CD8 T LYMPHOCYTE RESPONSES TO HUMAN HERPESVIRUS 8 LYTIC AND LATENCY PROTEINS

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Human herpesvirus 8 (HHV-8) is a lymphotropic gammaherpesvirus that causes Kaposi's sarcoma (KS), a vascular tumor of spindle-shaped endothelial cells. The importance of determining effective prevention and treatment for HHV-8 infection is evident in that KS continues to be the most common cancer among HIV-1 and AIDS patients. It is postulated that $CD8^+$ T cell responses play an important role in controlling HHV-8 infection and preventing KS. However, there are minimal data supporting the role for T cell immunity in control of HHV-8 infection. Therefore, I investigated $CD8^+$ T cell responses to HHV-8 lytic and latency proteins.

I used our dendritic cell-based system in an IFN-γ ELISPOT to identify novel epitopes in healthy, HHV-8 seropositive individuals, then further assessed new epitopes for immune mediators using polychromatic flow cytometry. I also used multimer complexes to directly detect HHV-8-specific $CD8^+$ T cells in blood. I investigated the effect of regulatory T cells (Treg) on these anti-HHV-8 T cell responses by depleting samples of Treg. I then applied these assays to patients in the Multicenter AIDS Cohort Study to longitudinally investigate the role of these responses during the progression to KS.

Through these studies, I identified 10 novel HLA A*0201-restricted epitopes, which activated both monofunctional and polyfunctional $CD8^+$ T cells producing various combinations of immune mediators. Responses were lower over many years prior to development of KS. Although $CD8^+$ T cell IFN-γ responses were modest, a low but consistent percent of HHV-8-

specific $CD8^+$ T cells were present in blood, suggesting a functional down-regulation of this response. In support of this, removal of Treg enhanced $CD8^+$ T cell responses to HHV-8 epitopes, and numbers of Treg increased prior to KS development.

Overall, these data support that T cell responses, in frequency, magnitude and quality, are essential for the control of HHV-8 infection to prevent disease development. They also indicate that these antiviral T cell responses are in part controlled by Treg. Involvement of $CD8^+$ T cells and Treg in control of HHV-8 infection has public health significance through implications for understanding the immunopathogenesis of HHV-8 needed for prevention and treatment of KS.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

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1.0 HUMAN HERPESVIRUS 8 INTRODUCTION

Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma associated herpesvirus (KSHV), is the etiologic agent of Kaposi's sarcoma (KS) (42), a neoplasm of endothelial origin that occurs in four distinct epidemiologic forms (57, 113): classic or Mediterranean KS, epidemic or acquired immune deficiency syndrome (AIDS)-related KS, endemic or African KS, and iatrogenic or organ transplant-associated KS. KS is the most common cancer associated with human immunodeficiency virus 1 (HIV-1) infection and AIDS (48). Although the incidence of KS in HIV-1 infected persons declined with the advent of antiretroviral therapy (ART) (81), KS can occur in persons on ART with suppressed HIV-1 infection (159). The success of ART in treating HIV-1 associated KS has been countered by the occasional occurrence of an immune reconstitution inflammatory syndrome (73). This is a severe, temporary enhancement of KS lesions due to an increase in inflammation and immunologic recovery after ART.

The discovery of HHV-8 and its causal role in KS development opened the potential for prophylaxis and treatment of the infection and cancer with antiviral drugs, and prevention of both with a vaccine. Strategies to achieve these ends require an intimate knowledge of the pathogenesis and immune control of HHV-8 infection. It is likely that host control of HHV-8 infection and development of KS is linked to T cell interactions with HHV-8 infected, professional antigen presenting cells (APC), i.e., dendritic cells (DC), monocytes/macrophages and B lymphocytes. Similarly, APC-T interactions are likely to be centrally involved in the

HHV-8 associated B cell neoplasms multicentric Castleman's Disease (MCD) (218) and primary effusion lymphoma (PEL) (35, 171).

HHV-8 has been reported to be transmitted to common marmosets and cause persistent infection with rare, KS-like skin lesions (41). However, there is as yet no consensus that this or other simian models (74, 115, 203) recapitulate human HHV-8 infection and development of KS or other cancers associated with this herpesvirus. Thus, although *in vitro* models are suspect to lacking certain *in vivo* characteristics, they are currently the best model of HHV-8 infection.

1.1 HHV-8 INFECTION OF PROFESSIONAL APC

As with the other human gammaherpesvirus, Epstein Barr virus (EBV) (179), HHV-8 targets APC both *in vivo* and *in vitro*. Indeed, the primary tropism of B cells by these gammaherpesviruses is uncommon among human virus infections. This sets the stage for development of their associated cancers both indirectly through alteration of host immunity dependent on APC function, and directly via neoplastic effects of the virus. HHV-8 is found in KS spindle cells, which are of mixed vascular and lymphatic endothelial cell and macrophage origin, monocytes that are found in proximity to KS lesions, and circulating B cells of KS patients (24, 30, 166, 194). In PEL, HHV-8 is found in immunoblastic cells expressing plasma cell markers, and in plasmablastic cells of a less terminally differentiated state in MCD (62). The intimate association of HHV-8 with such professional APC in the KS lesion and in other HHV-8 associated cancers suggests a major role for virus-APC interplay. Moreover, anti-HHV-8 T cell immunity, that presumably is critically dependent on such virus-APC interactions, is present in HIV-1 infected and uninfected persons who are seropositive for HHV-8 (202).

Achieving a better understanding of the role of HHV-8 in inducing associated cancers could greatly benefit from a yet-to-be-developed *in vitro* model of primary HHV-8 infection of a natural target cell that consistently reflects virus lytic, latent and reactivation infections. HHV-8 infection of APC could provide such a model.

1.1.1 HHV-8 receptors on APC

Infection of APC *in vitro* reveals different cycles of HHV-8 replication that are likely to relate to the pathogenesis of the virus. The first step in targeting and alteration of APC by HHV-8 is at the level of cell receptor. Herpesviruses use more than one receptor to infect the same cell (100). Use of these receptors by herpesviruses is hierarchical based largely on differential expression of the receptors in specific cell types and states of cell activation. Extensive *in vitro* evidence indicates that the ubiquitous cell surface proteoglycan heparan sulfate serves as an initial binding receptor for HHV-8 on endothelial cells and fibroblasts as well as APC (4, 5, 39, 119). Multiple integrins are subsequently involved in HHV-8 binding and entry (119). A third level of differential selection that has been identified from *in vitro* studies of the three major types of APC, i.e., monocyte-derived DC (MDDC), B cells and monocyte-derived macrophages (MDM), is the type II C-type lectin, DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN; CD209) (191, 192). A new entry receptor for HHV-8 on endothelial and epithelial cells (95), i.e., ephrin receptor tyrosine kinase A2, a tyrosine kinase that functions in neovascularization and oncogenesis, has not yet been assessed in HHV-8 infection of APC.

The relative contribution of each level of HHV-8 binding to viral infection of APC is not clear. For example, the Raji B lymphoblastoid cell line (LCL) and the myeloblastoid K562 cell line constitutively express little or no DC-SIGN or $\alpha 3\beta 1$ integrin (192). Thus, these cell lines do

not support detectable production of infectious virions (13, 23, 192). However, transfection of the cell lines with DC-SIGN renders them highly permissive for HHV-8 infection as measured by the production of viral proteins and DNA (192). Moreover, infection of these cell lines can be blocked by anti-DC-SIGN monoclonal antibody (mAb), soluble DC-SIGN and mannan, a natural ligand of DC-SIGN. Interestingly, six B cell and T cell lines (BLAB, Ramos, BCBL-1, JSC1, Jurkat and SupT1) were susceptible to infection through cell-mediated transmission with a doxycycline (Dox)- inducible cell line harboring recombinant HHV-8 (rKSHV.219), indicating that viral entry can be achieved despite lack of expression of a major HHV-8 receptor (170). There is also evidence that HHV-8 can infect $CD34^+$ stem cell precursors of DC *in vitro* by as yet undefined receptors (101, 131). It is likely that there are less prominent alternative receptors for HHV-8 that account for a small percentage of DC-SIGN negative APC and cell lines that can be infected by this virus.

1.1.2 B cell infection with HHV-8

Suggestive evidence that HHV-8 is B-cell tropic *in vivo* is that HHV-8 DNA is detected in B cells from patients with KS lesions (6) and some HIV-1/HHV-8 co-infected individuals (156). It is likely that this is related to DC-SIGN expression that is enhanced by an activated state in B cells. In support of this, previous work from our laboratory showed that once blood-derived B cells are activated to express DC-SIGN, HHV-8 can effectively establish infection and elicit full-cycle production of infectious virions in these cells (191). The fact that HHV-8 cannot infect Raji LCL and the K562 erythroleukemia cell line expressing DC-SIGN that lacks the transmembrane domain supports DC-SIGN-mediated endocytosis of viral entry. Moreover, infection can be blocked by pretreatment of the B cells with anti-DC-SIGN mAb or mannan, but

not an antibody specific for the amino acid transporter protein xCT (191). HHV-8 has been reported to use xCT for infection of surface adherent human cells (116), and in a post-entry stage of human endothelial cell infection as part of a complex of heterodimeric membrane glycoprotein CD98 and the α3β1 and αVβ3 integrins (239). Notably, HHV-8 infection is not restricted to blood-derived B cells, as tonsillar B cells constitutively express DC-SIGN and can be lytically infected with the virus *in vitro* (168, 191). It is probable that B cells in such tonsillar tissue are in an endogenously activated state resulting in enhanced expression of DC-SIGN.

HHV-8 infection of freshly derived blood and tissue B lymphocytes could provide an *in vitro* model for assessing HHV-8 lytic and latent infection. Our *in vitro* model for measuring HHV-8 infectivity and replication supports the concept previously put forth that DC-SIGN is a major receptor for this virus (39, 191, 192). This adds to the wealth of evidence that shows that in addition to certain integrins (3-5, 21, 239), DC-SIGN is required for highly efficient infection of the natural APC targets with HHV-8, which is in contrast to previous reports (84). However, there is still need for improved reliable, quantitative measures of HHV-8 replication to better define B cell infection with HHV-8. These measures should include combinations of real-time polymerase chain reaction (PCR) assays for cell-associated and non-cell associated copy numbers of HHV-8 encapsidated DNA, flow cytometry assays for the enumerating the number of mAb-stained cells expressing viral lytic and latency cycle proteins, and most important, cell culture-based assays, e.g., a 50% tissue culture infectious dose assay, for quantitating the number of infectious virus particles.

It is postulated that HHV-8 infection drives B cells to an early plasmablast-like state in MCD and a pre-terminal plasma cell stage of differentiation in PEL (2, 35-37, 63, 64, 80, 98, 123, 157, 164, 172). Hassman *et al.*, recently showed that latency associated nuclear antigen

(LANA, encoded by ORF73)-positive B cells express immunoglobulin (Ig) M and the λ light chain at 2.5-3.5 days post-HHV-8 infection. These cells are plasmablast-like with increased interleukin (IL)-6R expression and increased proliferative response to IL-6, with 7-36% expressing CD27 (98). This molecule is a member of the tumor necrosis factor (TNF)-receptor superfamily, and is involved in regulation of B cell activation. It is not known whether HHV-8 directly infects these IgM^+ memory B cells or a precursor of these cells. Also, there are no data on which subset of B cells supports a complete lytic cycle of replication with virion formation and death of the cell, or if this is abortive, leaving HHV-8 infected memory B cells that survive and maintain latent virus infection. It is possible that the infection of naïve and IgM^+ memory B cells leads to establishment of latency in a portion of cells, resulting in virus-driven plasmablast differentiation, while some cells support the viral lytic cycle. Activated B cells may support full lytic cycle replication, resulting in virion formation and cell lysis, or HHV-8 could abort the cycle prematurely and either enter latency or result in cell apoptosis.

Such definitive B cell targets for primary infection and lytic replication could be useful in studies of HHV-8 prophylactic and therapeutic vaccines. Currently, the main *in vitro* models to recapitulate HHV-8 infection in APC are cell lines persistently infected with the virus, particularly body cavity based lymphoma cells (BCBL)-1, a B cell line derived from PEL, which is latently infected with HHV-8 and EBV negative (35, 42). In such cell line models, HHV-8 lytic and latent infections cannot be defined conventionally starting with the total absence of infectious viral particles, as there is always a low level of persistent virus production. However, latency can be disrupted, triggering the lytic cascade of viral replication and lytic genes expressed sequentially as immediate early genes, early genes and late genes, resulting in production of encapsidated virions. Such lytic viral replication is largely irreversible (39, 247).

HHV-8 lytic gene profiling in these models has been extensively accomplished using tiling microarray (43), DNA microarray (2, 40, 147), and high-throughput real-time PCR (60, 71). However, most studies on latency-lytic reactivation of HHV-8 use various chemicals to induce viral replication (169). The question is whether such reactivation reflects natural HHV-8 viral lytic reactivation from latency, since chemical agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) have pleiotropic effects on host cell signaling and chromatin structure. How they affect cell signaling pathways is also unknown. Thus, the natural reactivated cascade of lytic transcripts of HHV-8 still waits to be revealed.

Instead of using chemical inducers, Nakamura *et al.* (173) developed an engineered BCBL-1 cell line that inducibly expresses the replication transactivator protein, RTA, encoded by open reading frame (ORF) 50, i.e., TREx-BCBL1-RTA. RTA has been shown to be necessary and sufficient for the switch between HHV-8 latency and lytic replication (59). In fact, mutation of the RBP-Jk sites within the RTA promoter is enough to enhance latency in transformed-293 cells and peripheral blood mononuclear cells (PBMC) (150). In the TREx-BCBL1-RTA cell line, RTA expression is under the control of a Dox-inducible promoter and treatment of TREx-BCBL1-RTA cells with Dox results in expression of RTA, which in turn induces viral replication (173). While the role of RTA in causing a switch from latency to viral replication has been demonstrated by several laboratories, the mechanisms regulating coordinate induction of expression of most of the HHV-8 lytic genes during this reactivation have not been evaluated in a systematic fashion.

Transcription of HHV-8 lytic genes occurs during either a primary infection of susceptible cells or during reactivation of latently infected cells. The question remains whether the kinetic gene activation in a chemically induced cell line (BCBL-1) or the naturally targeted

RTA (TREx-BCBL1-RTA) will reflect the cascade events of a natural infection of B cells or other APC. To identify the true gene transcription and reactivation events in HHV-8 infection, primary cells susceptible to HHV-8 should be used. Only then can the observations from TPA induced BCBL-1 and Dox induced TREx-BCBL1-RTA cell lines be validated.

1.1.3 Monocyte/macrophage infection with HHV-8

Macrophages in several body compartments naturally express DC-SIGN (91, 117), as well as integrins including α3β1 (7), which presumably renders them susceptible to HHV-8 infection. An early report showed that MDM from normal donors that are stimulated *in vitro* with allogeneic PBMC can be infected by HHV-8, but this rarely results in complete, lytic replication (23). In addition, treatment of blood monocytes from KS patients with proinflammatory cytokines *in vitro* results in HHV-8 persistence (166).

MDM become susceptible to HHV-8 infection *in vitro* after activation with IL-13, which results in enhanced DC-SIGN expression (192). IL-13 is an anti-inflammatory, T helper (Th) 2 cytokine that promotes differentiation of B cells into antibody-secreting plasma cells. Importantly, non-IL-13 activated MDM express α3β1 integrin yet are not infected by HHV-8 *in vitro*, supporting the requirement of multiple receptors for efficient infection of APC by HHV-8. Indeed, when DC-SIGN is blocked in IL-13-activated MDM or the monocytic cell line THP-1, HHV-8 can still bind using heparan sulfate but virus entry is reduced (119). HHV-8 establishes productive infection in THP-1 cells with an ordered expression of latency gene ORF73 and lytic gene ORF50. In fact, the HHV-8 genome was reported to persist for 30 days in these cells (119). Such limited expression of lytic genes together with the persistence of latency genes is believed to be unique for HHV-8 (124).

Of interest is that ORF K14 of HHV-8 encodes a surface glycoprotein vOX2 that is homologous to cellular OX2 (46), which inhibits macrophage function (78). The vOX2 glycoprotein could be central to HHV-8 immunopathogenesis in that it stimulates production of inflammatory cytokines IL-1β, IL-6, monocyte chemoattractant protein 1 (MCP-1), and TNF-α in primary monocytes, MDM and MDDC (46). Furthermore, expression of vOX2 on B cells stimulates monocytes to produce inflammatory cytokines. MDM transfected with vOX2 produce inflammatory cytokines and have enhanced phagocytic activity, while inhibiting the immunomodulatory effects of interferon gamma (IFN-γ) and down-regulating major histocompatibility complex (MHC) class I and class II expression on macrophages (206). It was recently reported that vOX2-transfected APC co-cultured with T cells results in suppressed IFN-γ production and mobilization of the cytolytic granule marker CD107a through inhibition of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (165).

1.1.4 HHV-8 infection of DC

Evidence of infection of human DC *in vivo* with HHV-8 has been limited (181, 196). However, there is no *a priori* reason why human DC should not take up HHV-8 and support at least abortive infection *in vivo*. When MDDC are infected *in vitro* with HHV-8, viral lytic proteins are produced with little viral DNA production (192), similar to abortive HHV-8 infection of vascular endothelial cells (4, 175, 189, 195, 241). Although HHV-8 infection does not significantly alter MDDC viability, it decreases MDDC function, i.e., lowers their capacity to activate antigen-specific $CD8^+$ T cell responses. Moreover, HHV-8 infected MDDC have impaired antigen uptake, with a significant decrease in endocytic capacity and DC-SIGN expression within 24 hours after infection. DC-SIGN internalization in MDDC is associated

with lytic HHV-8 gene expression (192). In addition to MDDC, HHV-8 *in vitro* infection of IL-13-treated MDM results in a loss of DC-SIGN surface expression, suggesting that HHV-8 binding to DC-SIGN triggers internalization. Hence, alteration of DC-SIGN expression could be a strategy used by HHV-8 to escape immune defenses and lead it to a non-robust immune response (244).

The skin and mucosa contain two major types of DC, Langerhans cells (LC) which reside in the epidermis in close contact with keratinocytes, and interstitial-dermal DC (iDDC) resident in the dermis and mucosal layers. LC and iDDC process cutaneous antigens and migrate to draining lymph nodes to present antigens to T and B cells. Because of the strategic position of LC and iDDC and their ability to capture pathogens, these cells could represent potential targets for HHV-8 infection. Furthermore, due to the expression of the C-type lectins, i.e., langerin (CD207) and DC-SIGN, on LC and iDDC, respectively, it is tempting to speculate that HHV-8 could utilize the same entry mechanisms as seen in MDDC (192). LC and iDDC can be generated from pluripotent cord blood $CD34^+$ cells (34) that could prove to be valuable tools to study HHV-8 infection and subsequent antigen process and presentation to T cells (50).

Plasmacytoid DC (pDC) are a lymphoid-lineage subset of APC that produce extraordinary amounts of the antiviral protein IFN-α in response to virus infection (145). DC-SIGN is not expressed by pDC, yet HHV-8 can infect human pDCs, upregulate expression of the activation molecule CD83 and T cell co-receptor CD86, and induce production of IFN-α (249). Induction of IFN-α by HHV-8 occurs through activation of Toll-like receptor 9 (TLR9) signaling in pDC.

1.1.5 HHV-8 infection and TLRs

Several types of TLR expressed on different APC are emerging as important factors in the innate and adaptive immune response to HHV-8. Notably, triggering of C-type lectins, including DC-SIGN, in combination with TLR triggering on DC induces signaling and cytokine responses, which in turn regulate T cell polarization that is central to host immune control of infections (238). In addition, TLR have also been implicated in reactivation of HHV-8. TLR 7/8 could control reactivation of HHV-8 from latency in B cells, as demonstrated by agonists specific for TLR7/8 reactivating latent HHV-8 and inducing viral lytic gene transcription and replication in latently infected PEL cell lines of B cell origin (92). This has important implications for host control of HHV-8 infection, as signaling through the TLR1/2/6 complex, TLR7, TLR9 and TLR10 affects multiple stages of B cell activation, proliferation, cytokine secretion, terminal differentiation and antibody secretion in response to T cell-dependent antigens (14).

1.2 CYTOKINES AND CHEMOKINES IN HHV-8 INFECTION

Cytokines and chemokines produced by inflammatory APC, as well as T cells, play a crucial role in HHV-8 replication and development of KS. Inflammatory changes occur early in KS, prior to the detection of the cancer (162). Proinflammatory processes drive early-stage KS to develop into mature, spindle cell lesions (197). Thus, KS tumors are comprised of spindle shaped cells of endothelial origin (194) in an environment rich in inflammatory cell infiltrates, including B cells, macrophages, monocytes and $CD8^+$ T cells (166). The infiltrating cells produce large amounts of Th1 polarizing, proinflammatory cytokines (e.g., IFN-γ, IL-1β, TNF-α and IL-6), chemokines

(e.g., IL-8), and growth factors (e.g., vascular endothelial growth factor [VEGF]), which can induce the KS-like phenotype observed in activated endothelial cells (70, 75, 166). IFN-γ is the earliest and most abundant inflammatory cytokine observed in KS (75) and can be detected in KS lesions before evidence of HHV-8 DNA (166). IL-6 is also found at very high levels in both KS lesions and in circulation of patients with MCD (6). In MCD, IL-6 induces B cell proliferation and causes inflammatory clinical symptoms (210). Observations from a transgenic mouse model demonstrate that mice expressing viral IL-6 but lacking mammalian IL-6 do not experience phenotypic changes (e.g., lymphoadenopathy, hypergammaglobulinemia, splenomagaly) associated with MCD (230). IL-6, as well as oncostatin M and IL-10, are also detected at high levels in PEL cells. Proliferation of PEL can be inhibited when receptors for the IL-6 pathway are blocked (61). Thus, an as yet minimally detailed imbalance in the Th1-Th2 milieu during HHV-8 infection appears to be closely linked to APC in driving the outgrowth of KS endothelial cells as well as PEL and MCD B cells.

Other cytokines and chemokines produced by APC, particularly IL-8 and MCP-1, are elevated in serum of KS patients and have been implicated in many cancers (160, 228). When bound to its CCR2 receptor on endothelial cells, MCP-1 results in chemotaxis and has been shown to mediate angiogenesis *in vitro* (83, 160). KS tumors are highly vascularized with abnormal angiogenesis, leading to enhanced blood flow to the tumor by expanding pre-existing blood vessels (162). IL-1β, TNF-α, IL-8 and IL-6 can also enhance tumor cell growth and vascularization (68, 69, 75) by inducing the expression of two angiogenic mediators, i.e., VEGF and fibroblastic growth factor (FGF) (49, 51, 68, 166). In addition to angiogenesis, inflammatory cells and cytokines can contribute to viral reactivation and replication. IFN-γ was shown to induce ORF59 expression in BCBL-1 (22) and reactivate latent HHV-8 in BC-3 PEL

cells by activation of Pim-family kinases (44). Mercader *et al.* showed oncostatin M, IFN-γ, and hepatocyte growth factor (HGF)/ scatter factor (SF) induced lytic cycle activation of BCBL-1 resulting in virion production (161). This principle has been demonstrated in HHV-8 infected PBMC, where inflammatory cytokines could maintain or increase viral load up to 10-fold higher when the infected cells were cultured in the presence of inflammatory cytokines (166).

In addition to cellular cytokines and chemokines, HHV-8 encodes several proteins involved in inflammation and angiogenesis that contribute to the inflammatory environment observed in KS. Cytokine and chemokine mimics encoded by HHV-8 have been the focus of numerous studies and reviews (86, 133, 162, 176, 205, 216). Thus, vIL-6 has 24% homology to human IL-6 and can induce expression of VEGF and MCP-1 (177). These in turn trigger angiogenic pathways. Elevated levels of vIL-6, as well as levels of human IL-6 and HHV-8 viral load, have been associated with a recently described syndrome of severe systemic inflammatory symptoms (237). The viral G-protein coupled receptor (vGPCR) is an early lytic phase gene homologous to the IL-8 receptor, CXCR-2 (10, 176). vGPCR constitutively signals and results in enhanced production of IL-1β, IL-8, MCP-1, IL-6 and VEGF that can have both autocrine and paracrine effects (87, 212). K1 and K15 are signal transducing proteins that induce VEGF, IL-6 and IL-8 (45). LANA and the viral FLICE [FADD-like interferon converting enzyme] inhibitory protein (vFLIP) have been linked to enhanced cytokine production via activation of the mitogen activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways, respectively (243). Viral interferon regulatory factor 3 (vIRF3) expression inhibits MHC class II expression as well as IFN-γ production (208). Finally, the viral macrophage inflammatory proteins (MIPs) (viral chemokine ligand 1 [vCCL1], vCCL2, vCCL3) share homology to MIP-1α and CCL5 and can induce monocyte chemotaxis

and signal transduction (10, 174, 176). Given the plethora of such data derived from highly manipulated molecular and cell line models, the challenge is to link these unique HHV-8 factors directly to HHV-8 infection and development of cancers in natural targets of the virus.

In epidemic or AIDS-related KS, the immune dysregulation and induction of inflammatory cytokines acts to further enhance KS tumor growth. When BCBL-1 cells that are latently infected with HHV-8 are cultured with HIV-1-infected $CD4^+$ T cells, soluble factors secreted by the T cells cause the virus to enter lytic reactivation (161). Inflammatory cytokines induced by both HIV-1-infected and HHV-8-infected cells promote expression of receptors for HIV-1 Tat, which acts as a progression factor in KS development (12, 67) and increases viral load (97). Indeed, serum and cell samples taken from KS lesions of HIV-1 infected individuals co-infected with HHV-8 show markedly increased levels of inflammatory cytokines, growth factors and angiogenic mediators (69, 186). Furthermore, treatment of KS patients with IFN-γ, IL-2 and TNF causes KS progression (166).

HHV-8 has a broad cellular tropism *in vivo* including B cells, endothelial cells, monocytes, keratinocytes and epithelial cells that could result in production of inflammatory mediators (38). In monocytes, production of interferon gamma-induced protein (IP)-10, IFN-β1, MCP-1 and IRF-1 occurs in conjunction with an upregulation of TLR3 expression (248). Our laboratory has previously demonstrated that *in vitro* HHV-8 infection of MDDC, which do not support lytic virus replication, secrete IL-6, TNF-α, IP-10, MIP-1α and MIP-1β (102). While IL-12p40 expression increases post-infection, bioactive IL-12p70 is not detected in HHV-8 infected MDDC. This suggests a virus-related inhibition of constitutive production of IL-12p35, or a defect in the complexing of these subunits into IL-12p70. Furthermore, the results support an

intentional skewing of cytokine production in HHV-8-infected MDDC towards induction of a Th2 response that could enhance development of KS.

It is possible that upon exposure of B cells to HHV-8, the virus binds to and activates cell surface receptors resulting in cytokine and chemokine production that is enhanced by HHV-8 entry and subsequent lytic replication. The cytokine and chemokine profiles in HHV-8-infected B cells are similar to the cytokine dysregulation observed in EBV-associated disease (88, 89, 122, 125). Elevated levels of IL-1β, TNF-α, IL-6, IL-8 and IL-10 are detected in the serum of patients with EBV-associated diseases, while a less favorable outcome correlates with increases of IL-6 and IL-10 in Hodgkins lymphoma (72). Common strategies between EBV and HHV-8, such as NF-κB signaling pathway alterations (56, 99) and the expression of virokines (216), imply that an imbalance of immune mediators is associated with the oncogenesis of these gammaherpesviruses.

1.3 APC-T CELL INTERACTIONS IN HHV-8 INFECTION

Little is known regarding HHV-8-specific T cell-APC interactions and their role in controlling viral infection and disease. A key challenge is to adapt *in vitro* models using cell lines and HHV-8 constructs to systems that allow deciphering of the basic steps of natural HHV-8 infection, and antigen processing and presentation, in various types of APC. The interactions of APC with T cells that underlie the generation of anti-HHV-8 T cell immunity begin with DC of myeloid origin that take up viral antigen at local sites of infection, then travel to the draining lymphatics and induce antiviral T cell responses (236). There are specialized subsets of DC that populate different tissue sites and have distinct virologic interactions and immunologic functions.

