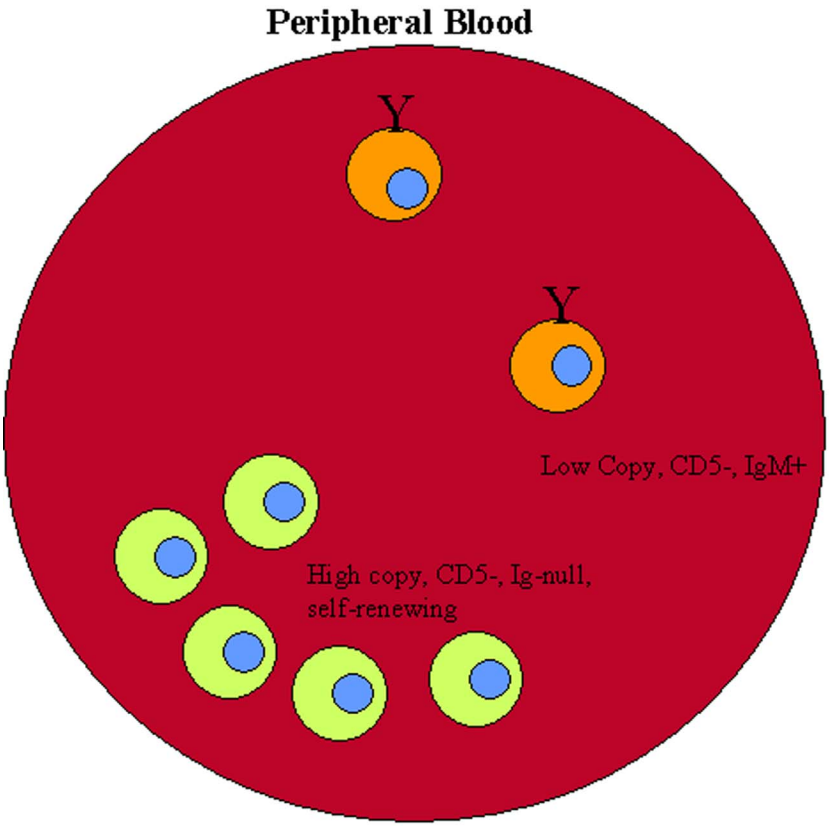

Figure 16. A model for Ig-null cells as B1 cells.

In this model, high copy cells originate as B1 cells. Upon infection, either directly or by bystanders, B1 cells either remain as IgM+, low copy cells with little or no activity, or begin to rapidly self-renew and amplify the viral genome to become high copy cells. Many of these cells undergo rearrangements of their heavy chain locus, although not during a germinal center reaction. Some of these rearrangements are non-productive. Survival signals may be provided by the LMP molecules, or alternatively, B1 cells may not require the same controls as B2 cells to survive in the periphery.

It is clear from the data presented here, along with evidence gathered from the presence of Ig-null cells in EBV-associated malignancies, that the relationship between the fate of the B cell and infection with EBV is complicated. The presence of additional factors, including immunosuppression complicates the picture further. It is unclear whether the Ig-null cell production preceded the development of a load composed of high copy number EBV genome carrying cells, or if B cells were infected before surface immunoglobulin expression was lost. Further studies will help clarify such issues. As Ig-null cells are cells that have lost one of two crucial survival signals and should not be present in the peripheral blood, these cells may ultimately be proven to be a hallmark for an increased risk of an EBV-driven complication following organ transplantation. Their elimination from the circulation of high load carriers would therefore be a great stride in decreasing the incidence of the morbidity and mortality that can be associated with transplantation.

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5. FUTURE DIRECTIONS

There has been much progress in the characterization of EBV-infected B cells in the peripheral blood of pediatric solid organ transplant recipients. Still, there is much room for growth, especially in light of the recent project in which aberrant cells were discovered and documented. Most of the future directions in which I see this project going involve being able to sort Ig-null cells from PBMC's with excellent purity. Fortunately this has been achieved. One big obstacle is cell number. With the volume of blood that so far has been obtained from Children's Hospital, we have been able to sort no more than 5,000 Ig-null cells from one sample at a time. For many of the proposed future experiments, this issue will need to be addressed.

The next logical step in this project is to determine what viral genes are expressed in Ig-null cells. It has been shown previously that low load patients have LMP2A transcripts, while high load patients demonstrate the presence of both LMP1 and LMP2A transcripts, by reverse-transcription PCR (RT-PCR) (2). It will be interesting to note if it is only the Ig-null cells in the peripheral blood of high load carriers that are expressing these transcripts or if they are also expressed by Ig positive cells. Ig-null cells are lacking at least one of the major survival signals necessary to avoid apoptosis, and in some cases, also lacking the second signal (CD40). These signals can be replaced by the LMP1 and LMP2A viral proteins. The first step would be to determine if these transcripts are present and whether the proteins are made. This project would involve sorting out both Ig-null and Ig-positive cells from PBMC's of high load patients, isolating the RNA, and performing RT-PCR.