Myeloid-derived LC and dermal DC populate the epidermis and dermis respectively, and are associated with KS lesions. Interstitial or interdigitating tissue DC are similar in phenotype and function to dermal DC, and are linked to systemic KS lesions. Other DC subsets such as $CD141^+$ DC, which are the human surrogates of mouse CD8α DC subsets (11, 114), could be natural targets for HHV-8. It is imperative to assess transcription of HHV-8 ORFs in natural targets of the virus, in comparison to well documented immunomodulatory properties of HHV-8 expressed in cell lines and artificial constructs, such as persistently infected BCBL-1 and its variants (52).

Interactions of HHV-8 with DC subsets could be critical at the site of virus replication, and be centrally involved in generating T cell responses to the virus. Efficient activation of HHV-8 epitope-specific $CD8^+$ T cells requires presentation by peptide-loaded, autologous, mature MDDC (244). This is similar to optimal activation of anti-EBV cytotoxic T lymphocyte (CTL) by peptide-loaded DC (143, 193, 224-227, 250). Other studies have revealed polyfunctional $CD8^+$ and $CD4^+$ T cell reactivity and new MHC class I epitopes for HIV-1 Gag and Nef using peptide-loaded DC (108). Importantly, our laboratory has used this DC model to map epitopes of HHV-8 lytic and latency proteins with libraries of synthetic, 15mer peptides overlapping by 11 amino acids (aa) (135). Nevertheless, it may be more practical to generate large numbers of CD40 ligand (CD40L)-activated, autologous B cells that favourably compare to DC as APC (211).

To date, relatively few $CD8^+$ and $CD4^+$ T cell epitopes within only 15 of the over 80 ORFs of HHV-8 have been identified, and most of these are restricted by human leukocyte antigen (HLA) A*0201 (202). Information is therefore needed on the broad range of potential antigenic sites in the virus that are restricted by other MHC class I and II haplotypes. Moreover,

no studies have yet established a hierarchy of naïve and memory $CD8^+$ or $CD4^+$ T cell responses to HHV-8 epitopes in control of HHV-8 infection. There also are minimal data on whether alterations in anti-HHV-8 T cell responses are related to development of KS (93) and whether the lower incidence of KS in HIV-1 infected persons receiving ART is related to increases in anti-HHV-8 T cell responses (19, 27). This information is important for development of prophylactic and therapeutic vaccines for HHV-8.

HHV-8 infection alters the capacity of DC to be recognized by and activate CTL. Both direct presentation using viral proteins endogenously produced in DC, and cross-presentation pathways using viral proteins from exogenous sources of virus are likely to be operative in HHV-8 infection. In fact, EBV does not replicate in MDDC, which instead activate anti-EBV $CD8^+$ T cells by an antigen cross-presentation pathway (103, 183, 226).

It is possible that HHV-8 infected, apoptotic endothelial cells, macrophages and B cells are recognized as "distressed" cells at local sites of infection and engulfed by LC and iDDC (236). These DC then migrate to local lymph nodes while processing the ingested viral proteins through alternative MHC class I pathways for presentation to $CD8^+$ T cells. Furthermore, several HHV-8 proteins, particularly those coded by ORFs K3 and K5, have intriguing properties of altering expression of MHC class I, T cell co-receptors and DC-SIGN. Interestingly, cytokines released by PELs can interfere with the *in vitro* differentiation of immature MDDC from $CD14^+$ monocytes (47).

An intriguing recent discovery is that activated $CD4^+$ T cells suppress HHV-8 lytic replication in tonsillar B cells (168). The suppressive activity requires cell-cell contact. However, it is not a classic CTL response, as it can be mediated by T cells from HHV-8 seronegative persons, is not MHC restricted and does not lyse the B cell targets. This is

proposed to be a pathway by which HHV-8 is driven into latency in B cells. These $CD4^+$ T cells are reminiscent of $CD8^+$ T cells that exhibit non-cytotoxic responses that suppress HIV-1 infection (120).

1.3.1 Altered HHV-8 antigen processing and presentation

Presentation of HHV-8 proteins to both $CD8^+$ (MHC class I restricted) and $CD4^+$ (MHC class II restricted) T cells is impaired by HHV-8 infection. Evidence suggests that anti-HHV-8 $CD8^+$ T cell responses can be inhibited by K3 and K5 proteins that down-regulate MHC class I expression (53, 111). Interestingly, K5 encoded modulator of immune recognition 2 (MIR2) down-regulates T cell co-stimulatory molecules intercellular adhesion molecule 1 (ICAM-1) and CD86 (54) and IFNγR1 (141), which could act to decrease T cell responses to HHV-8. Ishido *et al.* showed that K5 dampens natural killer (NK) cell-mediated cytotoxicity by down-regulation of ICAM-1 and CD86 (110). The NK activating receptor, NKG2D, responsible for detecting infected cells, is down-regulated by HHV-8 K5 (233) via the release of the tumor-associated prostaglandin E2 (PGE2) from KS cells (66). This also results in inhibition of IL-15-mediated NK cell activation and survival, adding to the immune escape tactics employed by this virus (66). Likewise, infection of primary fibroblasts results in limited NK cell activation and subsequent killing activity (158). Brander *et al.* (29) reported a decrease in lysis by HIV-1 peptide-specific CTL clones of cells infected with HHV-8. Thus, it is apparent that K3 and K5 potentially have multifactorial effects on immune control of HHV-8 infection. Of note is that the intracellular viral load of HHV-8 in infected endothelial cells is directly related to their loss of expression of MHC class I and ICAM-1, in association with expression of MIR2 (1). Interestingly, EBV infection also decreases recognition of latently infected cells by down-

regulation of MHC class I molecules, particularly in cells derived from Burkitt's lymphoma (106).

MHC class II recognition is dampened by HHV-8 infection. Sabbah *et al.* reported that LCL, with an intact MHC class II processing pathway, could present LANA peptides to LANA-specific $CD4^+$ T cell clones, whereas PEL cells were not recognized in an IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay (204). PEL express vIRF3, a known inhibitor of the MHC class II master regulator, CIITA (class II transactivator) (208). When CIITA function was restored in PEL, $CD4^+$ T cell clone recognition was also restored (204), supporting a role for HHV-8 in the reduction of MHC class II expression. Interestingly, IFN-γ inducible expression of CIITA results in MHC class II expression on endothelial cells, and is impaired after HHV-8 infection through induction of suppressor of cytokine signaling 3 (SOCS3) (32). This results in inhibition of the early events in the IFN-γ signaling pathway. Taken together, various HHV-8 proteins appear to play a significant role in the disruption of antigen processing and presentation. However, further data are needed to understand the extent of viral protein function in immunopathogenesis of HHV-8 infection in APC.

1.3.2 T cell responses to HHV-8

Although immunity to HHV-8 is far less well defined than that to EBV, T cell immunity to HHV-8 likely plays a similar, critical role in viral control. First, there is an increase in $CD4^+$ and $CD8^+$ expanded T cells in patients with classic KS that share a T cell receptor (TCR)-β variable subunit bias (82), a phenomenon observed in response to chronic viral infections (235, 255). Second, $CD8^+$ T cell immunity to HHV-8 proteins is present in HHV-8 seropositive, healthy individuals. $CD8^+$ T cells specific for five HHV-8 lytic cycle proteins are present in blood in the

first few months of primary HHV-8 infection of normal adults (245). This primary CTL and IFN-γ response to HHV-8 peaks within two years of infection, and wanes thereafter to low but detectable levels. Furthermore, KS does not commonly occur in individuals with a high $CD4^+$ T cell counts (223).

To date, however, there is little direct evidence for a role of T cell immunity in HHV-8 infection and control of KS (105). Lower $CD8^+$ T cell responses have been found in persons with KS compared to asymptomatic persons (93, 128). However, very modest increases in $CD8^+$ T cell responses to HHV-8 immunodominant peptides are found in persons on ART (27, 251). While progressive increases in HHV-8 viral load precede development of disease in HIV-1-infected persons (33, 130), evidence is lacking for a direct association between control of HHV-8 load and HHV-8 specific T cell immunity (93). Nevertheless, an increased incidence of KS in organ transplant recipients and HIV-1-infected persons (57) suggests a role for T cell immunity in prevention of KS, similar to T cell immunity in EBV-related cancers (90). Reduction of immunosuppressive regimens can result in spontaneous resolution of KS in organ transplant recipients (76). Similarly, the incidence of KS has declined after suppression of HIV-1 by ART (188), where T cell numbers and function are partially restored (15, 136, 199). There are also shorter incubation periods for development of KS after HHV-8 infection in HIV-1-infected men compared to men infected with HHV-8 prior to HIV-1 infection (85, 112). Primary infection with HHV-8 in immunosuppressed persons has a more severe outcome than reactivated HHV-8 infection. Finally, HHV-8 expresses many proteins that have immunomodulatory functions that could down-regulate T cell immunity (9).

The emerging biology of KS and HHV-8 infection presents intriguing factors that interrelate HHV-8 specific T cell immunity to control of the cancer. HHV-8 is found as a latent

infection in most of the spindle cells in the KS lesion (26, 65, 77, 167). Since replication of herpesviruses in susceptible cells results in cell death, latency must be established either very soon after infection or possibly following an abortive (non-productive) infection. A small percentage of endothelial and KS spindle cells express a complete replication library of HHV-8 proteins early in the disease, whereas the majority of the transformed cells ultimately express only HHV-8 latency proteins. Circulating B cells and monocytes can be positive for HHV-8 DNA (6, 24), and HHV-8-infected macrophages are present in KS tissues (24). Th1 cytokines have been implicated in reactivation and persistence of HHV-8 in B cells and monocytes from KS patients (217). T cell infiltrates are common in KS tissues (24, 75). $CD8^+$ T cells in KS tissues produce IFN-γ and express HLA DR (75, 217), suggesting that tumor-infiltrating lymphocytes are responding to HHV-8 antigens.

Comprehensive longitudinal studies are needed to accurately assess the role of anti-HHV-8 T cell immunity in the development of KS. T cell responses to HHV-8 could be directed at different lytic and latency proteins at different stages of infection and disease (90, 105). By comparison, the immediate early regulatory protein BMLF1 and other early and late lytic cycle proteins of EBV are targets for CTL during primary and latent infection (25, 106, 221). During mononucleosis, a primary symptomatic infection of EBV, both lytic and latency EBV-specific T cells are present, but responses to lytic epitopes tend to be stronger (149). In healthy EBV seropositive individuals, $CD8^+$ T cell responses are also found to be greater for lytic epitopes, with up to 3% of cells specific for a single lytic epitope and up to 0.5% for a single latency epitope (105). Anti-EBV CTL responses shift during latent infection to EBV nuclear antigens EBNA3 and LMP2, while still retaining specificity for some lytic cycle proteins (104). The hierarchy of CTL responses to immunodominant epitopes of EBV is related to a lower

expression of latency proteins in infected cells (185). Although HHV-8 does not have genes homologous to EBNA and LMP, HHV-8 LANA, kaposin (T0.7 or ORF K12) and K1 are putative latency and transforming proteins that are targets for CTL (28, 135, 182).

Host selection of $CD8^+$ T cell epitopes within HHV-8 proteins could be based in part on the relative expression and presentation of viral proteins by the MHC class I endogenous pathway, comparable to EBV (138). However, evidence from the anti-EBV CTL field indicates that CTL reactivity to this gammaherpesvirus varies as to the HLA haplotype, with different MHC class I haplotypes exhibiting different CTL reactivity to the same EBV proteins (106). Perhaps HHV-8 has mechanisms similar to the glycine-alanine repeat domain in EBNA1 (183) that inhibits proteosome processing of viral proteins through the MHC class I pathway (106, 137), thereby inhibiting generation of EBNA1-specific T cells. In fact, LANA-1 can inhibit protein processing *in cis* (126, 257). Bioinformatic analysis of HHV-8 sequences supports that latency proteins are likely to be poorer targets for CTL than immediate early or lytic proteins (240). However, it is not yet clear if the *in cis* function of LANA-1 is directly involved in down-regulation of CTL lysis of HHV-8 infected cells, including how it compares to other putative, *in trans* inhibitors of CTL function such as K3 and K5. Moreover, the EBNA1-CTL inhibition concept has undergone major revision. First, the GAr domains of EBNA1 can inhibit mRNA translation, which may be more critical to lack of CTL recognition than inhibition of proteosomal processing (256). Second, EBNA1 infected cells express EBNA1 peptides that can be recognized by CTL when assessed in more sensitive assays (134). This indicates that the effects of LANA-1 on pathways related to CTL function that use chimeric constructs, indicator cell lines, etc., need to be characterized in a natural context using CTL and natural targets that are specific for LANA-1.

Similar to EBV, $CD8^+$ T cell responses to HHV-8 tend to be directed more toward lytic antigens (200). While there are much fewer $CD8^+$ T cell epitopes known for HHV-8 than EBV, the majority of these epitopes are within the early and late-lytic proteins (201, 202). With regard to polyfunctionality, one study found that for both EBV and HHV-8, T cells specific for latency antigens were more polyfunctional than those specific for lytic antigens (20). The phenotype of these cells was also found to be different, with a greater proportion of effector memory T cells specific for latency antigens than lytic antigens for both EBV and HHV-8. In both EBV and HHV-8-associated malignancies, latency proteins are predominantly expressed, so it is thought that responses to latency proteins could be important in controlling these diseases (105, 231). Evidence suggests that there are higher levels of CTL specific for EBV and cytomegalovirus (CMV) than HHV-8 in the blood of seropositive individuals (93, 244). Higher T cell responses to EBV and CMV antigens could be related to their greater viral load in persistently infected persons, with more turnover of viral antigen from latent, persistent reservoirs that maintains a greater level of memory CTL precursors.

Antigen-specific $CD8^+$ T cells occupy a lineage of naïve and memory compartments that are involved in the expansion, effector and contraction phases of $CD8^+$ memory T cells (96). Central memory and effector memory T cells are contrasted based on expression of surface molecules related to migration and differentiation. Patients with MCD have more $CD45RA^-CCR7^-CD27^-CD8^+IFN\text{-}\gamma^+$ cells (a late memory T cell phenotype) and fewer $CCR7^-CD27^+CD45RA^-$ cells (early and intermediate T cell phenotype) than normal, HHV-8 seropositive controls. This phenotypic shift is not found for EBV-specific $CD8^+$ T cells. Interestingly, HHV-8 viral loads are negatively correlated with early and intermediate effector memory cells. The more differentiated T cell phenotype is associated with disease, rather than a

loss of HHV-8 specific $CD8^+$ T cells or polyfunctional activity, as the HHV-8 specific T cells are similar in function (secretion of IFN-γ, TNF-α, MIP1-β, and/or CD107a) in infected patients and healthy controls (94).

In healthy, HHV-8 seropositive individuals controlling infection, both monofunctional and polyfunctional $CD8^+$ T cells are present that are specific for HHV-8 proteins (135). This could have important implications in the immunopathogenesis of HHV-8 and for HHV-8-related disease development. In fact, patients who control KS have more polyfunctional $CD8^+$ T cells producing IFN-γ and TNF-α, while patients with progressive KS have weaker and less polyfunctional HHV-8-specific $CD8^+$ T cells (17). IFN-γ-producing CTL specific for some HHV-8 lytic and latency proteins also express CD107 and TNF-α (20). This is similar to polyfunctional CTL that produce multiple cytokines, such as IFN-γ, IL-2 and MIP-1β, that are associated with enhanced control of HIV-1 infection (16, 153, 222). Also, CTL specific for EBV lytic and latency proteins differ in phenotype, including expression of programmed death-1 (PD-1) (106). PD-1 expression could act as a negative regulator of HHV-8 specific $CD8^+$ T cells during disease progression.

While both monofunctional and polyfunctional antiviral $CD8^+$ T cells are present in healthy HHV-8 seropositive individuals, a week-long DC-enhanced system was required to reveal these responses to HHV-8 proteins (244). Overall, the immune response to HHV-8 is relatively non-robust compared to T cell reactivity to other herpesviruses such as EBV (20, 135). This suggests that the number and/or functional capacity of circulating anti-HHV-8 T cells is relatively low. However, using direct, multimer staining, we have found that there is an average of 0.05-0.10% circulating, $CD8^+$ T cells specific for single, immunodominant MHC class I

epitopes of HHV-8 in healthy, HHV-8 seropositive individuals (135). It is possible that these HHV-8-specific T cells are functionally down-regulated by T regulatory cells (Treg).

Treg are operative in peripheral tolerance and beneficial in preventing autoimmunity and tissue damage. These cells function through mechanisms including IL-10 and transforming growth factor (TGF)-β inhibitory cytokine secretion, granzyme cytolytic activity, metabolic disruption through the adenosine and IL-2 pathways, and suppression of DC function using the inhibitory receptor CTLA-4 (242). However, Treg can also inhibit immunity needed to resolve infections. While little is currently known about Treg during HHV-8 infection and disease development, these cells have been found to be important during other viral infections, including EBV (139) and HIV-1 (151). During primary EBV infection, patients with mononucleosis have less Treg than healthy seropositive individuals (253). In patients with Hodgkins lymphoma, Treg accumulated at tumor sites, and those patients with higher Treg ratios had shorter disease-free survival (154, 209). Additionally, in these patients, several EBV epitopes stimulate Treg, and the increases in Treg numbers are associated with decreased EBV-specific $CD8^+$ T cell IFN-γ production (155). In patients with nasopharyngeal carcinoma, large numbers of Treg are found both at tumor sites and in circulation (132, 140). During HIV-1 infection, HIV-1 specific $CD8^+$ T cell responses and cytolytic activity are repressed by Treg (121). Additionally, an increased Treg frequency negatively alters $CD8^+$ T cell polyfunctionality, which is restored with Treg depletion (152). As Treg could also be important in HHV-8-related disease development, such as KS, studies are needed to determine their exact role.

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1.4 HYPOTHESIS AND SPECIFIC AIMS

HHV-8 is a gammaherpesvirus that causes KS, PEL and MCD. The importance of determining effective prevention and treatment for HHV-8 infection is evident in that KS continues to be the most common cancer among HIV-1 and AIDS patients (48). $CD8^+$ T cell immunity likely plays a significant role during HHV-8 infection as these cells have been shown to be crucial in controlling infections caused by the other human gammaherpesvirus, i.e., EBV (105). *I hypothesized that monofunctional and polyfunctional $CD8^+$ T cells specific for HHV-8 lytic and latency proteins are important in controlling HHV-8 infection. Loss of this reactivity to immunodominant epitopes is due in part to the down-regulation of $CD8^+$ T cell responses by Treg, contributing to the development of KS.* In support of this hypothesis, our laboratory has shown that $CD8^+$ T cells produce IFN-γ in response to HHV-8 immunodominant epitopes in HHV-8 seropositive individuals (244). Polyfunctional T cells, i.e., single cells producing two or more immune mediators, have been linked to control of HIV-1 (16) and could play a role in controlling HHV-8 infection. Treg have been shown to modulate immune responses during other persistent viral infections (142), but it is currently unknown if they are important during HHV-8 infection. Identifying the viral regions targeted by the immune system and mechanisms that interfere with T cell responses could be critical for the development of effective treatments and vaccines for HHV-8-associated cancers such as KS. The specific aims of this project were:

1.4.1 Specific aim I

To define MHC class I epitopes that generate $CD8^+$ T cell responses to HHV-8 proteins and investigate the presence of polyfunctional T cell responses. T cells isolated from HHV-8

seropositive, HLA A*0201 donor PBMC were stimulated with autologous dendritic cells loaded with overlapping peptides derived from glycoprotein B (gB) and K8.1 lytic proteins, and LANA-1 and K12 latency proteins. To define novel immunodominant epitopes, T cell responses were evaluated using an IFN-γ ELISPOT. To investigate polyfunctional responses, positive peptides were then assessed for IFN-γ, IL-2, TNF-α, MIP-1β and CD107a production by flow cytometry. To directly detect HHV-8-specific T cells in the blood, multimer complexes were used.

1.4.2 Specific aim II

To determine the magnitude and breadth of $CD8^+$ T cell responses to HHV-8 proteins during the progression to KS. Longitudinal studies were performed using Multicenter AIDS Cohort Study (MACS) participants, including HHV8+/HIV- individuals as well as HHV8+/HIV+ individuals that did and did not develop KS. To compare $CD8^+$ T cell responses before and after HHV-8 seroconversion and the development of KS, multimer and phenotype staining were performed using known epitopes and epitopes defined in specific aim I.

1.4.3 Specific aim III

To investigate the role of Treg during the progression to KS. Longitudinal studies were performed using MACS participants from specific aim II. To compare $CD8^+$ T cell responses before and after HHV-8 seroconversion and the development of KS, ELISPOT assays were performed using known epitopes and epitopes defined in specific aim 1. Experiments were performed using PBMC and PBMC depleted of Treg**.**

2.0 DETECTION OF POLYFUNCTIONAL T CELL RESPONSES TO HHV-8

HHV-8 is the etiological agent of KS, PEL and MCD. It is postulated that $CD8^+$ T cell responses play an important role in controlling HHV-8 infection and preventing development of disease. As this is not clear, however, we investigated monofunctional and polyfunctional $CD8^+$ T cell responses to HHV-8 lytic proteins gB and K8.1, and latency proteins LANA-1 and K12. Based on our previous findings that DC reveal MHC class I epitopes in gB, we used a DC-based system to identify two gB, two K8.1, five LANA-1 and one K12 novel epitopes. These new HHV-8 epitopes activated both monofunctional and polyfunctional $CD8^+$ T cells that produced various combinations of IFN-γ, IL-2, TNF-α, MIP-1β and cytotoxic degranulation marker CD107a in normal, HHV-8 seropositive individuals. We were also able to detect HHV-8-specific $CD8^+$ T cells in the peripheral blood using HLA A*0201 pentamer complexes specific for one gB, one K8.1, two LANA-1 and one K12 epitopes. These immunogenic regions of viral lytic and latency proteins could be important in T cell control of HHV-8 infection.

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2.1 BACKGROUND

HHV-8, also referred to as KSHV, is a gammaherpesvirus that causes KS, PEL and MCD. The importance of developing effective prevention and treatment for HHV-8 infection is evident in that KS, a neoplasm of endothelial origin, continues to be the most common cancer among HIV-1-infected patients (48). It is also the leading cause of cancer in children in sub-Saharan Africa (31). Although the incidence of KS in HIV-1-infected persons declined with the advent of ART (81), KS can occur in persons on ART with suppressed HIV-1 infection and high $CD4^+$ T cell counts (159).

The immune responses responsible for controlling HHV-8 infection and preventing KS are not clear. $CD8^+$ T cell immunity likely plays a significant role in HHV-8 infection, as these cells have been shown to be crucial in controlling infection caused by the other human gammaherpesvirus, i.e., EBV (90, 105). In support of this hypothesis, our laboratory (244-246) and others (20, 27, 28, 93, 128, 148, 163, 180, 182, 198, 200, 220, 223, 251, 254) have shown that $CD8^+$ T cells produce IFN-γ in response to HHV-8 immunodominant epitopes presented by MHCI in HHV-8 seropositive individuals. Little is known about whether T cells produce other immune mediators in response to HHV-8 infection. Indeed, polyfunctional T cells, i.e., single cells producing two or more immune mediators, have been linked to control of HIV-1 and other persistent infections (16, 153, 184, 213), and could play a role in controlling HHV-8 infection. In one recent study, HHV-8 epitope-specific, polyfunctional T cells were detected in patients with MCD, but these did not differ in number from those in normal controls (94). Another study found that patients with controlled KS had HHV-8-specific $CD8^+$ T cells that secreted IFN-γ and TNF-α, while patients with progressive disease had weaker and less polyfunctional $CD8^+$ T cells (17).

HHV-8 epitope-specific, monofunctional and polyfunctional T cell immunity could be important in the development of HHV-8 vaccines that induce T cell responses that target these viral epitopes. We therefore investigated $CD8^+$ T cell responses to two HHV-8 lytic proteins, gB and K8.1, and two latency proteins, LANA-1 and K12. Our laboratory has previously shown that optimal induction of T cell reactivity to the HHV-8 protein gB required one week of stimulation with peptide-loaded, autologous, mature, MDDC (244). Using this enhanced DC-T cell stimulation system, we now have revealed several new epitopes for these four lytic and latency HHV-8 proteins in normal, HHV-8 seropositive individuals, which induce both monofunctional and polyfunctional $CD8^+$ T cells. These regions of HHV-8 could be critical in understanding HHV-8 immunopathogenesis and in vaccine development.

2.2 METHODS

2.2.1 Study subjects

Healthy, HIV-1 negative subjects were selected based on their HHV-8 antibody status and MHCI genotype, and written informed consent was obtained. Detection of HHV-8 serum antibodies specific for viral lytic antigens was done using an indirect immunofluorescence assay (244). High resolution, HLA molecular typing was conducted by the University of Pittsburgh Medical Center Tissue Typing Laboratory. The donors were classified into HLA A*0201, HHV-8 seropositive and HLA A*0201, HHV-8 seronegative groups.

2.2.2 Synthetic peptides

For initial studies, libraries of 15mer peptides overlapping by 11aa derived from K12, gB and K8.1 protein sequences were synthesized (PEPscreen, Sigma). For the larger protein LANA-1, a library of 15-20mer peptides overlapping by 11 aa was used. Protein sequences were obtained from the National Center for Biotechnology Information database, with LANA-1 accession number AAD46501, K12 number AAD46499, gB number ABD28851 and K8.1 number ABD28902. As LANA-1 contains a large repeat region, we used one set of representative peptides to span this region (peptide numbers 59-75). For epitope mapping studies, putative optimal 9mer peptides were synthesized based on anchor residues for HLA A*0201 (190), as well as peptides one N- or C-terminus amino acid shorter and longer than optimal (107). The following previously published 9mer, HLA A*0201-restricted, HHV-8 epitopes were also used: LANA-$1_{238-246}$ WATESPIYV (93), LANA-$1_{1116-1124}$ QMARLAWEA (93), $K12_{17-25}$ LLNGWRWRL (28), $gB_{492-500}$ LMWYELSKI (244) and $K8.1_{209-217}$ LVLILYLCV (27).

2.2.3 T cell stimulation

$CD14^+$ cells were isolated from donor PBMC using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotec). Immature DC were generated by culturing $CD14^+$ cells ($1x10^6$) for 7 days in AIM-V medium (GIBCO) containing 1,000 U/mL each of recombinant IL-4 (R&D Systems) and recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF) (Bayer Health Care) at 37°C in a 5% CO_2 atmosphere. Fresh IL-4 and GM-CSF were added every 2 days. On day 5, CD40L (1μg/mL, R&D) was added to induce maturation. Mature DC were loaded with peptide (2μg/mL) for 2 hours (h) at 37°C. Culture medium and peptide concentration were chosen based

on a comparison of assay conditions (see supplemental figures in Appendix B, Figure 16). To assess T cell reactivity, we used DC as APC and autologous $CD14^-$ cells as responders (244). Antigen-loaded DC or DC without antigen ($1x10^4$ cells) were mixed with $CD14^-$ cells ($1x10^5$) and co-cultured for 7 days in AIM-V at 37°C, with 100 U/mL IL-2 (Chiron) added on day 2. T cell stimulation was assessed in HHV-8 seronegative donors as controls.

2.2.4 Single-cell IFN-γ production

Following a 7 day co-culture of peptide-loaded mature DC stimulators and $CD14^-$ cell responders, our DC-enhanced, single-cell IFN-γ ELISPOT assay was performed based on our previously described method for assessing T cell reactivity (244). Briefly, nitrocellulose-bottomed, 96-well ELISPOT plates (Millipore) were pre-coated with an anti-IFN-γ mAb (10μg/mlL, Mabtech) overnight at 4°C then blocked for 2 h at 37°C with RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated human AB^+ serum (Gemini Bio-Products). Autologous PBMC ($1x10^4$) were loaded with peptide (2μg/mL) for 2 h at 37°C then combined with week-long T cell cultures described above to serve as APC. These samples were added to ELISPOT plates, cultured overnight at 37°C, and developed as previously described (50). Spots were counted using an ELISPOT reader (AID). The results were expressed as virus-specific spot-forming cells per million cells by subtracting the number of spots in T cell cultures stimulated by DC without peptide from the number of spots induced by DC with peptide. T cell responses were considered positive if the number of spots produced by cells stimulated with peptide was greater than the number of spots produced by cells not stimulated with peptide.

2.2.5 Generation of T cell lines

HHV-8 epitope-specific T cell lines were generated to confirm the novel epitopes and their HLA A*0201 restriction. Frozen PBMC were thawed, washed and resuspended in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS) (Cellgro). Cells were added to a 24-well plate at $2x10^6$ cells per well and kept at 37°C. Peptides were added at 10μg/mL. On day 3, IL-2 was added (100 U/mL), with fresh IL-2 added every 3 days for a total of 15 days. On day 15, a B cell line that only expresses one MHCI haplotype, HLA A*0201, was used as an APC; a B cell line that does not express HLA (null) was used as a control (214). The B cells were loaded with peptide (2μg/mL) for 2 h at 37°C and then used as APC with the 15 day cultured cells in an IFN-γ ELISPOT assay performed as described above.

2.2.6 MHCI binding assay

HLA A*0201 binding experiments were performed by ProImmune with their class I REVEAL binding assay. Briefly, the assay determines the binding of a peptide to an HLA molecule by its ability to stabilize the MHC-peptide complex. Binding is scored relative to a positive control of a known T cell epitope with very strong binding properties. A score is reported quantitatively as a percentage of the signal generated by the test peptide versus the positive control peptide.

2.2.7 Pentamer staining

Along with analysis of immune responses by IFN-γ ELISPOT, MHCI pentamers were also used to quantify epitope-specific $CD8^+$ T cells. Based on the results of the ProImmune class I

REVEAL binding assay described above, five high-affinity HLA-A*0201 binding peptides were used to generate MHC class I-peptide pentamer complexes (ProVE pentamers, ProImmune). Frozen PBMC (1-2x10^6 cells per well) were thawed, washed and then incubated for 10 minutes (min) with unlabeled pentamer (0.5µg per test). The cells were washed and then stained for 20 min in the dark with phycoerythrin (PE)-conjugated Pro5 Fluorotag (8µL per well, ProImmune), CD3- allophycocyanin-Cy7 (5µL per well, BD) and CD8-peridinin chlorophyll protein (PerCP)-Cy5.5 (20µL per well, BD). CD4-V450, CD19-V450 and CD14-V450 (all from BD) were also added at 5µL each per well for exclusion gating. Cells were washed and fixed with BD stabilizing solution and analyzed on an LSR II flow cytometer (BD Immunocytometry Systems).

2.2.8 ICS and polychromatic flow cytometry

Intracellular staining (ICS) for various immune mediators was performed as described (16, 129), with minor modifications. On day 7 of the DC-enhanced T cell cultures described above, cells were collected and plated (1-2x10^6 cells per well). The peptide stimulus (10µg/mL), costimulatory CD28/49d mAb (1µg/mL, BD), monensin (5µg/mL, Sigma), brefeldin A (5µg/mL, Sigma), and anti-CD107a-fluorescein isothiocyanate (FITC) (20µL per well, BD) were added. Unstimulated cells were also included. The cells were incubated for 6 h at 37°C, and then ethylenediaminetetraacetic acid (EDTA) (2mM) (GIBCO) was added. The cells were washed and resuspended in 100µL phosphate-buffered saline (PBS) (GIBCO) per well containing 1µL/well aqua viability dye (Invitrogen) for 30 min in the dark. The cells were washed and resuspended in 1x fluorescence-activated cell sorting (FACS) lysis solution (BD) and kept at 4°C overnight. Cells were washed and permeabilized using 1x Perm solution (BD), and then washed and stained for 45 min in the dark with the directly conjugated antibodies CD3- allophycocyanin

-Cy7, TNF-α-PE-Cy7, MIP-1β-PE, IL-2-allophycocyanin, IFN-γ-AF700, with 5μL each per well, and CD8-PerCP-Cy5.5 (all from BD) at 20μL per well. CD4-V450, CD19-V450, and CD14-V450 (all from BD) were also added at 5μL each per well for exclusion gating. Cells were washed and fixed with BD stabilizing solution and analyzed on an LSR II flow cytometer. Results were analyzed with Boolean gating to create the full array of possible cytokine combinations using BDFACS Diva (v6.0) software. Polyfunctional bar and pie charts for each peptide were created using Simplified Presentation of Incredibly Complex Evaluations (SPICE) software (v4.2.3, provided by Mario Roederer, VRC/NIAID/NIH).

2.2.9 Statistics

We used analysis of variance (ANOVA) and the Student *t* test for comparisons between groups.

2.3 RESULTS

2.3.1 Novel T cell epitopes in HHV-8 lytic and latency proteins

To define MHCI epitopes that generate $CD8^+$ T cell responses to HHV-8 proteins, a DC-enhanced IFN-γ ELISPOT assay was used. This was based on our previous finding that this method was necessary to reveal an epitope in HHV-8 gB due to the non-robust nature of the immune response to HHV-8 in normal, HHV-8 seropositive persons (244). We confirmed this finding with the donors used in the current study by comparing responses to previously published

epitopes in a standard, overnight and a DC-enhanced, 7 day ELISPOT assay (see supplemental figures in Appendix B, Figure 17). Therefore, T cells were stimulated with autologous mature DC that were loaded with overlapping peptides derived from HHV-8 LANA-1, K12, gB and K8.1, and single cell IFN-γ production was measured by an ELISPOT assay.

The IFN-γ ELISPOT results for four HLA A*0201, HHV-8 seropositive donors (A29, A34, A52 and A57) to peptides from each viral protein are displayed in Figure 1. For the larger proteins LANA-1 and gB, peptides containing possible epitopes, termed "hotspots" (Figure 1, circled), were defined as peptides that generated a positive response above the background of mock-stimulated cells in all donors tested, and with at least one donor responding in the highest category (red boxes). For the smaller proteins K12 and K8.1, we considered hotspots as those peptides that the majority of donors responded to above background, and at least one donor responded in the highest category. For LANA-1, out of 106 peptides tested, we found eight hotspots (Figure 1A). For K12, out of 13 peptides tested, we found one hotspot (Figure 1B). For gB, out of 235 peptides tested, we found nine hotspots, including one containing our previously described epitope (244) (Figure 1C). For K8.1, out of 60 peptides tested, we found three hotspots including two containing previously described epitopes (27, 251) (Figure 1D). Two HLA A*0201, HHV-8 seronegative donors did not respond above background to any of these hotspots from the four viral proteins. Thus, these hotspots represented regions of the four HHV-8 proteins that displayed positive reactivity associated with HHV-8 seropositivity.

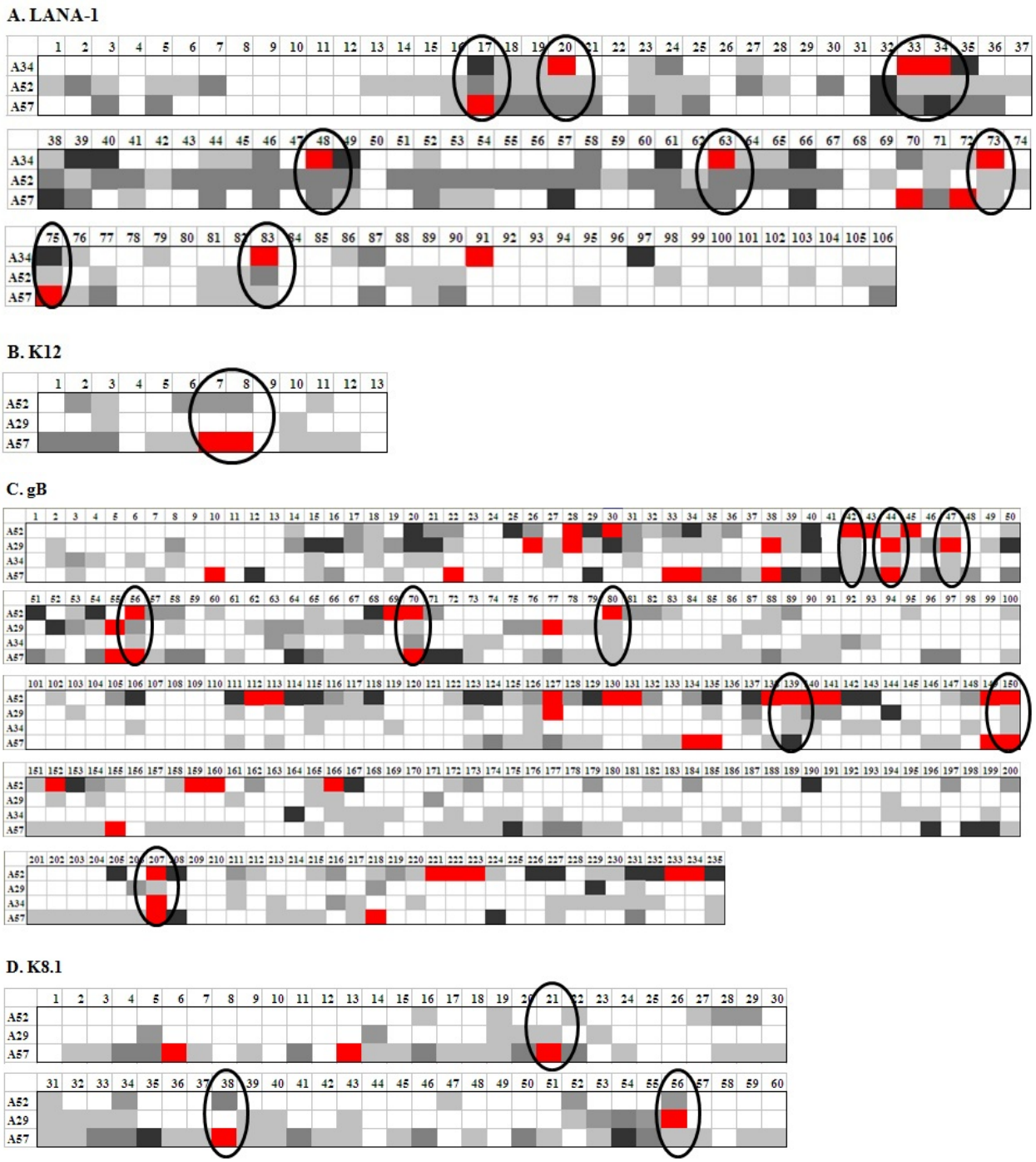

Figure 1. ELISPOT responses to four HHV-8 lytic and latency proteins

PBMC from HLA A*0201, HHV-8 seropositive, healthy donors were stimulated with autologous, mature DC that were loaded with overlapping 15-20mer peptides derived from each of the HHV-8 proteins LANA-1, K12, gB and K8.1. IFN-γ production was measured by a DC-enhanced ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. The donors (three to four donors used for each protein) are listed in each row, while the representative peptide numbers are listed in each column. The colors for the boxes represent the net IFN-γ spots per million cells as: □ <1, ▒ 1-199, ▒ 200-399, ▒ 400-599 and ■ ≥600 spots. Hotspots are circled for LANA-1 (A), K12 (B), gB (C), and K8.1 (D).

We next mapped minimal epitope sequences for 10 of these hotspot peptides. To determine the minimal, optimal epitope, we used peptide families consisting of the putative optimal 9mer based on anchor resides for HLA A*0201, along with peptides one N- or C-terminus aa truncation (8mers) and extension (10mers). Initially, we adapted the conventional method to define optimal T cell epitopes, i.e., T cell response to different concentrations of N- and C-terminal extensions and truncations of peptide in a standard overnight ELISPOT assay, to our 7 day, extended assay. However, stimulating PBMC for 7 days with DC loaded with five different, 10-fold concentrations of these peptide families did not differentiate a dominant peptide response (see supplemental figures in Appendix B, Figure 18). Therefore, we tested the peptide families for the optimal epitopes by stimulating the cells for 7 days with a single concentration of peptide followed by a conventional, 16 hour ELISPOT assay using the same peptide. We assessed five peptide families from LANA-1, one from K12, two from gB and two from K8.1. One of the hotspots we identified (K8.1 peptide 38, Figure 1D) contained a published 15mer epitope (251). Therefore, we included this peptide family to determine the minimal epitope sequence. Two of the hotspots (gB peptide 139, Figure 1C, and K8.1 peptide 56, Figure 1D) contained previously published 9mer epitopes (27, 244) that we included as controls. As shown in Figure 2, the DC-enhanced assay revealed positive IFN-γ responses for the known HHV-8 epitopes as well as for the peptide families of each protein. We defined the optimal epitope as the peptide from each family that generated the highest IFN-γ production above background (i.e., mock-stimulated cell cultures). By these approaches we were able to define five novel, minimal epitope sequences for LANA-1, one for K12, two for gB and two for K8.1 (Table 1).

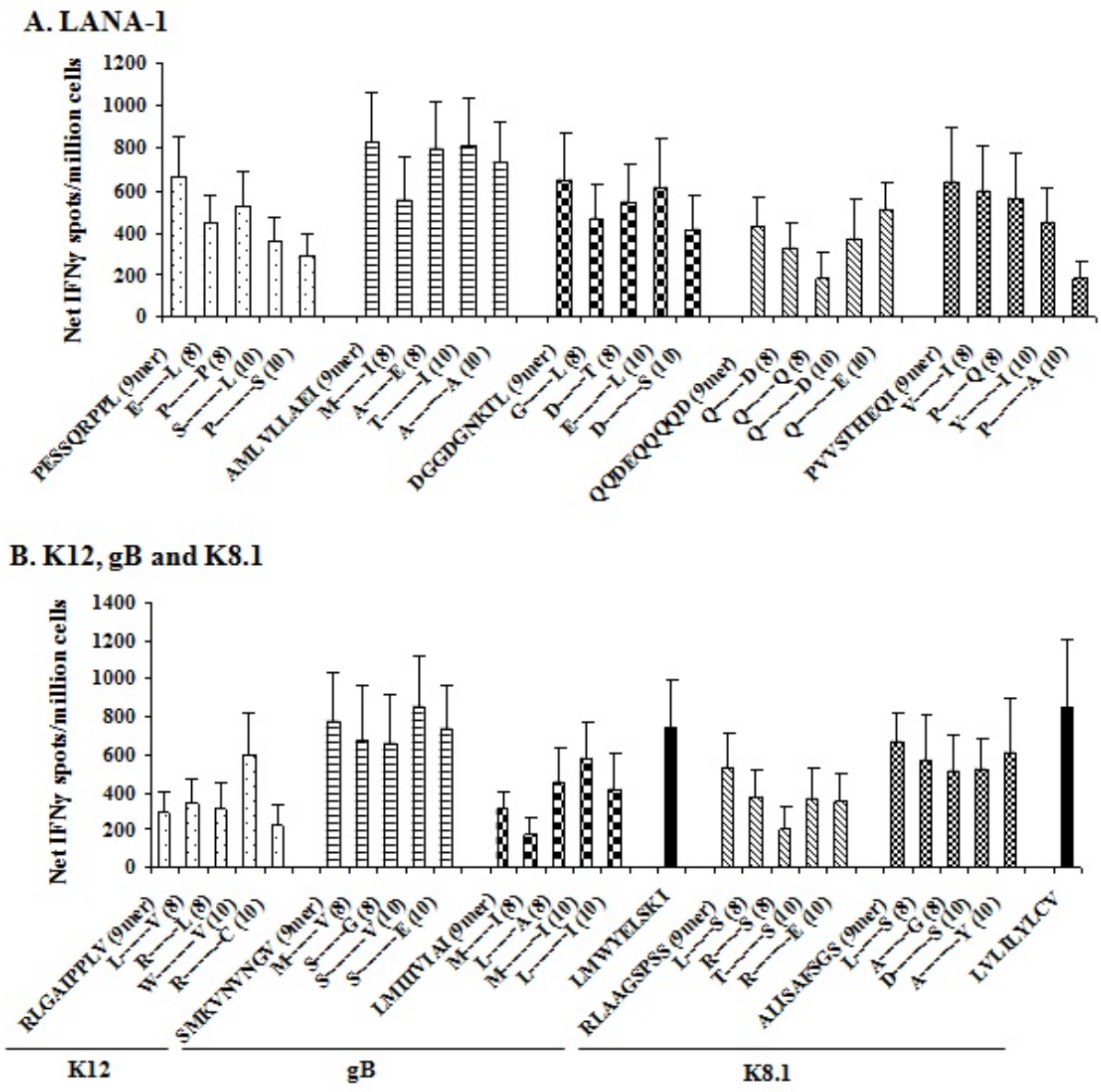

Figure 2. ELISPOT responses to peptide families derived from hotspots

PBMC from HLA A*0201, HHV-8 seropositive, healthy donors were stimulated for one week with autologous mature DC that were loaded with peptide. IFN-γ production was measured as in Figure 1. Mean + SE spot forming cells from 10 experiments using three donors in response to DC loaded with peptide families are shown for LANA-1 (A) and K12, gB and K8.1 (B), and known $gB_{492\text{-}500}$ and $K8.1_{209\text{-}217}$ epitopes (B).

Table 1. Novel HHV-8 HLA A*0201 epitopes identified in LANA-1, K12, gB and K8.1

Protein	Peptide number[a]	Amino acid position	Sequence
LANA-1	17	140-148	PESSQRPPL
LANA-1	33/34	281-289	AMLVLLAEI
LANA-1	48	417-425	DGGDGNKTL
LANA-1	63	688-697	QQDEQQQQDE
LANA-1	83	920-928	PVVSTHEQI
K12	7/8	23-32	WRLGAIPPLV
gB	44	159-168	SSMKVNVNGV
gB	207	736-745	MLMIIIVIAI
K8.1	21	73-81	RLAAGSPSS
K8.1	38	135-143	ALISAFSGS

[a] Representative peptide numbers from Figure 1.

2.3.2 MHCI restriction and binding of novel HHV-8 epitopes

To verify that the novel epitopes were HLA A*0201-restricted, we generated epitope-specific T cell lines and used B cells that only express HLA A*0201 (A2 cells) or do not express any HLA (null cells) as APC in a standard ELISPOT assay. Positive IFN-γ responses using A2 cells as APC were evident to our five novel LANA-1 epitopes, one novel K12 epitope, two novel gB epitopes and two novel K8.1 epitopes (Figure 3, black bars). IFN-γ responses were also detected when using A2 cells as APC for our previously determined HLA A*0201-restricted gB epitope (244) (Figure 3, black bars). For all peptides tested, responses were lower when the null B cells were used as APC (Figure 3, grey bars). Taken together, these results support that our novel epitopes from the four HHV-8 proteins are HLA A*0201-restricted.

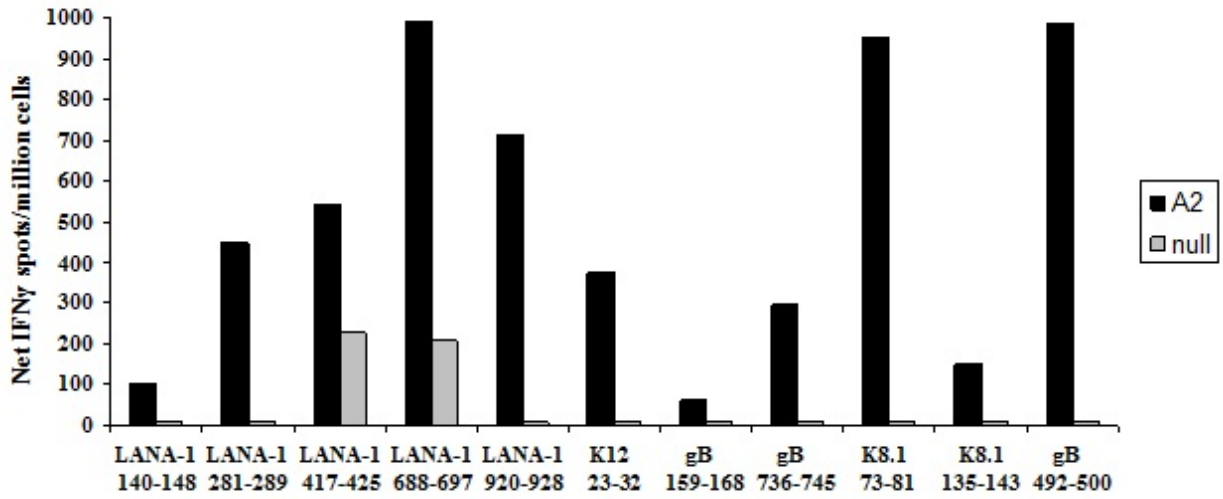

Figure 3. T cell line ELISPOT responses using HLA A2 cells as APC

PBMC from HLA A*0201, HHV-8 seropositive healthy donors were stimulated with peptide-loaded DC, with IL-2 added every 3 days. On day 15, B cell lines expressing only HLA A*0201 (A2 cells) or no HLA molecules (null cells) were used as APC. IFN-γ production was measured by ELISPOT as in Figure 1. Responses to novel epitopes from LANA-1, K12, gB and K8.1, and a known epitope from gB ($gB_{492\text{-}500}$) are shown for one representative donor out of two donors used.

Having demonstrated that these peptides are presented in the context of the HLA A*0201, we then tested them in an MHCI binding assay to confirm their specificity. To this end, ProImmune performed their class I REVEAL binding assay on our novel minimal epitopes as well as several known epitopes from the four HHV-8 proteins used in our study. The binding rates are reported as percent relative to the binding of a known, strong HLA A*0201 T cell epitope. The peptides that showed high binding (i.e., above the intermediate control of a known, weaker HLA A*0201 T cell epitope) were three known epitopes ($gB_{492\text{-}500}$, $K12_{17\text{-}25}$, LANA-$1_{1116\text{-}1124}$) as well as two of our novel epitopes (LANA-$1_{281\text{-}289}$, K8.$1_{135\text{-}143}$), which supports that all of these peptides are HLA A*0201-restricted, T cell epitopes (Figure 4). The remaining

novel epitopes were classified as weak binders, i.e., below the intermediate control (Figure 4). However, these binding scores are relative measures and general guidelines. Thus, peptides classified as weak binders are not necessarily poor T cell epitopes. Indeed, two known epitopes (LANA-$1_{238-246}$, K8.$1_{209-217}$) were classified as weak binders in this assay (Figure 4). Overall, we conclude that our peptides are novel HLA A*0201 epitopes.

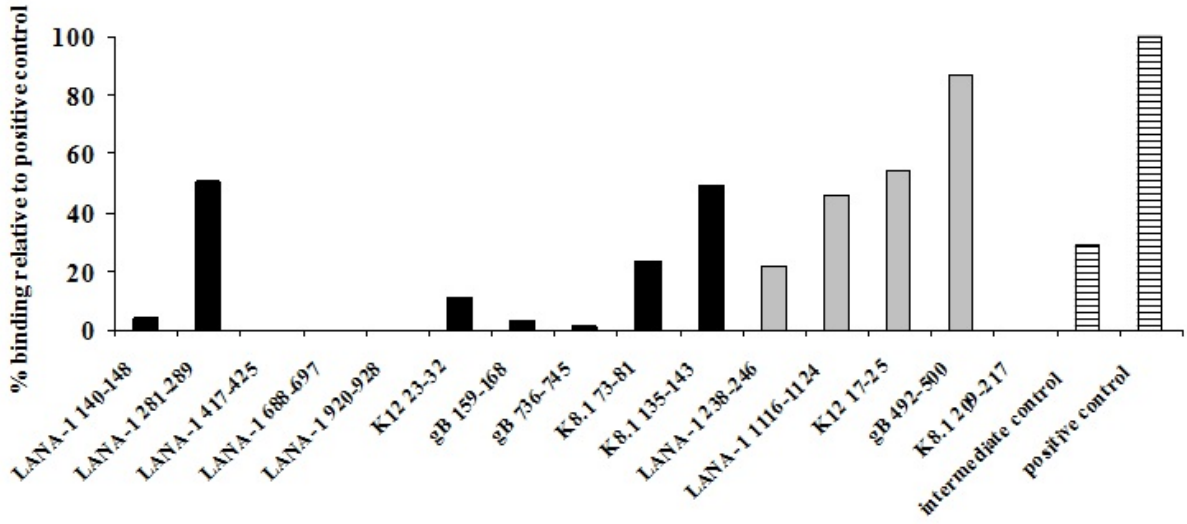

Figure 4. ProImmune class I REVEAL binding assay results
The binding score for each peptide is shown as relative to a positive control of a known T cell epitope with very strong HLA A*0201 binding properties. Binding scores are shown for our ten novel epitopes (black bars), five known epitopes (grey bars) and the positive and intermediate control peptides (striped bars).

2.3.3 Direct identification of $CD8^+$ T cells specific for HHV-8 epitopes

Based on the highest scores of the peptide-MHC class I binding results, we synthesized five HLA A*0201 pentamers for two novel epitopes, $LANA_{281-289}$ and $K8.1_{135-143}$, and three known epitopes, $gB_{492-500}$, $K12_{17-25}$ and LANA-$1_{1116-1124}$. As displayed in Figure 5, the mean (±standard error [SE]) percent of epitope-specific $CD8^+$ T cells in eight healthy, HLA A*0201, HHV-8 seropositive individuals was 0.048% (±0.010) for $LANA_{281-289}$, 0.050% (±0.012) for LANA-$1_{1116-1124}$, 0.053% (±0.018) for $K12_{17-25}$, 0.050% (±0.009) for $gB_{492-500}$ and 0.096% (±0.020) for $K8.1_{135-143}$. The mean levels of pentamer-positive $CD8^+$ T cells was 0.020% (±0.003) in three healthy, HLA A*0201, HHV-8 seronegative individuals (Figure 5). Overall, the level of pentamer-positive, $CD8^+$ T cells in the seropositive donors was significantly greater than that in the seronegative donors ($p\text{-value}<0.05$).

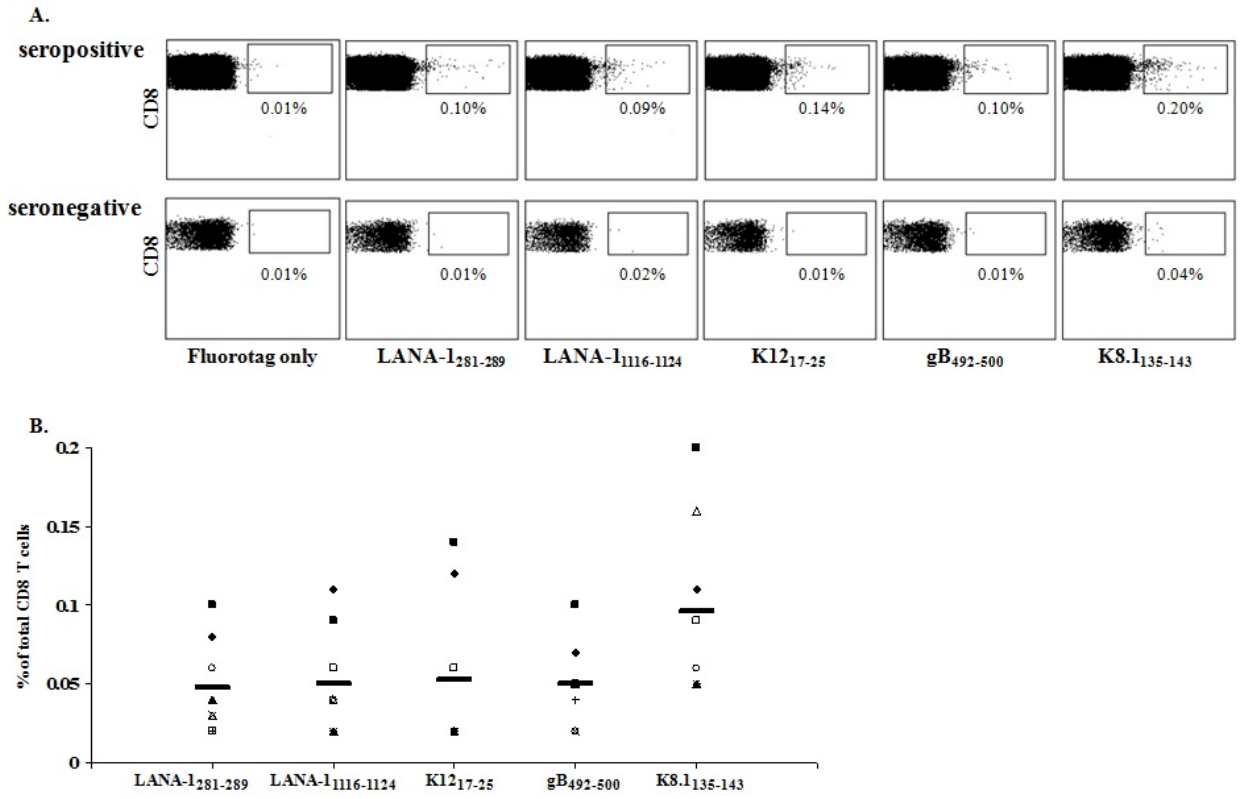

Figure 5. HLA A*0201 pentamer staining in peripheral blood samples

PBMC from HLA A*0201, HHV-8 seropositive donors were stained with fluorotag alone or MHC class I-peptide pentamer complexes specific for LANA-$1_{281-289}$, LANA-$1_{1116-1124}$, $K12_{17-25}$, $gB_{492-500}$ and $K8.1_{135-143}$. Results are shown for one representative HHV-8 seropositive and one representative seronegative donor (A) and for all eight HHV-8 seropositive donors tested (B, with averages indicated by the black bars).