It would also be advantageous to determine precisely what is preventing the expression of immunoglobulin on these cells. Sequence analysis of the rearrangements of the variable region genes in the heavy chain locus would shed light on the origin of the Ig-null cells and show

whether the null cell pool is mono-, oligo-, or polyclonal. Further, what types of somatic mutations are present and if they are crippling would also be determined. Examples of mutations that would lead to Ig-null cells include nonsense mutations, deleterious deletions, and stop codons in the coding region. To achieve this, single Ig-null cells would need to be isolated from high load carriers, either by laser capture microscopy or with the FACS Aria. The IgH sequences would be amplified from total genomic DNA with established primer combinations, with the PCR product cloned and sequenced. The changes from the germline in the IgH locus would then be analyzed to determine the ratio of the sum of replacement mutations to the sum of silent mutations (4). This ration determines if the cell is a normal antigen selected memory cell or if there is a non-antigen selected genotype. Also, the presence of deletions, stop codons, and nonsense mutations would be detected in this manner.

One assay that would provide beneficial information about these cells is a cDNA microarray, specifically the Affymetrix GeneChip human genome array. The level of expression of thousands of transcripts could be compared between Ig-null and Ig-positive cells. In such a manner, we would determine if the Ig-null cells have lost any of the B-lineage specific genes, as has been observed in the HRS cells of Hodgkin's Disease (3). In addition, the survival signals that are allowing Ig-null cells to avoid apoptosis might be identified in this manner. While this project would generate vast amounts of data, a large volume of blood from a high load carrier would need to be obtained in order to get the minimum cell number. One recent report suggests that a minimum starting number of 50,000 cells is needed to get reliable results with a microarray, with one round of RNA amplification (1). As we currently obtain about 3,000-4,000 Ig-null cells from each sample, a revision of the protocol is needed. Some ideas include pooling of samples from the one patient with blood obtained at different time points, obtaining a pint of

blood off one high load patient, and generation of an Ig-null cell line. Each of these has its own problems; pooling of samples would need to take into consideration any changes in the health status or medication of the patient. Further, obtaining a pint of blood from one high load patient would require special permission and consideration of the patient's health status. Finally, generation of an Ig-null cell line (see below) might induce changes in the gene expression of the Ig-null cells.

Outgrowth experiments in which cell lines from Ig-null cells are generated could solve the issue of low cell numbers. We suspect that trial and error would be necessary to encourage these cells to proliferate in culture. If such cell lines were established, a number of potential experiments could be proposed. In situ hybridization and immunofluorescense to confirm both the phenotype and high copy status of Ig-null cells would be one easily conducted experiment.

It is clear that with the discovery of Ig-null cells in the peripheral blood of pediatric solid organ transplant recipients, many more questions are generated. While they are beyond the scope of the current project, the potential for growth in this area is boundless. More information about Ig-null cells in transplant recipients could lead to advances in the prevention and treatment of EBV-associated PTLDs.

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APPENDIX A: List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
AILD	Angioimmunoblastic Lymphadenopathy with Dysproteinemia
ASM	American Society of Microbiology
BCR	B-cell receptor
BL	Burkitt's Lymphoma
C3d	complement protein C3 degradation fragment d
CD	cluster of differentiation
CHP	Children's Hospital of Pittsburgh
CMV	Cytomegalovirus
CNS	central nervous system
CTL	cytotoxic T lymphocyte
DIG	digoxigenin
DNA	deoxyribonucleic acid
DS	dyad symmetry element
dUTP	deoxyuridine triphosphate
EA	early antigen
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen - leader protein
EBV	Epstein-Barr Virus
EDTA	ethylenediaminetetra-acetic-acid
FACS	fluorescence activated cell sorting

FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FR	family of repeats
gp	glycoprotein
GVHD	Graft versus Host Disease
HCC	high copy cells
HD	Hodgkin's Disease
HHV4	human herpesvirus 4
HIV	Human immunodeficiency virus
HLA	human leukocyte antigen
HLC	high load carrier
HRS	Hodgkin and Reed Sternberg cells
HSCT	hematopoietic stem cell transplant
IF	immunofluorescence
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis
IR	internal repeat
kb	kilobase
kd	kilodalton
LCC	low copy cells
LCL	lymphoblastoid cell line
LCV	lymphocryptovirus

LLC	low load carrier
LMP	latent membrane protein
MAC	magnetic activated cell sorting
MoFLo	modular flow high performance cell sorter
ND	non-detectable
NK	natural killer cell
NPC	Nasopharyngeal carcinoma
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PEL	primary effusion lymphoma
PTLD	post-transplant lymphoproliferative disease
QC-PCR	quantitative-competitive polymerase chain reaction
RDV	rhadinovirus
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
SAGE	serial analysis of gene expression
sIg	surface immunoglobulin
SOT	solid organ transplantation
TR	terminal repeat
VCA	viral capsid antigen
XLP	X-linked lymphoproliferative disease

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