2.3.4 Monofunctional and polyfunctional T cell responses to HHV-8

There are many cytokines in addition to IFN-γ, as well as chemokines and cytotoxic molecules, that are important in antiviral T cell responses. Moreover, polyfunctional T cells, which are defined as a single cell producing two or more such immune mediators, are an important immune correlate of protection against HIV-1 disease progression (16). As it is currently not clear whether monofunctional and polyfunctional $CD8^+$ T cells play a role in controlling HHV-8 infection, we used a panel of markers for immune mediators to examine polyfunctional $CD8^+$ T cell responses. This panel included the cytokines IFN-γ, TNF-α and IL-2, the chemokine MIP-1β and the degranulation mobilization marker CD107a. We used a modified procedure with DC-enhanced T cell cultures for the ICS assay developed in our laboratory (109) to improve detection of production of these immune mediators (representative analysis in Figure 6).

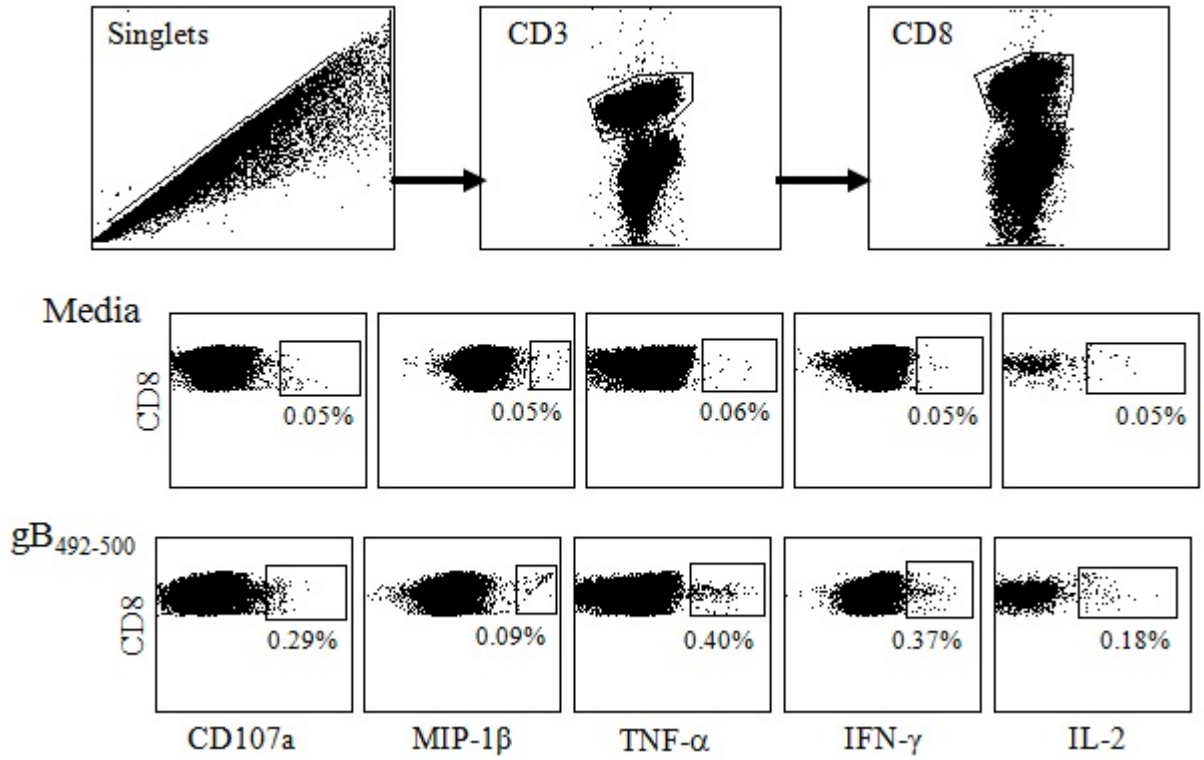

Figure 6. Representative gating strategy to define the expression of immune mediators

Cells were stimulated with media or the known epitope $gB_{492\text{-}500}$ and stained for T cell surface markers and the expression of CD107a, MIP-1β, TNF-α, IFN-γ, IL-2. Top, the progressive gating strategy used to define the $CD8^+$ population; middle and bottom, the results of the five functional responses of the $CD8^+$ T cells.

As shown in Figure 7, positive responses for a variety of immune mediators were detected to several known epitopes and to our novel minimal epitopes. The data are shown as bars representing the frequency of the listed mediator combination, with each color representing a different viral peptide. Each pie chart represents total responses to the listed peptide, with each color representing the number of cytokines produced. The ICS results revealed that in healthy HHV-8 seropositive individuals controlling infection, HHV-8 latency and lytic protein epitopes induced both monofunctional and polyfunctional $CD8^+$ T cell responses (Figure 7). Although all of the peptides displayed a trend of more monofunctional responses than polyfunctional responses, the differences were not significant. Taking into consideration all of the HHV-8 epitopes tested, all five immune mediators were produced by monofunctional T cells, with a predominance of IFN-γ, IL-2 and CD107a (Figure 7). Polyfunctional T cells producing two immune mediators were notable for the combinations of IFN-γ and IL-2 and for MIP-1β and IL-2. Predominant patterns of polyfuctional responses for three immune mediators included IFN-γ, TNF-α and CD107a, and MIP-1β, IL-2 and CD107a. Polyfunctional T cells producing four immune mediators included IFN-γ, TNF-α, MIP-1β and CD107a, and IFN-γ, TNF-α, IL-2 and CD107a.

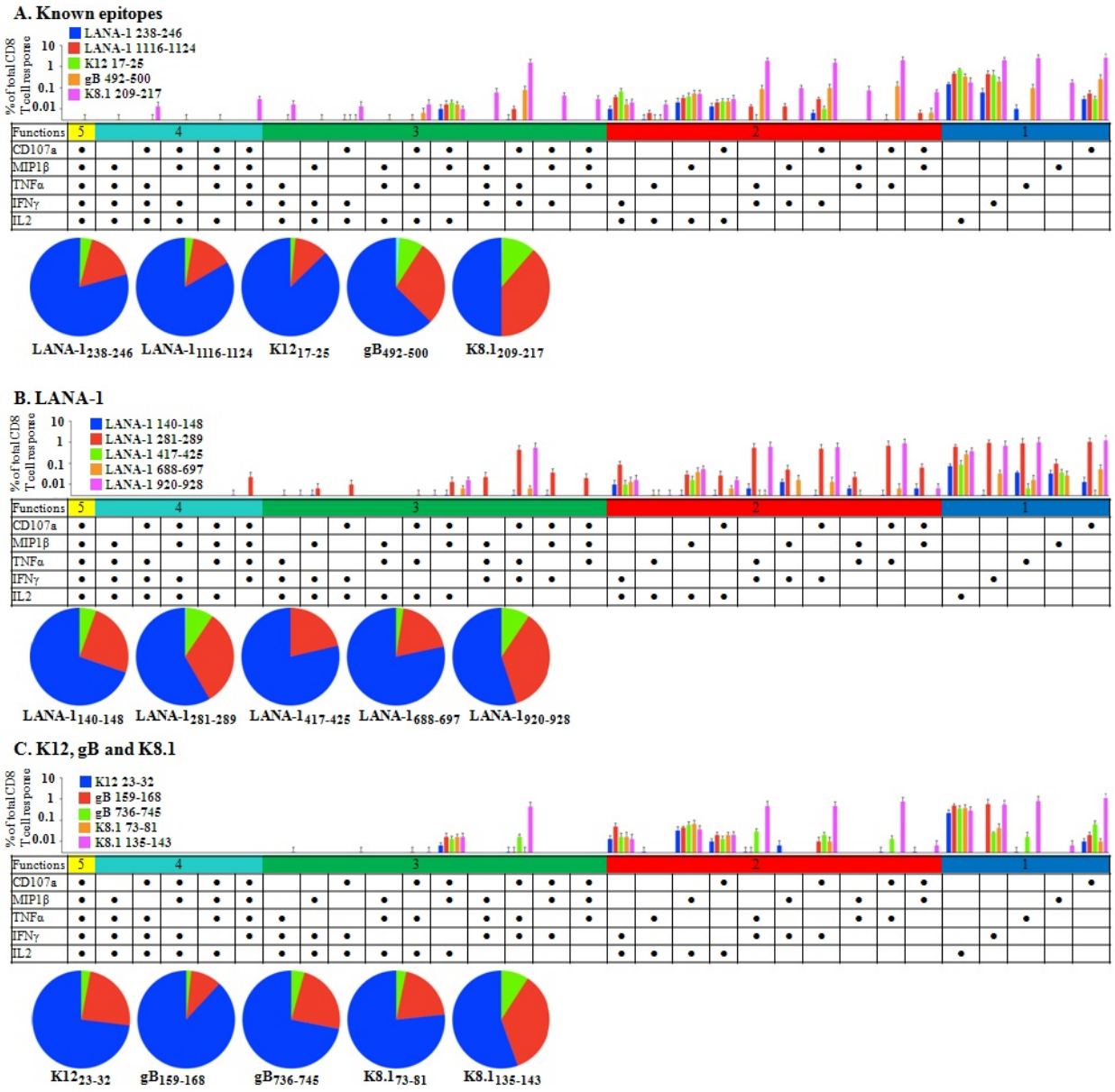

Figure 7. Polyfunctional CD8 T cell responses to known and novel HHV-8 epitopes

PBMC from HLA A*0201, HHV-8 seropositive healthy donors were stimulated for one week with autologous mature DC loaded with known HHV-8 epitopes (A) or novel epitopes from LANA-1 (B), K12, gB or K8.1 (C). Responses were measured by polychromatic flow cytometry and net responses were averaged for three donors (mean + SE). Diagrams were generated using SPICE.

Different HHV-8 epitopes induced different patterns of both monofunctional and polyfunctional responses. For example, the known epitope from gB ($gB_{492-500}$) induced notable monofunctional responses consisting of IL-2 or CD107a, and polyfunctional responses MIP-1β and IL-2, and IFN-γ, TNF-α and CD107a (Figure 7A). The known epitope K8.1 ($K8.1_{209-217}$) displayed notable monofunctional responses consisting of IFN-γ or TNF-α, and polyfunctional responses in the combinations of CD107a and TNF-α, MIP-1β, TNF-α and IFN-γ, and MIP-1β, TNF-α, IFN-γ and CD107a (Figure 7A).

The traditional method to map epitopes is through an IFN-γ ELISPOT assay. However, while analysis using one marker allows for the determination of response magnitude, the inclusion of several markers provides insight regarding the quality of the response. Therefore, along with our novel and known HHV-8 epitopes, we also investigated polyfunctional responses to one peptide family from each of the four HHV-8 proteins included in our study (Figure 8). While trends were evident, no single peptide had a significantly higher magnitude of responses or more polyfunctional responses than the other peptides within the peptide families examined. Taken together, these results show that the $CD8^+$ T cell epitopes from the four HHV-8 proteins presented by DC induced a variety of both monofunctional and polyfunctional T cell responses in HHV-8 seropositive healthy donors. The specificity of this T cell response is less evident in polyfunctional T cell determinations.

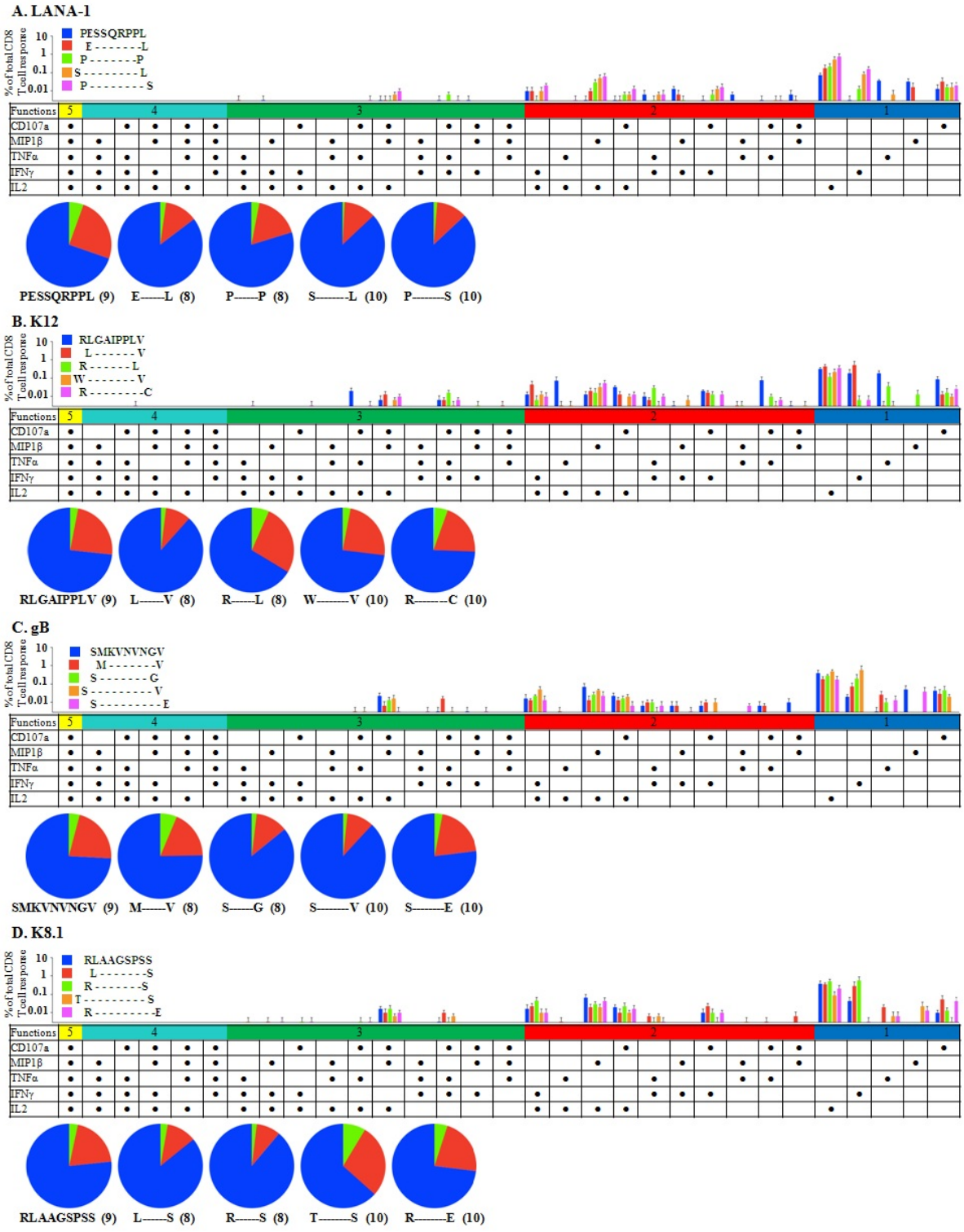

Figure 8. Polyfunctional CD8 T cell responses to HHV-8 peptide families

PBMC from HLA A*0201, HHV-8 seropositive healthy donors were stimulated for one week with autologous mature DC loaded with peptide. Peptide families are shown for LANA-1 17 (A), K12 7 (B), gB 44 (C) and K8.1 21 (D). Responses were measured by polychromatic flow cytometry and net responses were averaged for three donors (mean + SE). Diagrams were generated using SPICE.

2.4 DISCUSSION

It is postulated that $CD8^+$ T cell responses play a significant role in controlling HHV-8 infection and preventing development of KS (17, 93). A central issue in understanding the $CD8^+$ T cell immunity in HHV-8 infection is the identification of MHCI epitopes for the more than 80 ORFs of the virus. This has been difficult to do mainly because of relatively non-robust $CD8^+$ T cell responses to the virus (144). In this study, we employed a DC-based enhancement of $CD8^+$ T cell responses to four HHV-8 lytic and latency proteins modified from our previous approach (244) to provide a greater insight to immunopathogenesis of the virus. Using this DC-enhanced, IFN-γ ELISPOT assay, we have revealed five LANA-1, one K12, two gB and two K8.1, novel, HLA A*0201-restricted, $CD8^+$ T cell epitopes. We were also able to detect epitope-specific $CD8^+$ T cells directly in the peripheral blood using HLA A*0201 pentamer complexes specific for one gB, one K8.1, two LANA-1 and one K12 epitope. These results were expanded and confirmed by using a DC-enhanced ICS assay with polychromatic flow cytometry to detect multiple immune mediators. By this approach, we revealed that HHV-8 seropositive healthy donors controlling infection have circulating, monofunctional and polyfunctional $CD8^+$ T cells specific for an array of HHV-8 lytic and latency protein epitopes.

The novel epitopes in our study did not all correspond to published motifs for the preferred HLA A*0201 anchor residues at positions 2 and 9. Such lack of correspondence to MHCI allele-specific, peptide motifs has been recognized for T cell epitopes of other viruses. For example, the immunodominant, HLA A*0201-restricted, influenza A virus $M1_{58-66}$ epitope GILGFVFTL does not have the preferred HLA A*0201 anchor residue L or M in position 2 (215). Moreover, over half of the HLA A*0201-restricted epitopes for vaccinia virus do not fit the optimal peptide binding motif (215). Aside from binding to MHCI molecules, there are other

important factors that determine the T cell response to a peptide, such as the presence $CD8^+$ T cell precursors, interactions with T cell receptors and peptide transporters, and generation of peptides by different protease cleavage pathways (18, 163). Furthermore, our epitopes were determined by two different functional assays (ELISPOT and ICS), and represent $CD8^+$ T cell positive reactivity in HLA A*0201, HHV-8 seropositive individuals.

Given the large number of ORFs in HHV-8, we focused on ORFs that code for lytic and latency proteins that are considered important in HHV-8 pathogenesis and oncogenesis. We selected two latency cycle proteins, LANA-1 and K12, and two lytic cycle proteins, gB and K8.1. The lytic protein gB is a virion glycoprotein that binds cell surface heparan sulfate and induces signaling pathways, and the lytic protein K8.1 is a highly immunogenic (antibody inducing) virion glycoprotein that also binds cell surface heparan sulfate (244, 251). During latency, LANA-1 is a cell cycle regulatory protein important in anti-apoptotic functions and episome maintenance. The K12 (kaposin) latency protein has roles in B cell signaling, apoptosis and cell transformation induction (93, 163). While we do acknowledge that a limitation of our study is the small sample size, we found T cell reactivity to peptides of these lytic and latency proteins, indicating that immunity to each could be important in control of HHV-8 infection. In our next study, we then applied a battery of our newly identified and previously documented epitopes to assess T cell immunity to HHV-8 infection and progression to KS in the Multicenter AIDS Cohort Study.

Remarkably, given the need to use DC to reveal this T cell function, direct staining of PBMC with HLA A*0201 pentamer complexes for two lytic and three latency protein epitopes identified antigen-specific $CD8^+$ T cells circulating in peripheral blood. HHV-8 specific $CD8^+$ T cells have been previously detected in the blood of HHV-8 seropositive patients, including

transplant recipients, and those with AIDS-related and classical KS (128). However, to our knowledge, this is the first direct evidence of HHV-8-specific $CD8^+$ T cells in blood of healthy HHV-8 seropositive individuals. In our next study, we determined the memory phenotype of these circulating T cells, and investigated if there is a functional down-regulation of these cells.

Previous studies have shown that the central repeat region of LANA-1 inhibits proteasomal degradation and slows protein synthesis in order to inhibit cellular surveillance for $CD8^+$ T cell epitopes (126). This model predicts that host T cell reactivity to LANA-1 would be minimal. However, we detected $CD8^+$ T cell reactivity to several regions of LANA-1 in these normal, healthy HHV-8 seropositive subjects. Others have shown anti-LANA-1 $CD8^+$ T cell responses in patients with KS (93). Similarly, $CD8^+$ T cell recognition has been found for EBV-encoded nuclear antigen 1 (134, 232), which previously had been proposed to escape $CD8^+$ T cell recognition through *cis*-inhibition of synthesis or blockade of proteasomal degradation by its glycine-alanine repeat domain. Thus, it appears that $CD8^+$ T cells can mount functional responses to certain regions of LANA-1 regardless of *cis* effects on its production.

Polyfunctional $CD8^+$ T cells, i.e., cells producing more than one immune mediator, are associated with superior control of persistent viral infections such as HIV-1 (16, 153). A recent investigation found similar patterns of polyfunctional T cell responses in patients with MCD and normal controls (94). Other investigators have reported that patients with non-progressive KS have stronger and more frequent polyfunctional $CD8^+$ T cell responses than those with progressive KS (17). It is not known, however, whether polyfunctional $CD8^+$ T cell are involved in control of latent HHV-8 infection in healthy, HHV-8 seropositive persons. In the present study, we established that polyfunctional T cells specific for known and novel epitopes are present in the PBMC of such individuals. We found that in these healthy, non-

immunosuppressed subjects controlling HHV-8 infection, there was a trend of more monofunctional $CD8^+$ T cell responses compared to polyfunctional responses for all of the epitopes. Overall, our data suggest that the tight control of HHV-8 infection in normal individuals may not require a predominance of circulating, virus-specific, polyfunctional $CD8^+$ T cell reactivity. Furthermore, we found that different epitopes induced different patterns of monofunctional and polyfunctional responses. Similar results were found for T cell responses to influenza virus, where $CD8^+$ T cells specific for some but not all viral proteins produced a wide range of cytokines (127). This could be explained by differences in T cell avidity for the peptide-MHC complex.

Traditionally, the standard in monitoring $CD8^+$ T cell responses has been measuring single cell IFN-γ production in response to antigenic stimuli by an overnight ELISPOT assay. While analysis using one marker allows for the determination of response magnitude, the inclusion of several markers provides insight regarding response quality. Therefore, we examined ICS responses to families of putative optimal epitopes consisting of peptides with N- and C-terminal extensions and truncations from each viral protein to further delineate the minimal epitope sequences. We found that there were different patterns of T cell reactivity revealed by ICS as compared to the ELISPOT assay. This stresses the need to move beyond IFN-γ ELISPOT assays in defining T cell immunity to viral infections (16, 229). The data also suggest that $CD8^+$ T cells can mount diverse, functional reactivity to 8-10mer variants of an MHCI 9mer epitope when presented by DC. This is supported by a recent finding in our laboratory that DC are able to generate efficient, monofunctional and polyfunctional T cell responses against 8-10mer N- and C-terminal variants of HIV-1 Gag and Nef epitopes (108).

This enhancement of T cell responses by DC to peptide variations could also explain the broad spectrum of ELISPOT responses evident in our HHV-8 epitope mapping study.

In conclusion, we have revealed several novel HLA A*0201 minimal epitopes in both HHV-8 lytic and latency proteins. We have also shown the presence of antigen-specific $CD8^+$ T cells in the peripheral blood specific for two lytic and three latency protein epitopes. We have demonstrated that epitopes within these HHV-8 lytic and latency proteins induced both single and multiple immune mediators in $CD8^+$ T cells from healthy, HHV-8 seropositive individuals. The likely involvement of $CD8^+$ T cells in the immune response to HHV-8 infection has implications for the prevention and treatment of HHV-8-associated cancers. These targeted regions of the virus that induce immune responses by $CD8^+$ T cells could be critical in HHV-8 immunopathogenesis and the progression to KS.

2.5 ACKNOWLEDGMENTS

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3.0 T CELL RESPONSES DURING THE PROGRESSION TO KS

$CD8^+$ T cells are considered important in controlling HHV-8 infection, the causative agent of KS and some B cell lymphomas. However, these T cell responses are relatively non-robust, suggesting that they are under tight regulatory control. We have conducted a longitudinal study of the effect of Treg on $CD8^+$ memory T cell reactivity to HHV-8 lytic and latency proteins after HHV-8 infection, with and without HIV-1 coinfection, and subsequent development of KS in the MACS. Direct staining of PBMC with MHCI multimers showed a relatively high frequency of circulating, HHV-8 lytic and latency antigen-specific $CD8^+$ T cells, but negative or very low IFN-γ production by ELISPOT assay. Removal of Treg significantly enhanced IFN-γ production of $CD8^+$ T cells against HHV-8 lytic and latency antigens throughout the course of HHV-8 infection, with or without HIV-1 coinfection. Anti-HHV-8 and anti-HIV-1 T cell activity were lower during the years prior to development of KS compared to subjects who did not develop KS. This was related to a higher number of circulating Treg but less enhancement of the antiviral $CD8^+$ T cell response after removal of Treg prior to KS. These data show for the first time that Treg suppress $CD8^+$ T cell responses to HHV-8 lytic and latency antigens, effectively masking more robust, underlying anti-HHV-8 T cell responses. During HIV-1 coinfection, decreases in these antiviral T cell responses, even after removal of the Treg effect, are related to subsequent development of KS. The involvement of $CD8^+$ T cells and Treg in control of HHV-8

infection has important implications for understanding the immunopathogenesis of HHV-8 infection and development of vaccines to prevent KS.

3.1 BACKGROUND

HHV-8 is the etiologic agent of KS, a neoplasm of endothelial origin, which is the most common cancer associated with HIV-1 infection and AIDS (48). While the incidence of KS in HIV-1 infected individuals has decreased with the use of effective ART (81), KS still occurs in those on ART with suppressed HIV-1 infection (159). The success of ART in treating HIV-1 associated KS has been countered by the occasional occurrence of immune reconstitution inflammatory syndrome (73). This is a severe, temporary enhancement of KS lesions due to an increase in inflammation and immunologic recovery after ART. The development of effective treatments of the infection and cancer with drugs, or the prevention of both with a vaccine, requires an intimate understanding of the pathogenesis and immune control of HHV-8 infection.

Although immunity to HHV-8 is far less well defined than that to the other human gammaherpesvirus, EBV, $CD8^+$ T cells likely play a similar, critical role in viral control (105). In support of this hypothesis, our laboratory has previously found that $CD8^+$ T cells specific for several HHV-8 lytic proteins are present in the blood in the first few months of primary infection, which peaks within two years of infection then decreases to low but detectable levels (245). We have also shown that HHV-8 monofunctional and polyfunctional $CD8^+$ T cells are present in healthy, HHV-8 seropositive adults controlling infection (135). To date, there are minimal data on whether alterations in anti-HHV-8 T cell responses are directly related to KS development (93, 128), and if the lower incidence of KS in HIV-1 infected individuals on ART

is related to increases in anti-HHV-8 T cell responses (19, 27). Therefore, comprehensive longitudinal studies are needed to accurately assess the role of anti-HHV-8 T cell immunity in the development of KS.

While $CD8^+$ T cell responses to both HHV-8 lytic and latency proteins are evident in HHV-8 seropositive individuals, the immune response to this virus is relatively non-robust compared to T cell reactivity to other human herpesviruses, such as EBV (20, 135). This suggests that the number and/or functional capacity of circulating anti-HHV-8 T cells is relatively low. However, using direct, multimer staining, we have found that a reasonably high level of these circulating T cells specific for HHV-8 proteins is present in seropositive individuals (135). Therefore, we hypothesized that anti-HHV-8-specific $CD8^+$ T cells are functionally down-regulated by Treg. In support of this hypothesis, these regulatory cells have been found to play a role during other viral infections, including EBV (139) and HIV-1 (151). However, it is currently unknown if these cells are important during HHV-8 infection and disease development.

To provide insight into the importance of $CD8^+$ T cell responses and the effect of Treg on the development of KS, we conducted a non-concurrent, prospective, multiparameter study of T cell immunity in HHV-8 infected participants in the MACS who did or did not develop KS. We found that anti-HHV-8 T cell reactivity was under control of Treg in healthy HHV-8 seropositive persons. During HHV-8/HIV-1 coinfection, decreases in these antiviral T cell responses, even after removal of the Treg effect, were related to development of KS. The involvement of $CD8^+$ T cells and Treg in control of HHV-8 infection has important implications for our understanding of HHV-8 immunopathogenesis and development of vaccines to prevent KS.

3.2 METHODS

3.2.1 Study participants and samples

Participants were chosen from the Pittsburgh site of the MACS, a national longitudinal study of the natural history of HIV-1 infection in men who have sex with men that began in 1984 (118). Approximately every 6 months, participants visit the clinics to provide epidemiologic information and biologic samples, which are obtained with informed consent and following protocols approved by institutional review boards. Clinical information is recorded in the MACS database, and biologic samples are cryopreserved and stored in local and national repositories.

Participants were chosen based on HIV-1 and HHV-8 infection status, as well as clinical diagnosis of KS (112). Additionally, participants were chosen based on their MHC class I type, as known HLA A*0201-restricted epitopes were used in the study assays. An indirect immunofluorescence assay was used to detect HHV-8 serum antibodies (112). HHV-8 viral load was determined by PCR as previously described (178, 187, 219). HIV-1 viral load was determined using Roche Ultrasensitive RNA PCR assay (Hoffman-LaRoche). T cell counts were determined using flow cytometry (207). The participants were classified into HIV-1-positive/HHV-8-positive/KS-negative or KS-positive groups, with 10 participants in the KS-negative group and five participants in the KS-positive group. Four HIV-1-negative/HHV-8-positive individuals were included to investigate the natural history of HHV-8 infection without the presence of HIV-1 co-infection. Three HIV-1-negative/HHV-8-negative were included as controls. PBMC and serum samples were chosen within a year of KS diagnosis and the corresponding time point for those that did not develop KS. Four additional time points were chosen of 8-12, 6-8, 4-6 and 2-4 years prior to KS diagnosis. Average T cell counts and HIV-1

and HHV-8 viral loads at the chosen visits are shown below. All participants were Caucasian men, with an average age of 35 (range 22-64) at study entry.

3.2.2 Dextramer staining

To determine the frequency of epitope-specific $CD8^+$ T cells, PE-conjugated HLA A*0201 dextramers were used (Immudex). Dextramers were specific for five HHV-8 epitopes (LANA-$1_{281-289}$ (135), LANA-$1_{1116-1124}$ (93), $K12_{17-25}$ (28), $gB_{492-500}$ (244) and $K8.1_{135-143}$ (135)). As controls, detraxmers specific for an immunodominant HIV-1 (SL9, $p17_{77-85}$) epitope (146), an immunodominant CMV (N9V, $pp65_{495-503}$) epitope (252), and an HLA A*0201 negative dextramer were also included. Staining was performed according to the manufacturer's protocol. Briefly, frozen PBMC (1-2 million cells per dextramer well) were thawed, washed and resuspended in 100µL PBS (GIBCO) per well containing 1µL per well aqua viability dye (Invitrogen) for 30 min at room temperature in the dark. The cells were washed with PBS containing 5% FCS (Cellgro) and incubated with 10µL of dextramer for 10 min at room temperature in the dark. Cells were then stained for 30 min in the dark at 4ºC with CD8-PerCP-Cy5.5 (BD, 20µL per well), CD3-allophycocyanin-Cy7 (BD, 5µL per well), CD45RA-PE-Cy7 (BD, 5µL per well), CD57-FITC (BD, 20µL per well), CCR7-allophycocyanin (R&D, 10µL per well) and CD27-PC5 (Beckman Coulter, 10µL per well). CD4-V450, CD19-V450, and CD14-V450 (all from BD, 5µL each per well) were also added for exclusion gating. Cells were washed twice and fixed with PBS containing 1% paraformaldehyde (PFA). Samples were analyzed with an LSR II flow cytometer (BD Immunocytometry Systems), and results were analyzed using BDFACS Diva (version 6.0) software.

3.2.3 Treg staining

To determine the frequency of Treg, frozen PBMC (1-2 million cells per well) were thawed, washed and resuspended in 100µL PBS per well containing 1µL per well aqua viability dye for 30 min at room temperature in the dark. The cells were washed and then stained for 30 min in the dark at room temperature with CD4-V450, CD8-PerCP-Cy5.5, CD3-allophycocyanin-Cy7 and CD25-allophycocyanin (BD, 5µL per well). Samples were then washed twice and fixed with PBS containing 1% PFA. Samples were analyzed with an LSR II flow cytometer, and results were analyzed using BDFACS Diva software.

3.2.4 Treg depletion and cell stimulation

To investigate the effect of Treg on $CD8^+$ T cell function, frozen PBMC samples were thawed and depleted of Treg using a Treg $CD4^+CD25^+$ isolation kit (Miltenyi Biotec). Briefly, this two-step column-based kit uses magnetic beads to first negatively select $CD4^+$ cells by labeling all the non-CD4 cells. Treg are then positively selected from the $CD4^+$ fraction using anti-CD25 mAb-coated magnetic beads. Treg-depleted samples consisted of the non-CD4 fraction from the first step plus the $CD4^+CD25^-$ non-Treg fraction from the second step. To assess HHV-8 T cell reactivity, PBMC and PBMC that were depleted of Treg were stimulated with four different peptide pools. An HHV-8 latent antigen pool consisted of nine HLA A*0201 eptiopes (LANA-$1_{140\text{-}148}$ (135) , LANA-$1_{238\text{-}246}$ (93), LANA-$1_{281\text{-}289}$, LANA-$1_{417\text{-}425}$ (135), LANA-$1_{688\text{-}697}$ (135), LANA-$1_{920\text{-}928}$ (135), LANA-$1_{1116\text{-}1124}$, $K12_{17\text{-}25}$, $K12_{23\text{-}32}$ (135), all from Sigma). An HHV-8 lytic antigen pool consisted of six HLA A*0201 epitopes ($gB_{159\text{-}168}$ (135), $gB_{492\text{-}500}$, $gB_{736\text{-}745}$ (135) , K8.$1_{73\text{-}81}$ (135), K8.$1_{135\text{-}143}$, K8.$1_{209\text{-}217}$ (27), all from Sigma). As controls, a CEF peptide

pool was used (32 MHCI T cell epitopes from CMV, EBV and FLU (55), National Institutes of Health [NIH] AIDS Reagent Program). A pool consisting of 12 HLA A*0201 epitopes from HIV-1 was also used ($p17_{77–85}$, $p2p7p1p6_{70–79}$, $p24_{19-27}$, $PR_{76–84}$, $RT_{33–41}$, $RT_{179–187}$, $RT_{309–317}$, $Vpr_{59–67}$, $gp160_{311–320}$, $gp160_{813–822}$, $Nef_{136–145}$, $Nef_{180–189}$, (146), all from Sigma). Cells stimulated with RPMI 1640 medium (GIBCO) supplemented with 10% FCS were used as a negative control.

3.2.5 Single-cell IFN-γ production

To assess T cell reactivity, a standard overnight IFN-γ ELISPOT assay was performed. Briefly, nitrocellulose-bottomed, 96-well ELISPOT plates (Millipore) were precoated with an anti-IFN-γ mAb (10 µg/ml, Mabtech) overnight at 4°C and then blocked for 2 h at 37°C with RPMI 1640 medium supplemented with 10% heat-inactivated human AB^+ serum (Gemini Bio-Products). PBMC (100,000 cells per well) or PBMC depleted of Treg (100,000 cells per well) were stimulated with peptide pools (10µg/mL) or medium and added to ELISPOT plates, cultured overnight at 37°C, and developed as previously described (50). Spots were counted using an ELISPOT reader (AID). The results were expressed as net numbers of virus-specific spot-forming cells per million cells by subtracting the number of spots in cultures stimulated with medium from the number of spots induced by peptide.

3.2.6 Serum biomarker assay

Frozen serum samples were thawed and tested for the presence of several biomarkers relating to Treg function and KS development using an electrochemiluminescence Meso Scale Discovery

(MSD) multi-array assay. Samples were tested using ultra-sensitive kits for human IFN-γ, IL-10, IL-2, IL-6, IL-8, TNF-α, IL-1β, IL-12, IL-13, IL-4, IL-5, MIP-1β, MIP-1α and TGF-β, and a human serum kit for VEGF (MSD). All assays were performed according to the manufacturer's protocol for serum samples. Both samples and standards were performed in duplicate. Plates were read on a SECTOR Imager 2400 electrochemilumuinescence machine (MSD) and data were analyzed using the Discovery Workbench (version 3, MSD).

3.2.7 Statistics

We used ANOVA and the Student *t* test for comparisons between groups. Differences were considered significant with p-value<0.05.

3.3 RESULTS

3.3.1 Levels of HHV-8 specific $CD8^+$ T cells over many years prior to KS

We have previously shown that both monofunctional and polyfunctional $CD8^+$ T cells specific for HHV-8 lytic and latency proteins are present in healthy, HHV-8 seropositive adults controlling infection (135). In order to investigate the role these cells play in controlling HHV-8 infection to prevent disease development, we applied multimer staining to PBMC from MACS subjects obtained many years prior to KS development. Subjects who were co-infected with HIV-1 and HHV-8 and developed KS had significantly lower $CD4^+$ T cell counts than HHV-8/HIV-1 co-infected non-KS controls 4-6 years prior to KS (p=0.023) that persisted through KS

diagnosis ($p<0.001$ at both 2-4 and 0-1) (group averages in Figure 9B, individual donors in Appendix C). Levels of $CD3^+$ T cells ($p=0.014$) (Figure 9A) and $CD8^+$ T cells ($p=0.013$) (Figure 9C) were also significantly different between groups within a year of KS. This immunologic deficit was not associated with differences in plasma viral load for HIV-1 until one year prior to KS ($p=0.002$) (Figure 9D). Although not significant, plasma viral load for HHV-8 was also higher in the KS cases within a year of diagnosis of KS (Figure 9E).

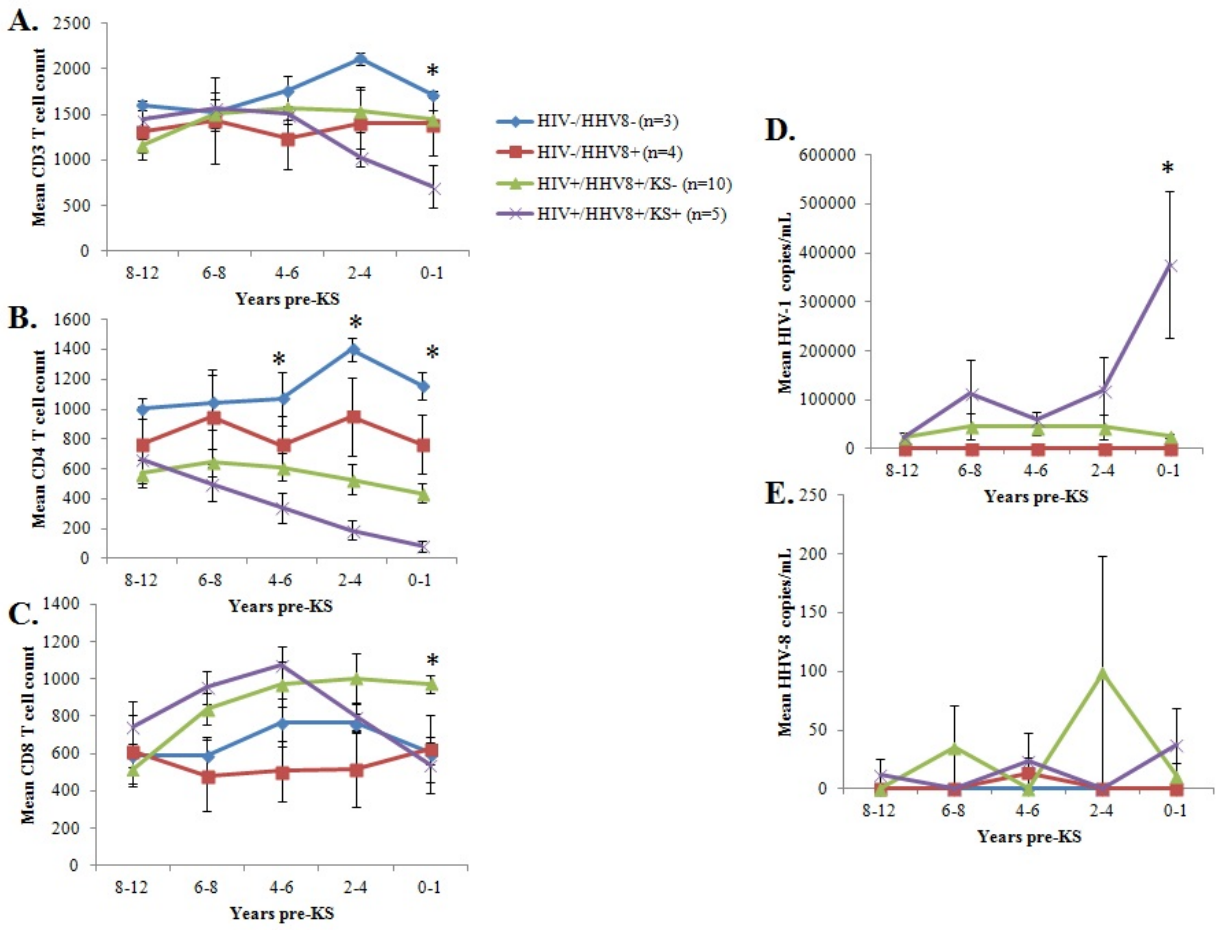

Figure 9. T cell counts and viral loads in MACS subjects

Three HIV-/HHV8-, four HIV-/HHV8+, 10 HIV+/HHV8+/KS- and five HIV+/HHV8+/KS+ subjects were selected from the Pittsburgh MACS. Five visits were chosen, with one visit within a year of KS development in the cases and the corresponding visit in the controls, a second visit 2-4, a third visit 4-6, a fourth visit 6-8 and a fifth visit 8-12 years prior to KS development. Mean (±SE) $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C) T cell counts at each visit were determined by flow cytometry. Mean (±SE) HIV-1 (D) and HHV-8 (E) plasma viral load at each visit were determined by PCR. (*= $p<0.05$ by ANOVA)

To directly investigate the frequency of HHV-8-specific $CD8^+$ T cells in the blood during the progression to KS, HLA A*0201 multimers were used for three latency epitopes (two LANA-1, one K12) and two lytic epitopes (one gB, one K8.1). In our previous studies, we used pentamers to define epitope-specific $CD8^+$ T cells (135). However, in our current studies, we used dextramers, as these have more MHC and fluorochrome molecules than pentamers. This produces higher affinity staining, which results in brighter epitope-specific staining and lower non-specific background staining. We confirmed this through a direction comparison of a CMV-specific pentamer and dextramer (see supplemental figures in Appendix B, Figure 19).

Using dextramer staining, higher levels of HHV-8-specific $CD8^+$ T cells were found in HHV-8 seropositive subjects than seronegative subjects (Figure 10). For all groups, relatively constant levels of HHV-8, CMV and HIV-1 epitope-specific $CD8^+$ T cells were found over time (Figure 10). The average frequency of HHV-8 latentcy and lytic epitope-specific $CD8^+$ T cells ranged from 0.1-0.3% in HIV-/HHV8+ subjects (Figure 10A), 0.05-0.2% in HIV+/HHV8+/KS- subjects (Figure 10B) and 0.01-0.2% in HIV+/HHV8+/KS+ subjects (Figure 10C). Additionally, cells specific for HHV-8 lytic epitopes tended to be higher than those specific for latency epitopes in all groups (Figure 10A-C). When comparing averages among groups, there was a significant difference of HHV-8 epitope-specific T cells as early as 6-8 years prior to KS development ($p<0.001$), which continued up to a year within KS ($p=0.002$ at 4-6 years, $p=0.001$ at 2-4 years and $p=0.001$ at 0-1 year) (Figure 10D). Levels were highest in the HIV-/HHV8+ group, lower in the HIV+/HHV8+/KS- group and lowest in those who developed KS (Figure 10D).

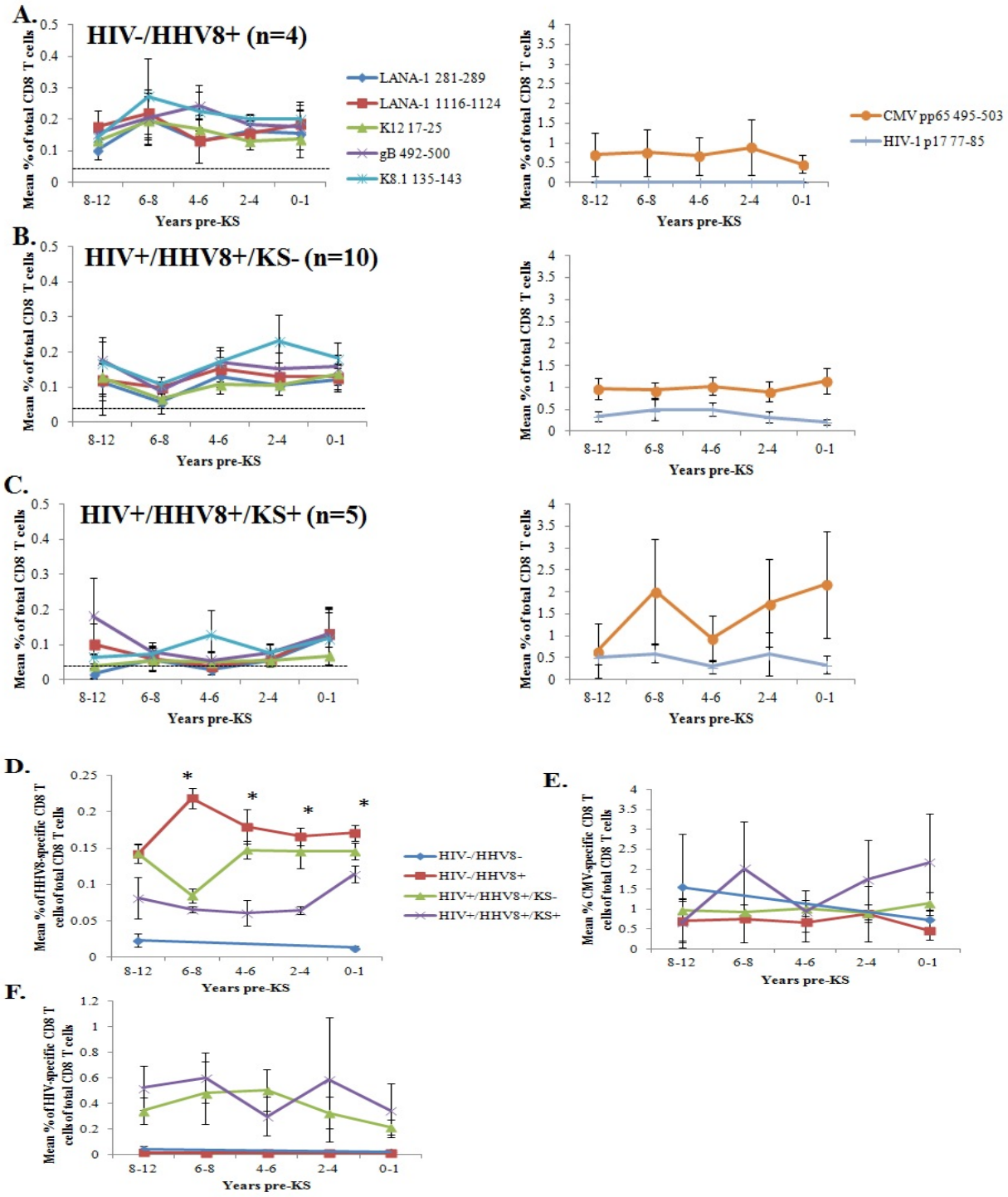

Figure 10. HHV-8 epitope-specific CD8 T cells over many years prior to KS

MACS subjects were used as described in Figure 9. Staining assays were performed using dextramers specific for five HHV-8 epitopes, one HIV-1 epitope and one CMV epitope. A negative HLA A*0201 dextramer was used as a control to set the staining gates. Mean (±SE) levels of epitope-specific cells are shown for HIV-/HHV8+ (A, n=4), HIV+/HHV8+/KS- (B, n=10) and HIV+/HHV8+/KS+ (C, n=5) subjects over time. Mean levels for HIV-/HHV8- subjects (n=3) are shown by the dotted line. Overall means compared by group are shown for HHV-8 (D), CMV (E) and HIV-1 (F) epitopes. (*= $p<0.05$ by ANOVA)

In all groups, frequencies of HIV-1-specific T cells were higher than HHV-8-specific cells, and frequencies were highest for CMV-specific cells (Figure 10). The average frequency of CMV epitope-specific $CD8^+$ T cells ranged from 0.4-0.9% in HIV-/HHV8+ subjects (Figure 10A), 0.9-1.2% in HIV+/HHV8+/KS- subjects (Figure 10B) and 0.6-2.1% in HIV+/HHV8+/KS+ subjects (Figure 10C). The average frequency of HIV-1 epitope-specific $CD8^+$ T cells ranged from 0.2-0.5% in HIV+/HHV8+/KS- subjects (Figure 10B) and 0.3-0.6% in HIV+/HHV8+/KS+ subjects (Figure 10C). Differences among groups were less evident for CMV-specific cells (Figure 10E) and HIV-1-specific cells (Figure 10F) than for HHV-8-specific cells.

Overall, these data show that HHV-8-specific $CD8^+$ T cells are lower many years prior to the development of KS, and continue to decrease until development of KS. T cell reactivity to HHV-8 lytic epitopes was higher than to latency epitopes throughout the time course of the study.

3.3.2 Phenotypes of $CD8^+$ T cells for virus-specific and nonspecific cells

As the state of differentiation of $CD8^+$ T cells is important for immune control of infections, we investigated the phenotype of epitope-specific and epitope-nonspecific $CD8^+$ T cells. We found similar patterns of phenotypes in both KS-negative and KS-positive subjects over time for both virus-specific and virus-nonspecific $CD8^+$ T cells (Figure 11A-C). Lowest levels of cells were found for naive and central memory cells, with higher levels of effector memory and terminally differentiated T cells. Over time, levels of effector memory T cells increased, while terminally differentiated T cells decreased. Although similar patterns were evident for HHV-8, CMV and HIV-1 specific cells, levels of terminally differentiated cells specific for HHV-8 (first column)

and CMV (second column) were initially higher than HIV-1 specific (third column) cells, while effector memory cells specific for HIV-1 were initially higher compared to the other viruses. Levels of naive and central memory cells were found at higher levels for virus-nonspecific cells (last column) compared to the virus-specific cells.

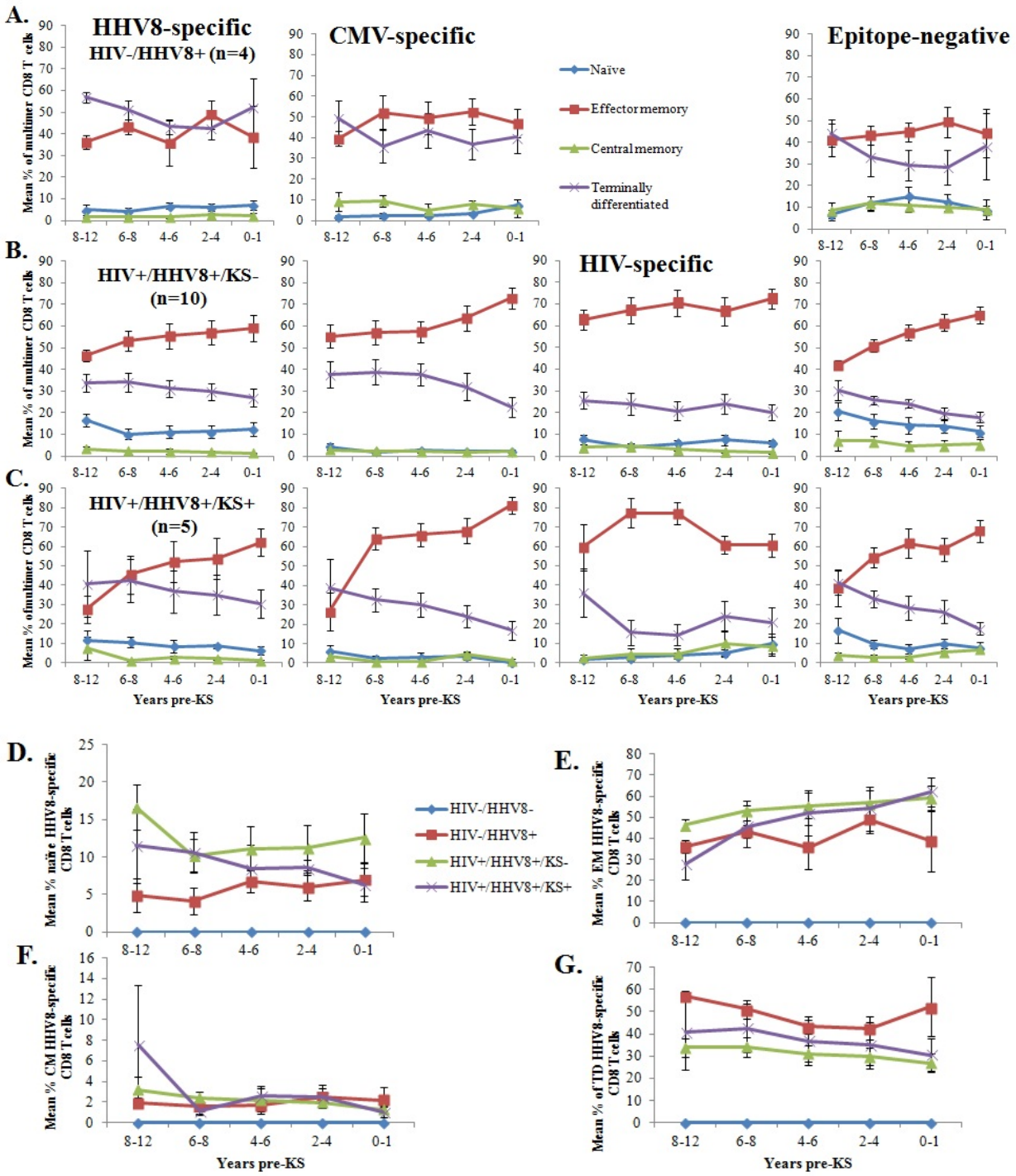

Figure 11. CD8 T cell phenotypes of virus-specific and non-specific cells

MACS subjects were used as described in Figure 9. Epitope-specific and epitope-nonspecific cells as defined by multimers were stained for phenotype markers. Mean (±SE) levels of each phenotype are shown for HIV-/HHV8+ (A, n=4), HIV+/HHV8+/KS- (B, n=10) and HIV+/HHV8+/KS+ (C, n=5) subjects over time for HHV-8-specific (first column), CMV-specific (second column), HIV-1-specific (third column) and epitope-negative (last column) $CD8^+$ T cells. Overall means compared by group are shown for HHV-8 epitope-specific cells that are naïve (D), effector memory (E), central memory (F) and terminally differentiated (G).

When comparing phenotypes of HHV-8-specific T cells among groups, the HIV+/HHV8+/KS- group had the highest levels of naive (Figure 11D) and effector memory (Figure 11E) cells, while the HIV-/HHV8+ group had the lowest levels. Similar levels of central memory cells were evident in all groups, although levels were initially highest in the HIV+/HHV8+/KS+ group (Figure 11F). For terminally differentiated cells, highest levels were in the HIV-/HHV8+ group, and lowest levels in the HIV+/HHV8+/KS- group (Figure 11G).

Overall, these data show similar patterns of $CD8^+$ T cell phenotypes for both virus-specific and non-virus-specific cells. However, the data on non-epitope-specific T cells suggest that this is an effect of aging over the years of the study.

3.3.3 Numbers of circulating Treg over many years prior to KS

While we have shown that both monofunctional and polyfunctional $CD8^+$ T cells specific for HHV-8 lytic and latency proteins are present in HHV-8 seropositive individuals, these T cell responses are relatively non-robust compared to other herpesviruses (135, 244). This suggests that either the number and/or functional capacity of circulating anti-HHV-8 T cells is low. However, we found these $CD8^+$ T cells are present in seropositve individuals (Figure 10). This led us to investigate if HHV-8-specific $CD8^+$ T cells are functionally down-regulated by Treg.

In HIV-/HHV8- (Figure 12A), HIV-/HHV8+ (Figure 12B) and HIV+/HHV8+/KS- (Figure 12C) subjects, a low percentage of Treg was present that remained stable over time. In HIV+/HHV8+/KS+ subjects (Figure 12D), a low percentage of Treg was present that began to increase 2-4 years prior to KS, which continued to increase within a year of KS. Higher percentages of $CD8^+$ compared to $CD4^+$ T cells were present in HIV-1 seropositive subjects (Figure 12C-D).

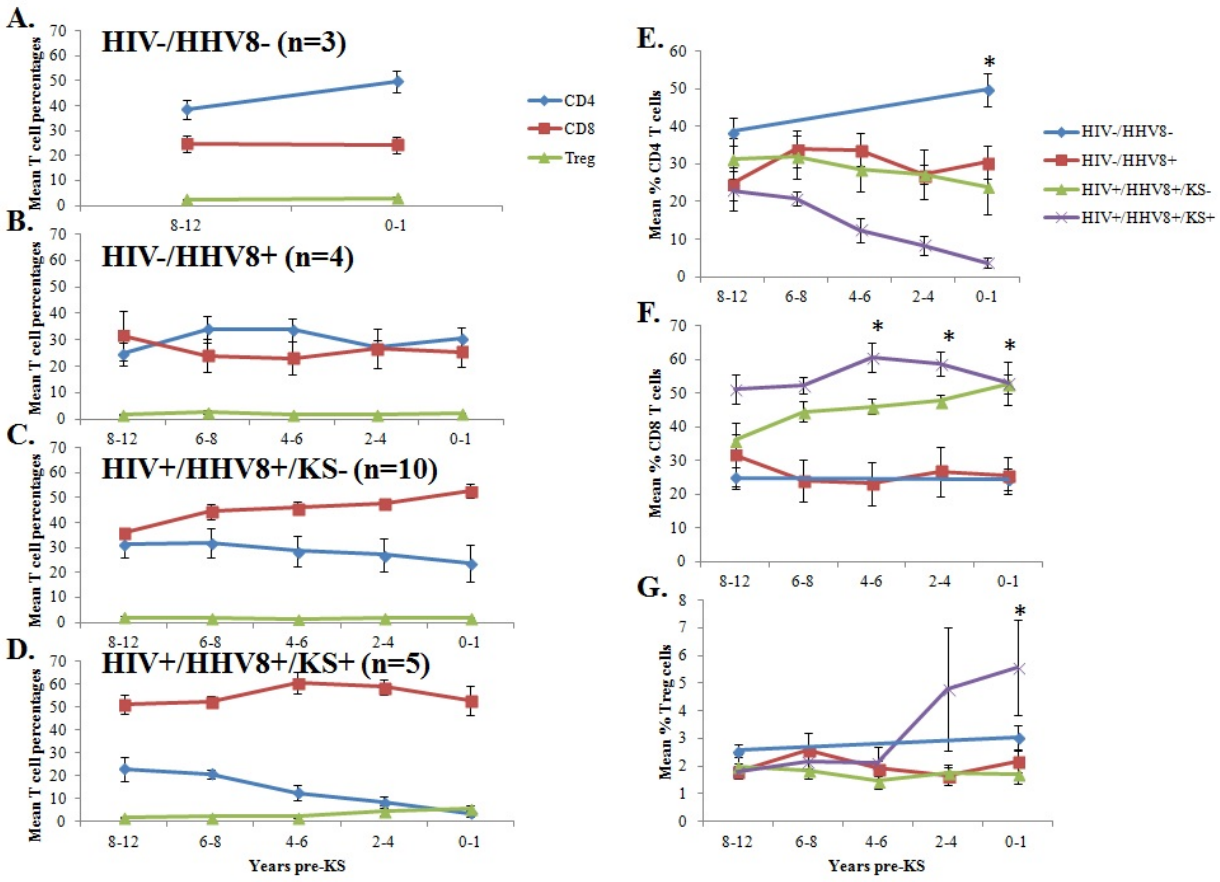

Figure 12. Numbers of circulating Tregs over many years prior to KS

MACS subjects were used as described in Figure 9. Mean (±SE) $CD4^+$ T cell, $CD8^+$ T cell and Treg percentages were determined by flow cytometry for HIV-/HHV8- (A, n=3), HIV-/HHV8+ (B, n=4), HIV+/HHV8+/KS- (C, n=10) and HIV+/HHV8+/KS+ (D, n=5) subjects over time. Overall means compared by group are shown for $CD4^+$ T cells (E), $CD8^+$ T cells (F) and Treg (G). (*= $p<0.05$ by ANOVA)

When comparing T cell percentages among groups, the lowest $CD4^+$ T cell percentages were present in the KS-positive group, with significantly lower levels within a year of KS development ($p=0.012$) (Figure 12E). $CD8^+$ T cell percentages were significantly different as early as 4-6 years prior to KS ($p<0.001$) that persisted through KS development ($p<0.001$ at both 2-4 and 0-1) (Figure 12F). Levels of Treg began to increase in KS-positive subjects 2-4 years prior to KS, with significantly higher levels compared to the non-KS groups within a year of KS diagnosis ($p=0.021$) (Figure 12G). Taken together, these data suggest that Treg could play a role in KS development as their numbers increase in the years leading to KS.

3.3.4 Effect of Treg depletion on anti-HHV-8 $CD8^+$ T cell responses

We initially investigated the effect of Treg on anti-HHV-8 T cell responses using healthy, HHV-8 seropositive individuals using single peptides from HHV-8, CMV and FLU. As we used DC-enhanced, 7 day cultures in our previous studies (135), we first compared these cultures with standard, overnight cultures in an ELISPOT assay. Responses of PBMC cultured with peptide were compared to responses of PBMC that were depleted of Treg prior to culture with peptide in an ELISPOT assay. We found that we were able to detect responses against HHV-8 epitopes when we removed Treg in standard, overnight cultures (see supplemental figures in Appendix B, Figure 20). Based on these results and due to large cell numbers required in DC-enhanced cultures, we used standard, overnight cultures in further studies. We next verified these overnight Treg depletion results in additional healthy, HHV-8 seropositive subjects. We found that removal of Treg enhanced anti-HHV-8 T cell responses in healthy seropositive, but not seronegative, subjects (see supplemental figures in Appendix B, Figure 21). Therefore, we next

examined Treg function in MACS subjects over many years prior to KS development. Pools of HHV-8 latency peptides, HHV-8 lytic peptides, CEF peptides and HIV-1 peptides were used.

We found that removal of Treg from PBMC enhanced IFN-γ production in all subject groups and for all peptide pools over many years (Figure 13A-D). We confirmed that this effect was not attributable to an activation of T cells during the bead and column depletion method, or to a proportional increase in number of $CD8^+$ T cells after removal of Treg (see supplemental figures in Appendix B, Figure 22). Differences for PBMC compared to PBMC depleted of Treg were significant in the HIV-/HHV8+ group for the HHV-8 lytic pool at 6-8 years ($p=0.012$) and the CEF pool at 0-1 year ($p=0.029$) (Figure 13B). Differences were significant in the HIV+/HHV8+/KS- group at 4-6 and 0-1 years for both the HHV-8 latency ($p=0.028$ for 4-6 and $p=0.021$ for 0-1) and lytic pools ($p=0.005$ for 4-6 and $p=0.014$ for 0-1) (Figure 13C). Differences in this group were also significant at 8-12 ($p=0.011$) and 2-4 years ($p<0.001$) for the CEF pool and 4-6 years ($p=0.008$) for the HIV-1 pool (Figure 13C). For the HIV+/HHV8+/KS+ group, differences were significant 4-6 years prior to KS development for both the HHV-8 latency ($p=0.022$) and lytic pools ($p=0.038$) (Figure 13D).

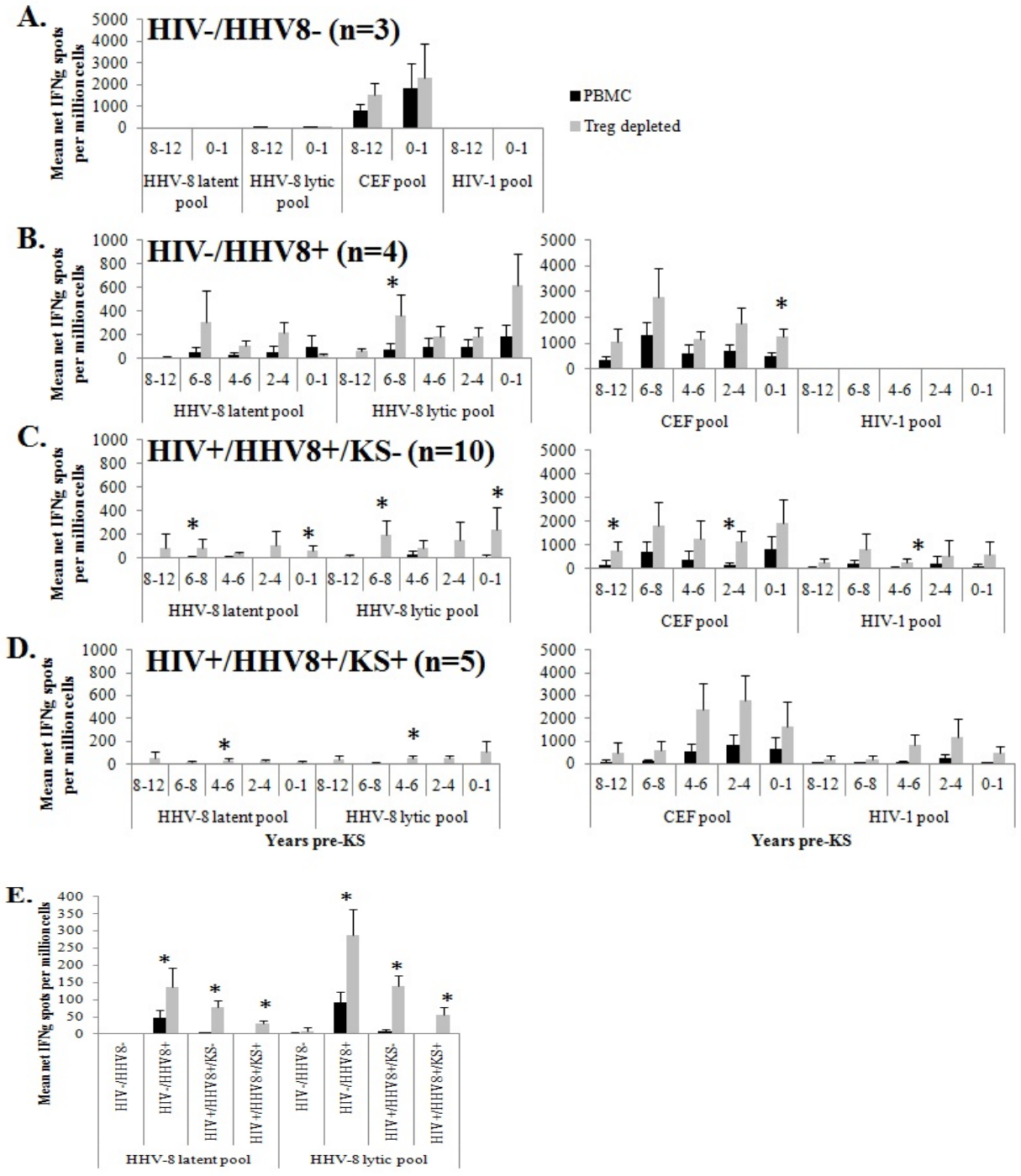

Figure 13. Effect of Treg depletion on anti-HHV-8 CD8 T cell responses

MACS subjects were used as described in Figure 9. PBMC or PBMC depleted of Treg by a magnetic bead depletion system were cultured with an HHV-8 latency peptide pool, an HHV-8 lytic peptide pool, a CEF peptide pool or an HIV-1 peptide pool overnight in a standard IFN-γ ELISPOT assay. Responses of cells cultured with medium alone were subtracted from peptide-induced responses to give net number of spots produced per million cells. Net mean (±SE) responses are shown for HIV-/HHV8- (A, n=3), HIV-/HHV8+ (B, n=4), HIV+/HHV8+/KS- (C, n=10) and HIV+/HHV8+/KS+ (D, n=5) subjects over time. Responses for HHV-8 latency and lytic pools are displayed in the left column, and CEF and HIV-1 pools are in the right column. Overall means from all time points combined compared by groups are shown for the HHV-8 latency and lytic pools (E). (*= $p<0.05$ by t-test)

Averaged over all time points of study, responses for Treg depleted samples were significantly enhanced for HHV-8 latency and lytic pools compared to PBMC for HIV-/HHV8+ ($p=0.044$ for latency and $p=0.011$ for lytic), HIV+/HHV8+/KS- ($p<0.001$ for both latency and lytic) and HIV+/HHV8+/KS+ ($p=0.002$ for latency and $p=0.007$ for lytic) groups (Figre 13E). Additionally, IFN-γ production was higher in response to the HHV-8 lytic pool compared to the HHV-8 latency pool (Figure 13E). When comparing between groups, overall responses as well as Treg enhancements were greatest in the HIV-/HHV8+ group and lowest in the HIV+/HHV8+/KS+ group (Figure 13E).

Overall, these data suggest that there is a functional down-regulation of anti-HHV-8 $CD8^+$ T cell responses by Treg, which is most evident in HIV-1 seronegative individuals.

3.3.5 Levels of serum biomarkers within a year of KS development

As differences in $CD8^+$ T cells were found during progression to KS, we next examined levels of several biomarkers in the serum of MACS participants to investigate which factors may play a role in KS development. We also looked for several Treg markers, including IL-10, TGF-β and IL-2, to provide insight into possible mechanisms by which Treg could be exerting their suppressive effects on HHV-8-specific $CD8^+$ T cells.

Significant differences between groups were not evident over many years prior to KS development (see supplemental figures in Appendix B, Figure 23). We found the greatest differences between groups for these markers within a year of KS diagnosis (Figure 14). For the markers present at the lowest levels, notable differences were found in IL-10, IFN-γ, IL-8, TNF-α and MIP-1α (Figure 14A). Higher levels of IL-10 and TNF-α were found in subjects co-infected with HIV-1 and HHV-8 compared to those infected with only HHV-8 or seronegative

for both viruses. Levels of IFN-γ, IL-8 and MIP-1α were higher the KS-positive group compared to those without KS. For markers found at higher levels, differences were evident for VEGF (Figure 14B). This marker was found at higher levels in the KS-positive group compared to the KS-negative groups. While TGF-β was found at the highest levels, differences were not as evident between groups (Figure 14C).

As B cells are one of the main targets of the virus, we also looked for a subset of these markers in B cells from KS patients. Along with elevated levels in the serum, we found that B cells from KS cases had elevated RNA levels of IL-6, MIP-1α, IL-8 and VEGF compared to non-KS controls (see Appendix D). Taken together, these data show that several biomarkers are increased at KS development.

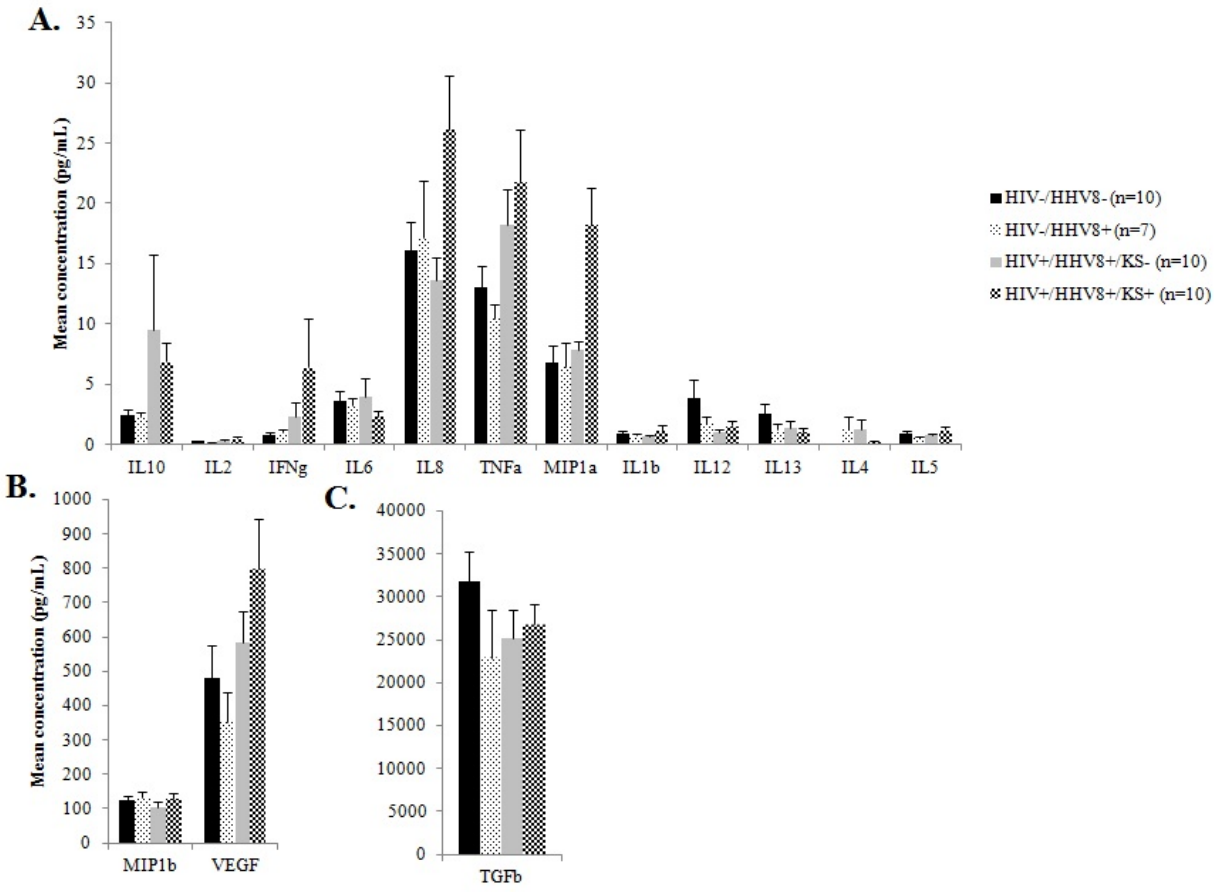

Figure 14. Levels of serum biomarkers within a year of KS development

Serum samples from MACS subjects were tested for the presence of biomarkers at KS using an electrochemiluminescence MSD assay. Mean (±SE) concentrations (pg/mL) are shown for HIV-/HHV8- (n=10), HIV-/HHV8+ (n=7), HIV+/HHV8+/KS- (n=10) and HIV+/HHV8+/KS+ (n=10) subjects within a year of KS development.

3.4 DISCUSSION

There is need to develop effective prevention and treatment for HHV-8 infection and associated diseases, as KS continues to be the most common cancer among HIV-1-infected persons despite advances in ART (48). $CD8^+$ T cell responses to HHV-8 lytic and latency proteins could play a major role in controlling HHV-8 infection to prevent KS (17, 93). To address this, a greater understanding of the immunopathogenesis of HHV-8 infection is needed. We therefore performed longitudinal immunologic studies of MACS subjects who did and did not develop KS.

Our most striking finding is that based on multiparameter immunologic assessments, HHV-8-specific $CD8^+$ T cell immunity was lower beginning up to 12 years prior to diagnosis of KS in cases compared to those who did not develop KS. This was evident in lower numbers of HHV-8 multimer-specific $CD8^+$ T cells and less $CD8^+$ T cell IFN-γ production specific for HHV-8 lytic and latency proteins. The basis of this profound immune dysfunction so early in HHV-8/HIV-1 co-infection is unclear. There is no clear evidence for a genetic predisposition to HIV-1-associated KS. Notably our study subjects were all HLA A*0201 due to the availability of HHV-8 immunodominant T cell epitopes associated with this MHC class I allele (202).

Although very intriguing, most studies to date regarding the presence, frequency and phenotype of HHV-8 specific T cells in HHV-8 negative, HHV-8 infected and HHV-8/HIV-1 co-infected persons are primarily cross sectional. This lacks the power of more revealing, multi-dimensional longitudinal analyses of anti-HHV-8 T cell responses. In support of this, it was recently demonstrated that a decline in the quality of HIV-1-specific $CD4^+$ and $CD8^+$ T cells during HIV-1 infection, including functional cytokine production and a shift toward a memory cell phenotype, occurred over a 7 year period (58). This evidence supports the need for longitudinal studies focusing on the reactivity of $CD8^+$ T cells to HHV-8 lytic and latency

proteins in those individuals that control infection versus those who develop KS. Therefore, we used MACS participants in such longitudinal studies to provide insight on the role of these effector responses and how they contribute to the prevention of KS.

In this study, we observed lower T cell counts and higher HIV-1 and HHV-8 viral loads in MACS subjects who developed KS. We found the highest levels of HHV-8 epitope-specific $CD8^+$ T cells in healthy, HHV-8 seropositive subjects who were not infected with HIV-1. Over many years prior to KS, significantly lower levels of HHV-8-specific T cells were evident in the cases, which suggests that these $CD8^+$ T cells specific for HHV-8 lytic and latency proteins are important in controlling infection to prevent disease. In the KS cases, HHV-8-specific $CD8^+$ T cells were lower even at the first visit approximately 10 years prior to disease development, indicating a very early suppressive effect on HHV-8 T cell responses. While $CD8^+$ T cell responses to HHV-8 were low many years prior to KS, we did not find an association with increases in HHV-8 plasma viral load. We only found increases in HHV-8 in plasma within a year of KS diagnosis, which suggests that there may be additional factors along with $CD8^+$ T cells that affect development of disease. Furthermore, we found lower levels and less functional reactivity for HHV-8-specific $CD8^+$ T cells in all groups compared to CMV or HIV-1-specific cells. This trend has also been found in other studies (93, 244), and could be related to the greater viral load of CMV and HIV-1 in persistently infected individuals, with more turnover of viral antigen that maintains a greater level of memory cytotoxic T lymphocyte precursors.

In this study, all subjects were seroprevalent for both HHV-8 and HIV-1 upon study entry. In future studies, we plan to include individuals who seroconverted to the viruses after study entry, which will provide insight about these responses during primary infection. This will also allow us to further investigate these responses when HHV-8 infection occurs both before

and after HIV-1 co-infection. In future studies, we also plan to include a subset of longitudinal visits in these subjects post-ART initiation, as the visits used in this study were all pre-ART. This will provide further insight on the effect of ART on anti-HHV-8 $CD8^+$ T cell responses.

T cell responses to HHV-8 are likely directed against different lytic and latency proteins at different stages of infection and disease, similar to EBV-associated diseases (90). During EBV infection, the hierarchy of $CD8^+$ T cell reactivity to immunodominant epitopes is related to a lower expression of latency proteins compared to lytic proteins in infected cells (185). During mononucleosis, a primary symptomatic infection of EBV, both lytic and latent EBV-specific T cells are present, but responses to lytic epitopes tend to be stronger (149). This trend towards lytic responses is also evident in healthy, EBV seropositive individuals (105). Similarly, $CD8^+$ T cell responses to HHV-8 have been found to be directed more towards lytic compared to latency antigens (200, 201). One recent study found that virus-specific T cells against LANA-1 in classic KS patients were associated with persistent KS, while K8.1-specific T cells were inversely correlated with KS occurrence (8). Another study reported a novel late-lytic glycoprotein ORF28-P29 epitope that was recognized in 7% of HIV+/HHV8+ individuals (201), compared to an immunodominant HLA A*0201 late-lytic glycoprotein K8.1 epitope recognized in 71% (27). In the present study, we have found that both the frequency and function, through multimer staining and ELISPOT assays, respectively, tend to be greater for the lytic epitopes in gB and K8.1 than the latency epitopes in LANA-1 and K12 both in the presence and absence of HIV-1 co-infection.

Antigen-specific $CD8^+$ T cells occupy a lineage of naive and memory compartments that are involved in the expansion, effector and contraction phases of the $CD8^+$ T cell immune response (96). The naive, central memory, effector memory and terminally differentiated

phenotypes can be classified based on the expression of surface markers related to migration and differentiation. The phenotype of $CD8^+$ T cells likely plays a role in controlling infection to prevent disease. In support of this, patients with MCD, another malignancy associated with HHV-8 infection, were found to have more cells with a late memory phenotype, compared to seropositive, healthy controls who had more cells with an early and intermediate phenotype (94). Interestingly, this MCD study found that HHV-8 viral loads were negatively correlated with early and intermediate effector memory cells and that the more differentiated T cell phenotype is associated with disease, rather than a loss of HHV-8 specific $CD8^+$ T cells. In one study of KS patients, those who controlled KS had greater percentages of HHV-8-specific terminally differentiated cells, while those who did not control KS had more effector memory cells (128). Another HHV-8 study found that the phenotype for $CD8^+$ T cells specific for an HHV-8 epitope consisted mainly of effector memory cells, and also notable levels of terminally differentiated cells (201). In regards to other virus-specific cells, the phenotype of HIV-1-specific cells tends to consist of higher levels of effector memory cells, while CMV-specific cells are mainly terminally differentiated (96). In our study, we found similar patterns of phenotypes for HHV-8, CMV and HIV-1 specific T cells, as well as for epitope nonspecific cells, in the presence and absence of HIV-1 co-infection and for both KS-negative and KS-positive subjects. As this study looked at responses over many years, the patterns in phenotypes may be an effect of aging, which alters the phenotype of T cell subsets (79).

Despite the presence of $CD8^+$ T cells at a relatively high frequency in HHV-8 seropositive subjects, the immune response to HHV-8 epitopes was found to be very low as measured by IFN-γ ELISPOT assays. Therefore, it is possible that HHV-8-specific immune responses are down-regulated by Treg, which are a $CD4^+$ T cell subset that function to maintain

peripheral tolerance and are also capable of immune suppression (151). These cells are beneficial in preventing autoimmunity and tissue damage, but can also inhibit immunity needed to resolve infection and prevent disease. Treg alter the immune response to many viruses, including EBV and HIV-1. For example, in patients with Hodgkins lymphoma, Treg accumulated at tumor sites and those patients with higher Treg ratios had shorter disease-free survival (154, 209). Additionally, in these patients, increases in Treg numbers were associated with decreases in EBV-specific $CD8^+$ T cell IFN-γ production (155). In patients with nasopharyngeal carcinoma, large numbers of Treg are found both at the tumor site and in circulation (132, 140). During HIV-1 infection, HIV-1 specific $CD8^+$ T cell responses and cytolytic activity are suppressed by Treg (121), and an increased Treg frequency negatively affected $CD8^+$ T cell polyfunctionality, which was restored with Treg depletion (152).

To our knowledge, this study is the first to explore Treg in relation to HHV-8 infection and disease development. Interestingly, we found an increase in Treg frequency in KS cases compared to controls, which could mean that these cells are contributing to disease development through immune modulation. We found that depletion of Treg significantly increased HHV-8-specific IFN-γ responses in HHV-8 seropositive subjects, which was evident to a greater extent in HHV-8 seropositive, HIV-1 seronegative subjects. While this effect was present in all subjects, it was less apparent during HIV-1 co-infection without KS, and the lowest in magnitude for those that developed KS. Of note, responses were only detected in HIV-1 seropositive, HHV-8 seropositive KS-negative and KS-positive subjects when Treg were depleted from the PBMC samples. Additionally, this effect was evident even during early infection many years prior to the development of KS. Compared to HHV-8, responses to the CEF pool as well as to HIV-1 were greater in both the presence and absence of Treg. We also plan to look at the effect

of Treg in seroconvertors to investigate these responses during primary infection as well as HHV-8 infection before and after HIV-1 infection. We also plan to examine Treg at post-ART visits to investigate if therapy alters the effects of Treg on anti-HHV-8 T cell responses. In addition, we also plan to look at these responses in ICS assays to provide further insight on the quality of response through the inclusion of more immune mediators.

Treg are known to have several mechanisms of suppression, including production of inhibitory cytokines such as IL-10 and TGF-β. Therefore, we also looked for these factors in the serum of the study subjects. However, significant differences in these markers were not evident at KS development for cases compared to controls. It is possible that Treg in KS patients function through other mechanisms, such as use of inhibitory receptors, cytolytic activity or metabolic disruption (151). The exact role and mechanism of Treg during HHV-8 infection needs to be further investigated as these cells could play an important role in disease development. As we did not see major increases in responses when Treg were removed in KS-positive subjects yet we found a significant increase in Treg numbers prior to KS, it is also likely that other mechanisms of suppression play a role in the inability of $CD8^+$ T cells to control HHV-8 infection. For example, PD-1 expression was found to be increased on HIV-1-specific $CD8^+$ T cells, which was associated with an impairment of function through a reduced ability to produce cytokines and effector molecules as well as decreased proliferation (234). This also could be important during HHV-8 and HIV-1 co-infection and needs to be examined in future studies.

In addition to the levels of $CD8^+$ and $CD4^+$ T cells, cytokines and chemokines produced by T cells as well as other types of cells, such as APC, likely play a crucial role in disease development. Inflammatory changes occur early in KS, prior to the detection of the cancer

(162). KS tumors are known to be rich in inflammatory cell infiltrates, including $CD8^+$ T cells, B cells, macrophages and monocytes (166). These infiltrating cells produce large amounts of proinflammatory cytokines (e.g., IFN-γ, IL-1β, TNF-α and IL-6), chemokines (e.g., IL-8), and growth factors (e.g.,VEGF) (70, 75, 166, 186). We therefore examined serum samples for the presence of several biomarkers over many years prior to KS development, but found the greatest differences between cases and controls within a year prior to KS. Increases in IFN-γ, IL-8, TNF-α, MIP-1α and VEGF were noted in the cases compared to controls. These factors may contribute to creating an environment that promotes the spread of HHV-8 infection and subsequent KS development.

In conclusion, we have revealed several important differences in the immune response to HHV-8 infection in KS cases compared to those that did not develop KS over many years leading to disease development. Specifically, the frequency of circulating $CD8^+$ T cells specific for both HHV-8 lytic and latency proteins was lower over many years in the cases prior to KS. These cells have a predominantly effector memory and terminally differentiated phenotype. Numbers of Treg increased in the cases prior to the development of KS, and these cells suppress HHV-8-specific T cell responses. The present study supports the hypothesis that T cell responses, in frequency, quality and magnitude, are essential for control of HHV-8 infection to prevent development of disease.

3.5 ACKNOWLEDGMENTS

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4.0 CONCLUSION

Professional APC are critically important in the recognition of an invading pathogen and presentation of antigens to the T cell-mediated arm of immunity. HHV-8 targets these APC for infection, altering their cytokine profiles, manipulating their surface expression of MHC molecules and altering their ability to activate HHV-8-specific T cells. HHV-8 infection of professional APC could demonstrate an evolutionary mechanism to establish viral latency in cell types responsible for initiating T and B cell adaptive immune responses. Subsequently, T cell responses to HHV-8 antigens are not very robust as compared to the other human gammaherpesvirus, EBV. In some instances, APC support lytic HHV-8 infection, and the replication cycles directly affects viral pathogenesis and progression to KS and certain B cell lymphomas. Given that KS lesions are common in the skin and mucosa which are heavily populated with APC, the interaction of APC with HHV-8 and the subsequent effect on T cell responses is of central importance. As KS continues to be the most common cancer associated with HIV-1 infection, understanding the immunopathogenesis of this virus to develop treatments and vaccines for HHV-8-associated diseases has significant public health implications.

To succeed, a pathogen must be able to evade immune surveillance. HHV-8 infects APC and has revealed two distinct replication patterns of HHV-8 in APC, i.e., non-productive and productive, which could have direct consequences on viral pathogenesis. The direct effect of HHV-8 infection of professional APC and its indirect effect on T cell control of infection need to

be tied together in a more revealing fashion to define the magnitude and breadth of T cell responses to HHV-8 antigens. It is likely that T cell responses, in both quality and magnitude, are essential for control of HHV-8 infection. Although evident in HHV-8 infection, it is not clear whether polyfunctional T cells are required to control progression of associated diseases. Furthermore, T cell responses to HHV-8 antigens are not very robust as compared to EBV and CMV. This dampened immune response could be related to down-regulation by Treg. A delicate balance exists between protective immunity involving cytokine and chemokine production by host cells, and virus-driven induction of cytokines and chemokines that aid in the dissemination and spread of infection and mediate pathogenesis. The role of each cytokine and chemokine in HHV-8 infection and KS likely varies depending on their quantity and origin of production. Several of the mediators that are essential to the immune response and activation of lymphocytes can exacerbate infection and cause clinical symptoms when over produced in response to HHV-8 infection.

My studies have provided valuable, novel insight into the immune response to HHV-8. This is important as the immunity to HHV-8 is much less well defined than to EBV, yet HHV-8 has a significant public health impact, especially on the HIV-1-infected population. In initial studies, I used healthy, HHV-8 seropositive subjects to explore $CD8^+$ T cell responses to HHV-8. I identified 10 novel, HLA A*0201 epitopes in two lytic and two latency proteins. A major limitation in studying HHV-8 immune responses to date is lack of known epitopes in viral proteins, so this work greatly increases the number of epitopes that can be used in future studies of the complex immune response to this virus. In these healthy subjects, I found that lytic and latency epitopes induced various combinations of both monofunctional and polyfunctional T cell responses. Polyfunctional responses have been shown to be crucial in control of other viruses,

including HIV-1. This was the first study to show the presence of polyfunctional T cells specific for HHV-8 lytic and latency epitopes in healthy, HHV-8 seropositive individuals. This suggests that response quality, as examined through polyfunctional T cells producing multiple immune mediators to respond against the virus, is important in controlling the spread of HHV-8 infection. While the immune responses to this virus are relatively non-robust, this was also the first study to directly show the presence of $CD8^+$ T cells specific for lytic and latency proteins in the blood of healthy, HHV-8 seropositive individuals controlling infection. Overall, these studies in healthy, HHV-8 seropositive individuals are important because they provide insight on the immune responses to regions of the virus that may be critical in controlling the spread of infection and disease development.

The development of effective treatments and vaccines for KS requires an understanding of the differences in immune responses for those that did and did not develop the disease. My next study therefore involved MACS participants who were co-infected with HIV-1 and HHV-8 that did and did not develop KS. A major strength of the study was the longitudinal aspect in examining responses over many years prior to disease, as the majority of the studies completed to date have been cross-sectional. In fact, this was the first longitudinal comprehensive investigation of $CD8^+$ T cell responses to HHV-8 lytic and latency proteins and their relation to progression of HHV-8 infection and development of KS in homosexual men in the MACS. Higher levels of cells specific for HHV-8 lytic epitopes were evident compared to cells specific for HHV-8 latency epitopes. This may be due to higher levels of lytic proteins present during infection as compared to latency proteins, and cells specific for lytic epitopes may be important in controlling the spread of infection when the virus is reactivated. Overall, I found highest levels of $CD8^+$ T cells specific for HHV-8 lytic and latency proteins in subjects only infected

with HHV-8, and lower levels in those co-infected with HHV-8 and HIV-1. Notably, lowest levels of $CD8^+$ T cells were evident in KS patients, even many years prior to disease development. This suggests that $CD8^+$ T cells are important in preventing KS development during HIV-1 and HHV-8 co-infection, as levels of these cells were higher in those that did not develop disease and may represent the effective control of infection to prevent KS.

I also investigated the effect of Treg on $CD8^+$ T cell responses to HHV-8 proteins. Removal of Treg showed that the relatively low functional reactivity of $CD8^+$ T cells to HHV-8 in healthy, HHV-8 seropositive individuals was due to the immunosuppressive effects of Treg, as responses to HHV-8 proteins were enhanced following removal of Treg. This is the first evidence that Treg down-regulate anti-HHV-8 T cell responses. This effect was also present in those co-infected with HIV-1 and HHV-8 that did and did not develop KS, but was most evident in those only infected with HHV-8. Notably, numbers of Treg significantly increased prior to KS development, suggesting that they may play a role in promoting disease development through immune modulation. However, as removal of Treg in KS subjects resulted in the lowest levels of enhancement despite the highest Treg numbers, it is likely that other mechanisms of immune regulation are also important during KS development. Overall, these data in MACS participants support that T cell responses, in frequency, magnitude and quality, specific for HHV-8 lytic and latency proteins are centrally involved in controlling HHV-8 infection to prevent KS development (see model in Figure 15).

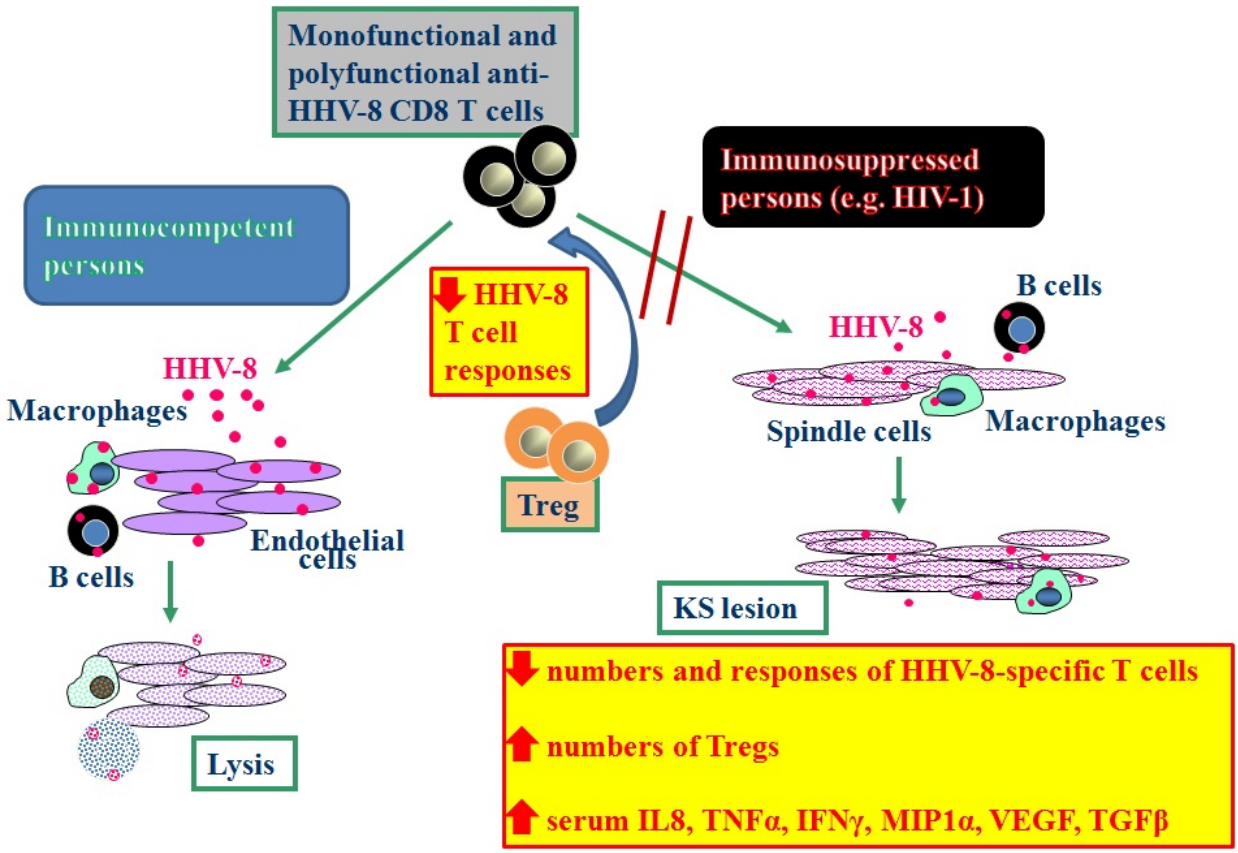


Figure 15. Hypothetical role of anti-HHV-8 T cells in control of HHV-8 infection

We hypothesized that in immunocompetent individuals, monofunctional and polyfunctional $CD8^+$ T cells specific for HHV-8 lytic and latency proteins can kill cells that are infected by the virus to control the spread of infection and prevent disease development. However, in those that are immunosuppressed, such as during HIV-1 co-infection, these anti-HHV-8-specific $CD8^+$ T cells might not be functioning properly, which leads to the spread of infected cells and disease development, such as KS. Furthermore, Treg may play a role in modulating these $CD8^+$ T cell responses. Overall, the data of my project support this initial hypothesis, with my findings highlighted in the yellow boxes.

To provide further insight into these responses, future studies should include subjects that seroconverted to these viruses after study entry to examine responses during primary infection. Additionally, subjects should be included that were infected with HIV-1 prior to and after HHV-8 to look at the impact of the order and timing of infection. As viral lytic reactivation from latency could be affecting these responses, longitudinal HHV-8 antibody titers should be examined to determine if they correlate with responses of $CD8^+$ T cell during disease development. Additionally, correlating HHV-8 antibody titers to Treg levels and responses may provide insight as to whether HHV-8 infection induces Treg. $CD8^+$ T cell and Treg responses should also be examined in subjects post-ART to explore the impact of therapy on these responses. Polyfunctional responses for additional immune mediators should also be included in future studies to further investigate the quality of $CD8^+$ T cell responses during the progression to KS, as well as the effect of Treg on the quality of immune responses. Levels of markers of immune activation, such as CD38 and HLA-DR, should be examined to determine the effect of immune activation on $CD8^+$ T cell and Treg responses. Another future direction of this project should be to look at other possible mechanisms that Treg may be using to suppress anti-HHV-8 T cell responses, such as through inhibitory receptors or metabolic disruption. Additionally, as Tregs are likely not the only factor influencing disease development, other suppressive factors that may be important in disease development should be explored, such as PD-1.

Taken together, the work generated throughout this project has important public health implications. We have revealed novel data regarding the immune response to HHV-8 in healthy, seropositive individuals controlling infection, as well as highlighted some of the differences in responses between those that are co-infected with HHV-8 and HIV-1 who did and did not develop cancer. These studies therefore help to provide insight as to why certain individuals

develop KS while others do not develop the cancer, so it is possible that these protective immune responses could be enhanced through therapies and vaccines. Understanding both the immunopathogenesis of HHV-8 infection and the immune responses that control infection to prevent disease progression are essential for the development of therapeutic and preventative vaccine strategies. If successful, effective vaccines to prevent HHV-8 infection and treatments for KS could improve the lives of many people around the world by decreasing the burden of the most common cancer currently associated with HIV-1 infection.

APPENDIX A. ABBREVIATIONS

aa: amino acids
AIDS: acquired immune deficiency syndrome
ANOVA: analysis of variance
APC: antigen presenting cells
ART: antiretroviral therapy
BCBL: body cavity based lymphoma cells
CCL: chemokine ligand
CD40L: CD40 ligand
CIITA: class II transactivator
CMV: cytomegalovirus
CTL: cytotoxic T lymphocyte
DC: dendritic cells
DC-SIGN: DC-specific ICAM-3 grabbing nonintegrin
Dox: doxycycline
EBV: Epstein Barr virus
EDTA: ethylenediaminetetraacetic acid
ELISPOT: enzyme-linked immunosorbent spot
ERK: extracellular signal-regulated kinase
FACS: fluorescence-activated cell sorting
FCS: fetal calf serum
FGF: fibroblastic growth factor
FITC: fluorescein isothiocyanate
FLICE: FADD-like interferon converting enzyme
gB: glycoprotein B
GM-CSF: granulocyte-monocyte colony-stimulating factor
GPCR: G-protein coupled receptor
h: hours
HGF/ SF: hepatocyte growth factor/ scatter factor
HHV-8: human herpesvirus 8
HIV-1: human immunodeficiency virus 1
HLA: human leukocyte antigen
ICAM: intercellular adhesion molecule
ICS: intracellular staining
iDDC: interstitial-dermal DC
IFN: interferon
Ig: immunoglobulin

IL: interleukin
IP: interferon gamma-induced protein
IRF: interferon regulatory factor
KS: Kaposi's sarcoma
KSHV: Kaposi's sarcoma associated herpesvirus
LANA: latency associated nuclear antigen
LC: Langerhans cells
LCL: lymphoblastoid cell line
mAb: monoclonal antibody
MACS: Multicenter AIDS Cohort Study
MAPK: mitogen activated protein kinase
MCD: multicentric Castleman's disease
MCP: monocyte chemoattractant protein
MDDC: monocyte-derived DC
MDM: monocyte-derived macrophage
MHC: major histocompatibility complex
min: minutes
MIP: macrophage inflammatory protein
MIR: modulator of immune recognition
MSD: Meso Scale Discovery
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NIH: National Institutes of Health
NK: natural killer
ORF: open reading frame
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PD: programmed death
pDC: plasmacytoid DC
PE: phycoerythrin
PEL: primary effusion lymphoma
PerCP: peridinin chlorophyll protein
PFA: paraformaldehyde
PGE2: prostaglandin E2
SE: standard error
SOC: suppressor of cytokine signaling
SPICE: simplified presentation of incredibly complex evaluations
TCR: T cell receptor
TGF: transforming growth factor
Th: T helper
TLR: Toll-like receptor
TNF: tumor necrosis factor
TPA: 12-O-tetradecanoylphorbol-13-acetate
Treg: T regulatory cell
VEGF: vascular endothelial growth factor
vFLIP: viral FLICE inhibitory proteins

APPENDIX B. SUPPLEMENTAL FIGURES

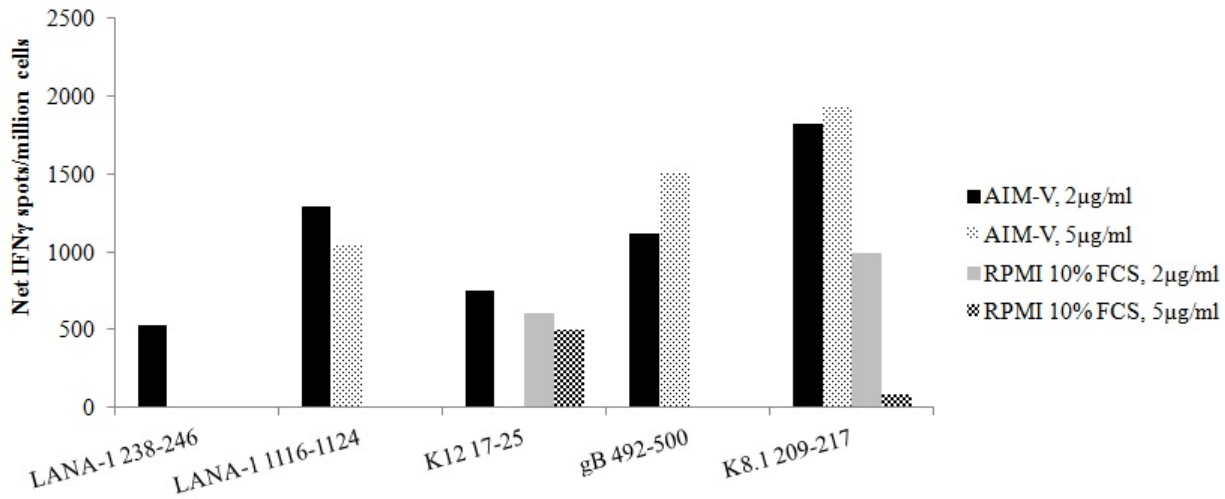


Figure 16. Medium and peptide concentration comparison

PBMC were stimulated for one week with autologous, mature DC that were loaded with previously published HHV-8 epitopes. To compare assay conditions, we used AIM-V and RPMI supplemented with 10% FCS, with peptide concentrations of 2µg/ml and 5µg/ml. IFN-γ production was measured by an ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net responses are shown for a representative healthy, HHV-8 seropositive subject.

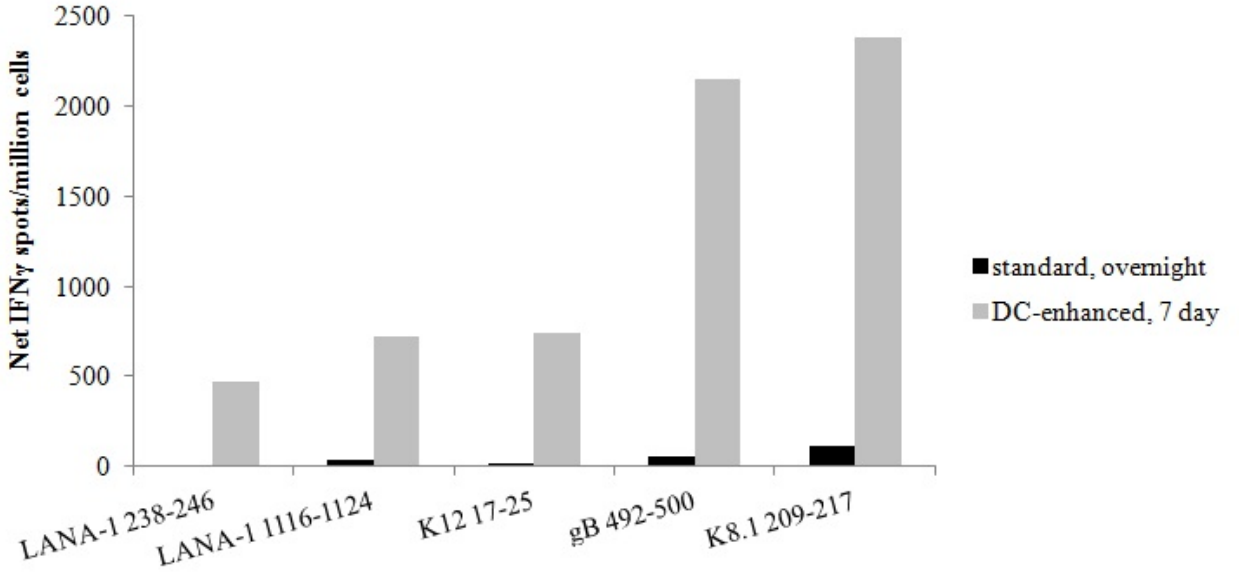


Figure 17. Comparison of DC-enhanced, 7 day and standard, overnight ELISPOT cultures
PBMC were stimulated for one week with autologous, mature DC that were loaded with previously published HHV-8 epitopes. PBMC were also cultured overnight with peptide. IFN-γ production was measured by an ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net responses are shown for a representative healthy, HHV-8 seropositive subject.

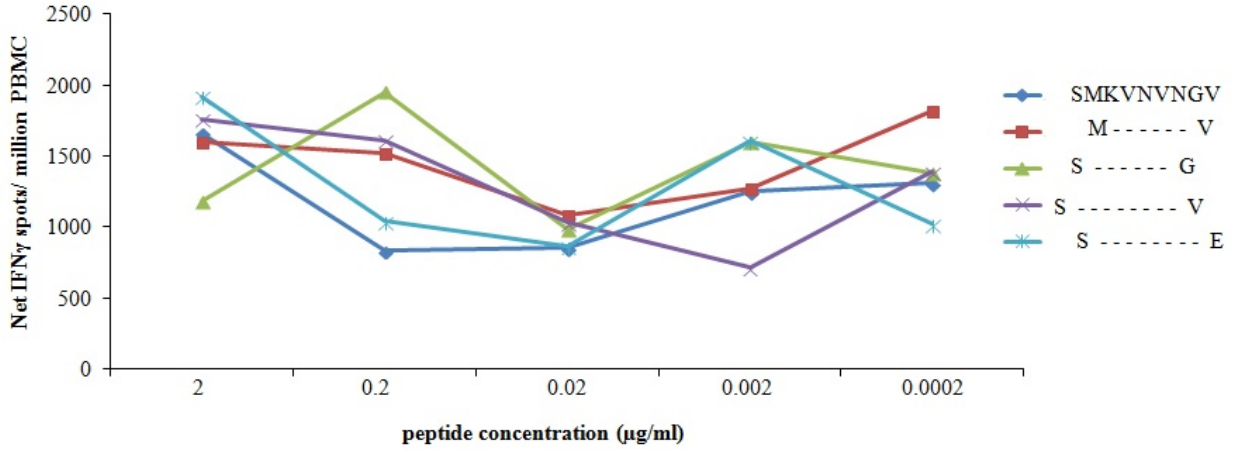


Figure 18. ELISPOT responses a hotspot family using varying peptide concentrations

PBMC were stimulated for one week with autologous, mature DC that were loaded with five different, 10-fold concentrations of peptide from a hotspot family. IFN-γ production was measured by an ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net responses are shown for a representative healthy, HHV-8 seropositive subject in response to a peptide family from gB.

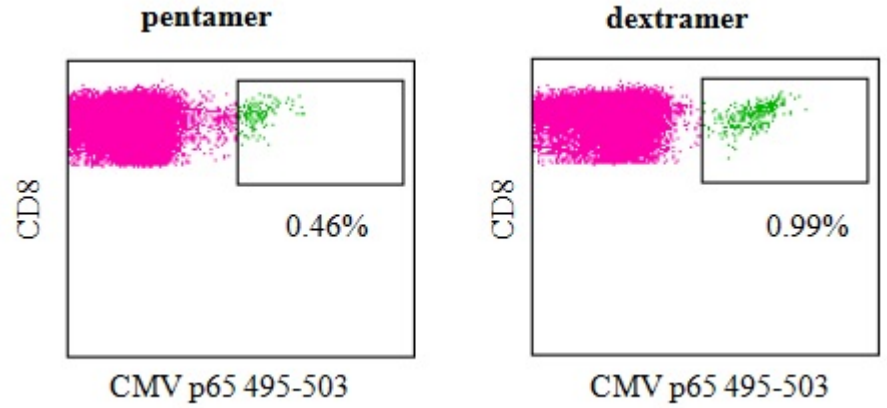


Figure 19. Multimer comparison of a pentamer and dextramer

PBMC were stained with an MHC class I-peptide pentamer or dextramer complex specific a CMV epitope. Results are shown for a representative healthy, HHV-8 seropositive subject.

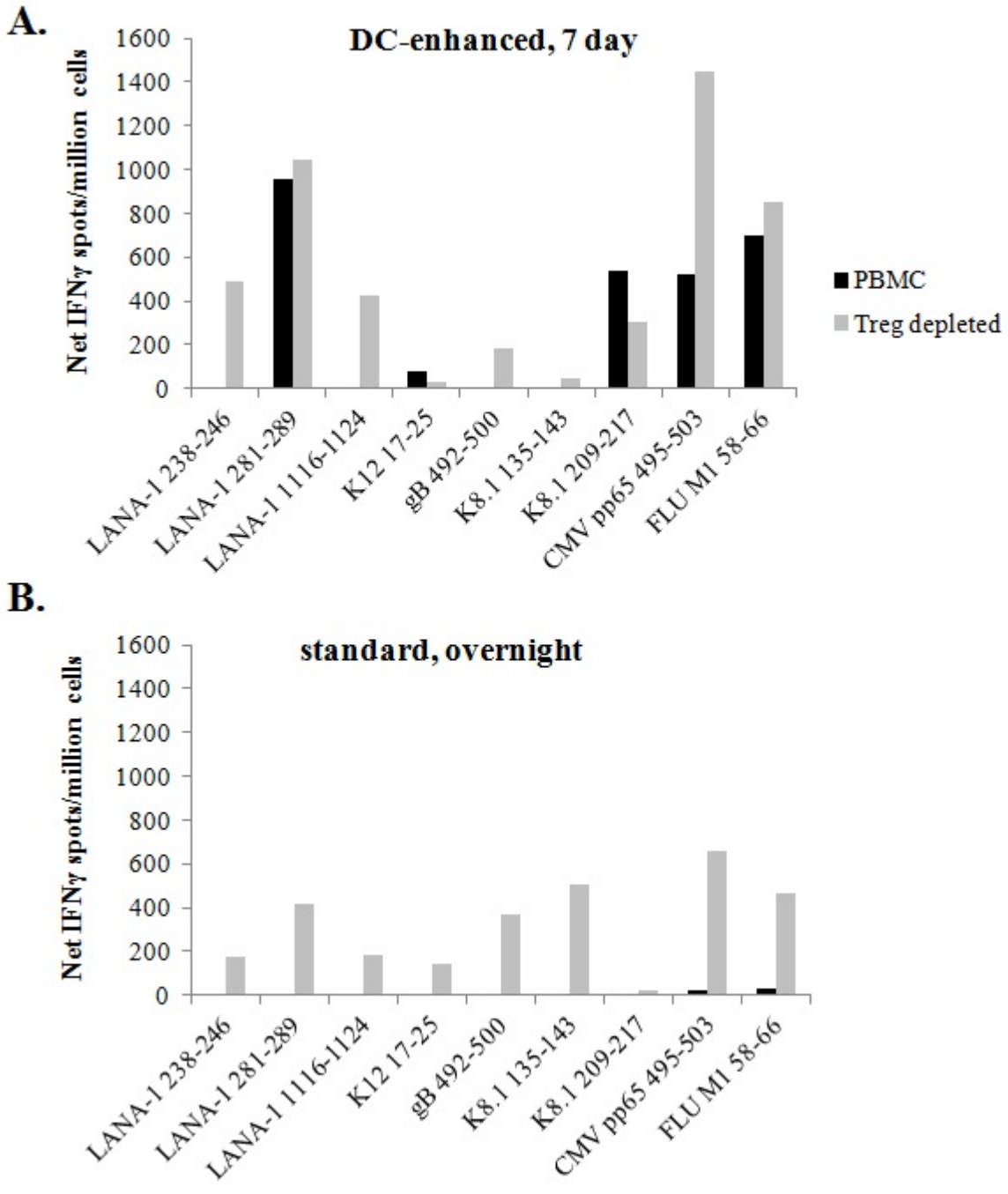


Figure 20. Comparison of ELISPOT cultures with Treg depletion

PBMC or PBMC depleted of Treg by a magnetic bead depletion system were stimulated for one week with autologous, mature DC that were loaded with HHV-8, CMV and FLU peptides (A). PBMC or PBMC depleted of Treg were also cultured overnight with peptide (B). IFN-γ production was measured by an ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net responses are shown for a representative healthy, HHV-8 seropositive subject.

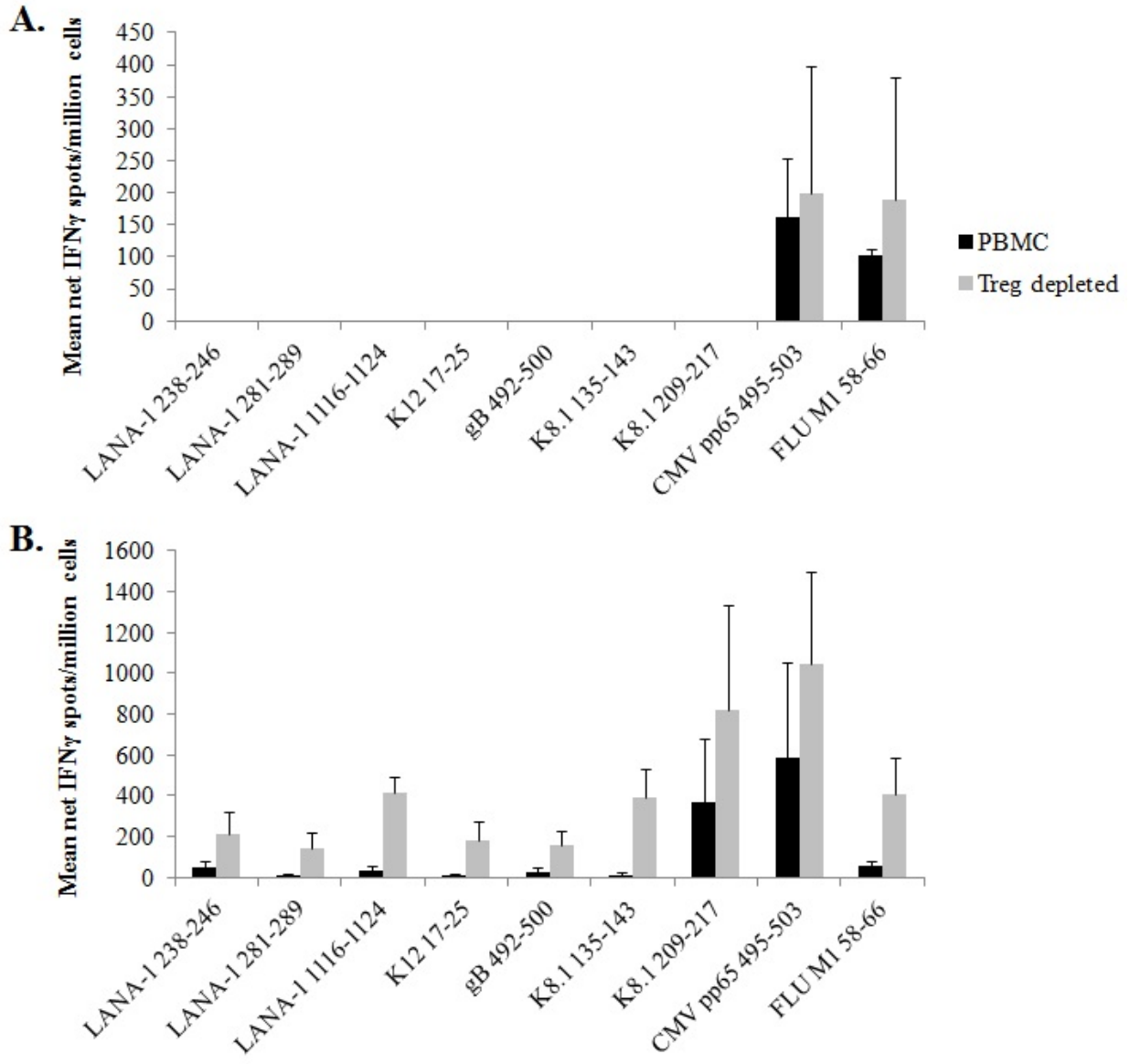


Figure 21. Effect of Treg depletion on anti-HHV-8 CD8 T cell responses in healthy subjects

PBMC or PBMC depleted of Treg by a magnetic bead depletion system were cultured overnight with HHV-8, CMV and FLU peptides. IFN-γ production was measured by a standard ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net mean (±SE) responses are shown for three healthy, HHV-8 seronegative subjects (A) and five healthy, HHV-8 seropositive subjects (B).

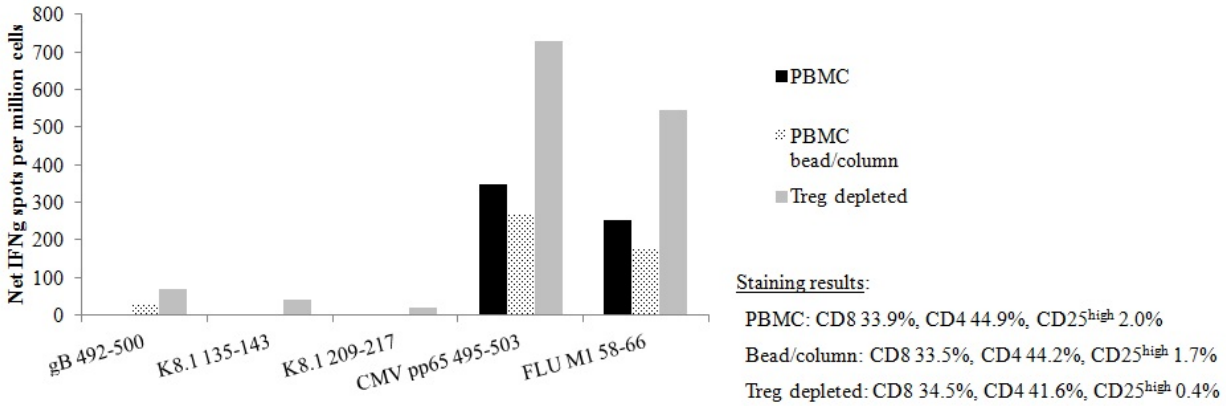


Figure 22. Treg depletion method comparison with staining

PBMC, PBMC that were passed through the magnetic bead depletion column system but not depleted of Treg, or PBMC depleted of Treg were cultured overnight with HHV-8, CMV and FLU peptides. IFN-γ production was measured by a standard ELISPOT assay, and the number of spots produced by cells without peptide was subtracted from spots produced by cells with peptide to give net values of spots. Net responses are shown for a healthy, HHV-8 seropositive subject. These samples were also stained for the surface markers CD8, CD4 and CD25.

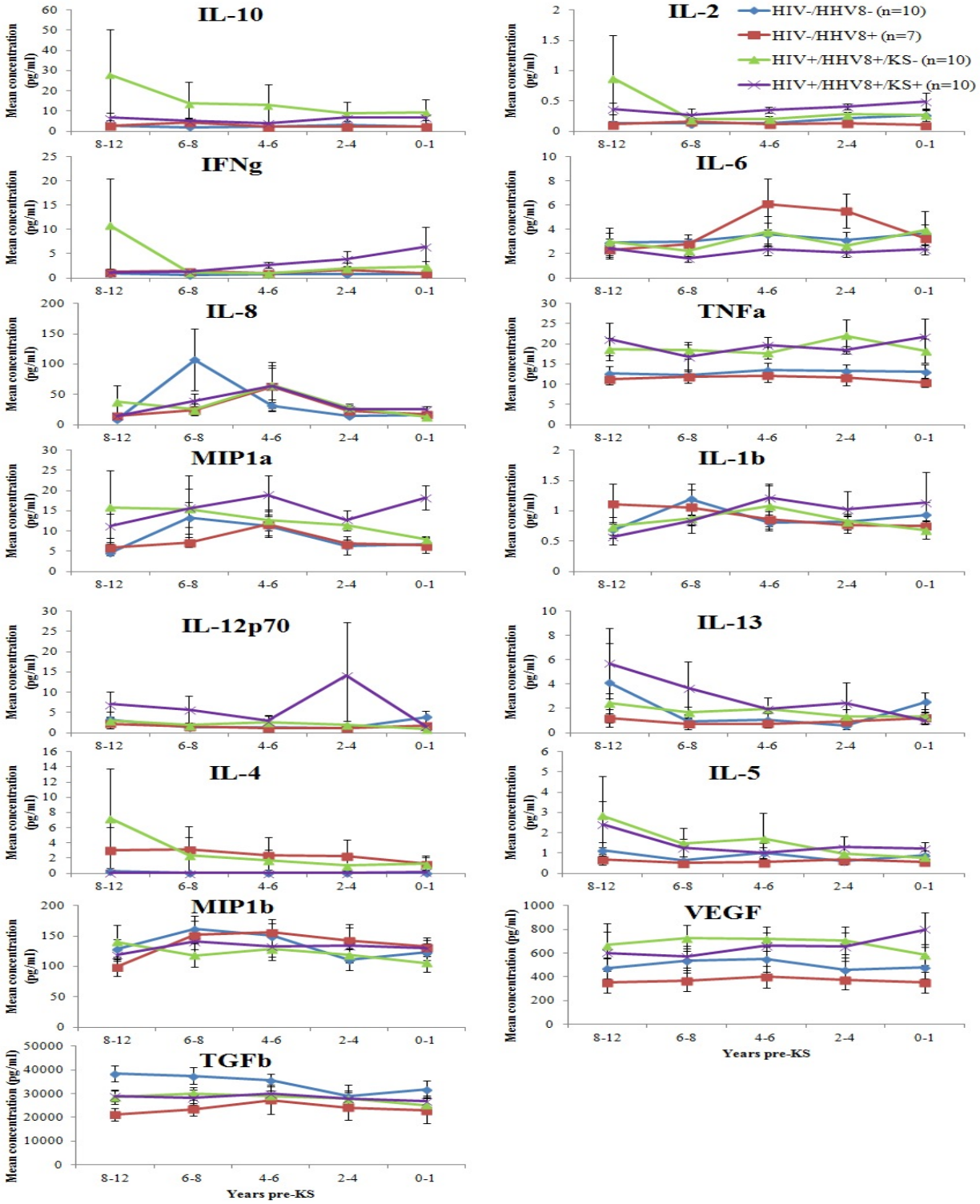


Figure 23. Levels of serum biomarkers over many years prior to KS

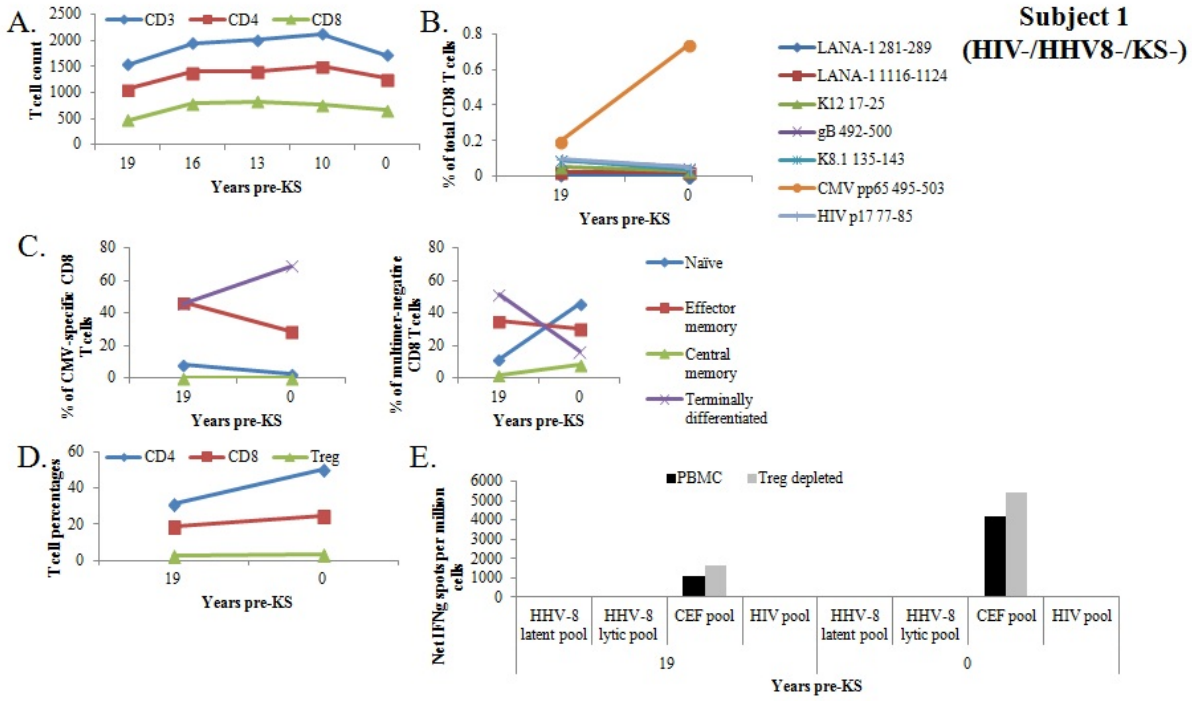
Serum samples from MACS participants were tested for the presence of biomarkers using an electrochemiluminescence MSD assay. Mean (±SE) concentrations (pg/ml) are shown for HIV-/HHV8- (n=10), HIV-/HHV8+ (n=7), HIV+/HHV8+/KS- (n=10) and HIV+/HHV8+/KS+ (n=10) subjects over many years prior to KS diagnosis.

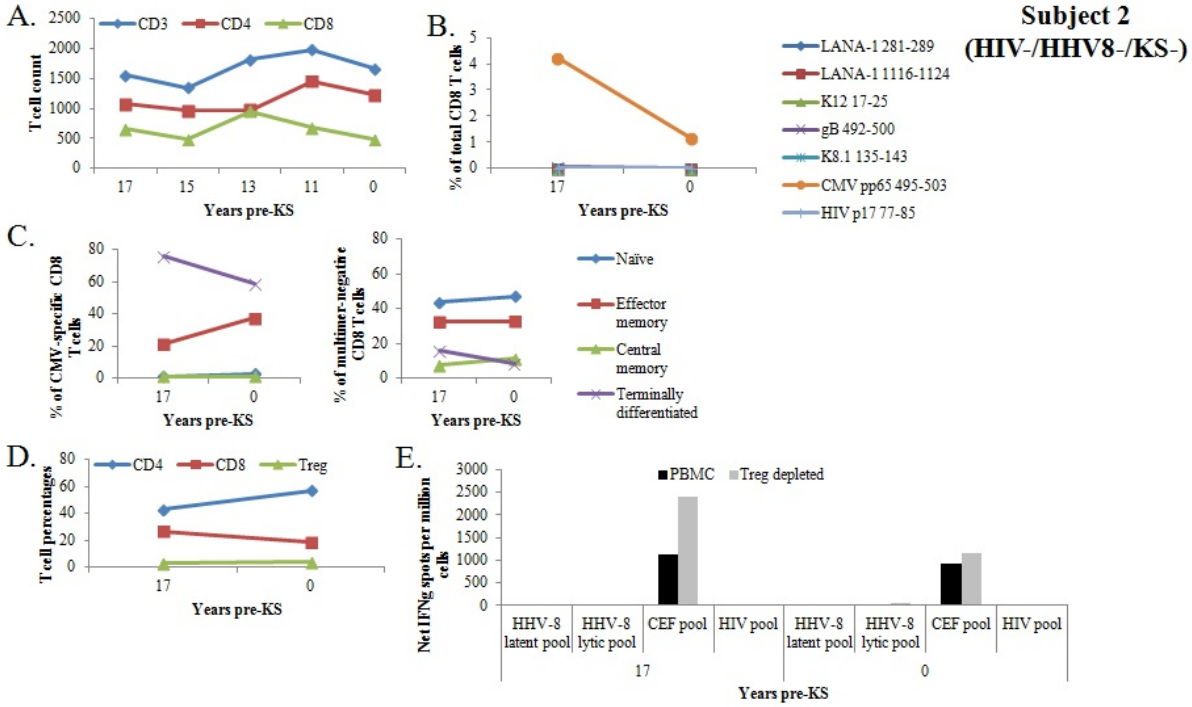
APPENDIX C. MACS SUBJECTS FOR LONGITUDINAL STUDY

As described in chapter 3.0, we investigated the importance of $CD8^+$ T cell responses and the role of Treg during the progression to KS using MACS subjects that did or did not develop the cancer. We performed longitudinal studies over many years of study visits for 10 HIV+/HHV8+/KS- and five HIV+/HHV8+/KS+ subjects. We also included four HIV-/HHV8+/KS- subjects to investigate the natural history of HHV-8 infection without the presence of HIV-1 co-infection. As controls, we also included three HIV-/HHV8-/KS- subjects. Graphs displaying the overall averages for each group are shown in the chapter above, and individual subject data are shown below.

C.1 HIV-/HHV8-/KS-

In three HIV-/HHV8-/KS- subjects (Figure 24), we measured blood T cell counts by flow cytometry (A), levels of HHV-8, CMV and HIV-1 epitope-specific $CD8^+$ T cells by dextramer staining (B) and their phenotype by flow cytometry (C), frequency of Treg by flow cytometry (D) and IFN-γ ELISPOT responses to HHV-8, CEF and HIV-1 peptide pools using PBMC or PBMC that were depleted of Treg with a magnetic bead depletion system (E) over many years of study.


Subject 1
(HIV-/HHV8-/KS-)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
C.
% of CMV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
D.
T cell percentages
E.
PBMC
Treg depleted
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool


Subject 2
(HIV-/HHV8-/KS-)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
C.
% of CMV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
D.
T cell percentages
E.
PBMC
Treg depleted
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool

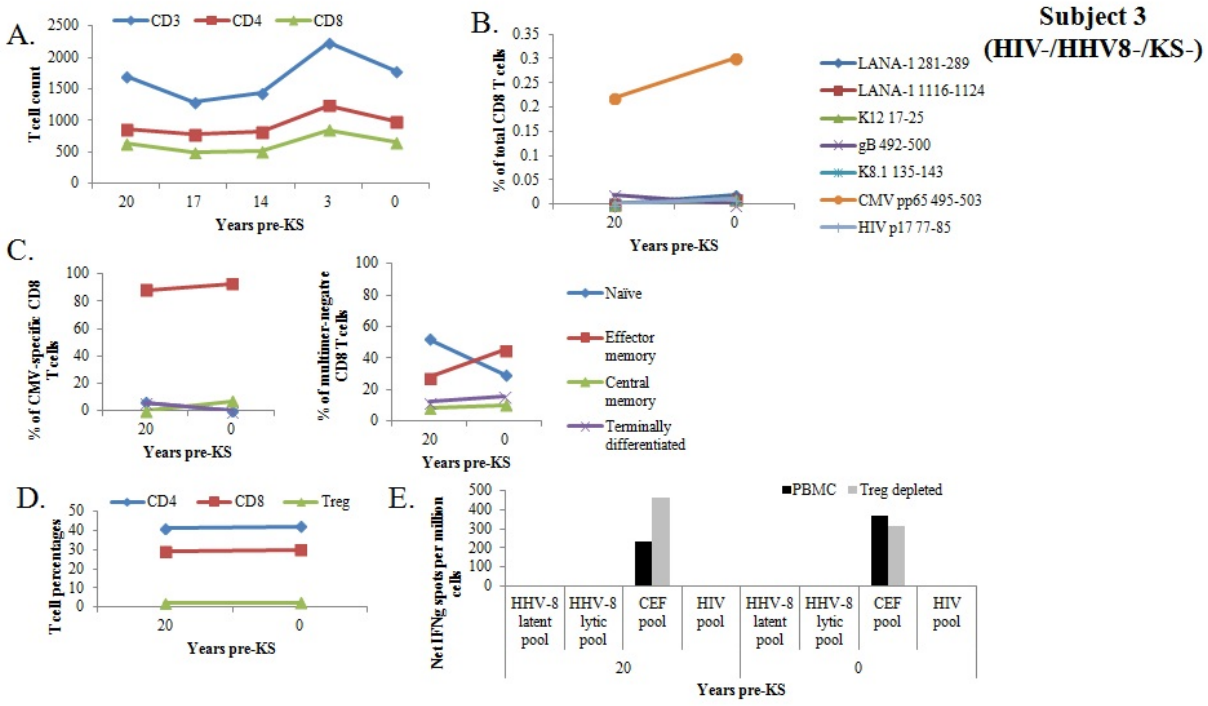
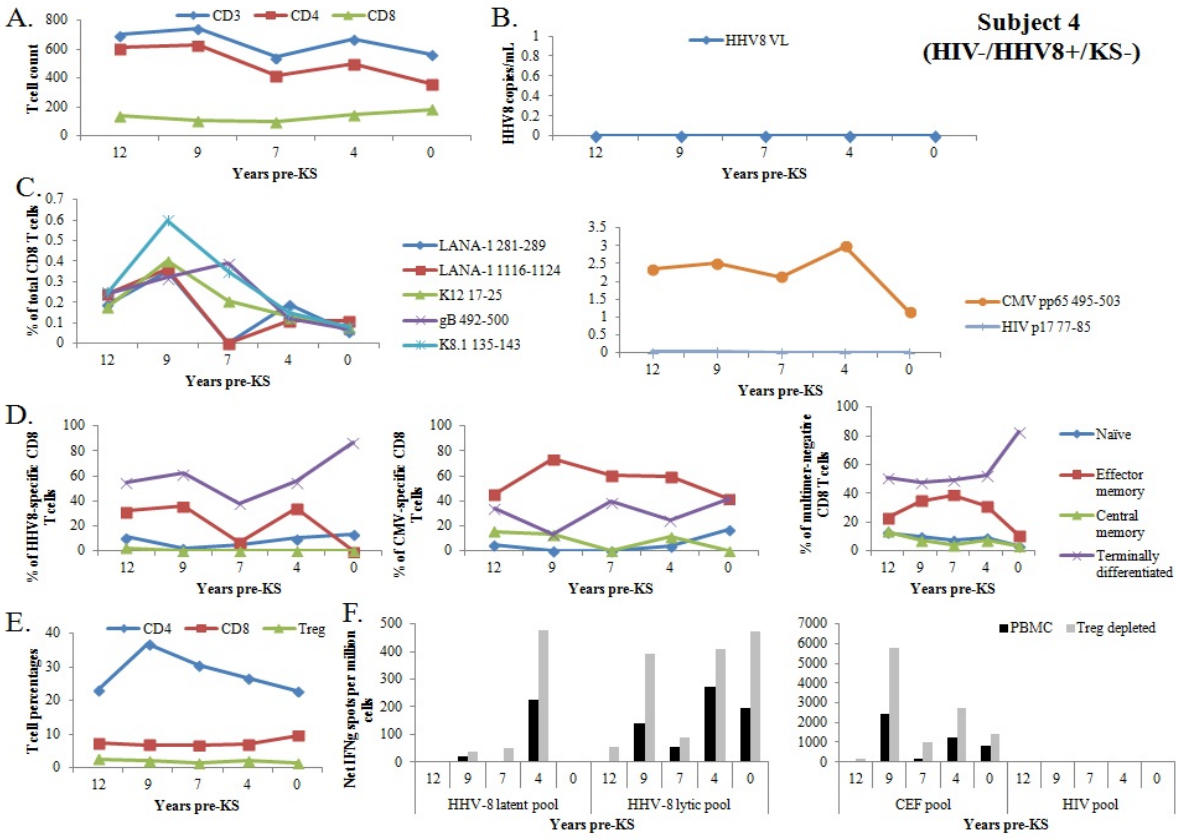
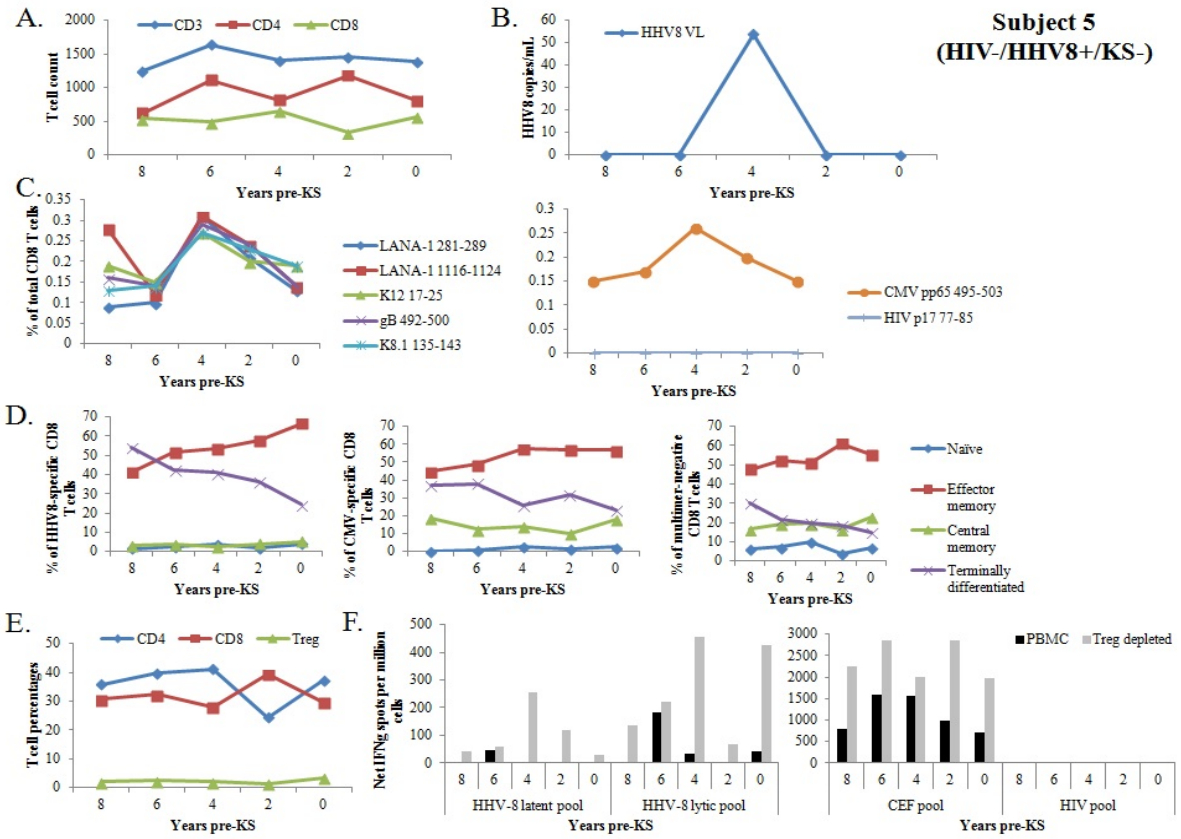


Figure 24. HIV-/HHV8-/KS- subjects

C.2 HIV-/HHV8+/KS-

In four HIV-/HHV8+/KS- subjects (Figure 25), we measured blood T cell counts by flow cytometry (A), HHV-8 plasma viral load by PCR (B), levels of HHV-8, CMV and HIV-1 epitope-specific $CD8^+$ T cells by dextramer staining (C) and their phenotype by flow cytometry (D), frequency of Treg by flow cytometry (E) and IFN-γ ELISPOT responses to HHV-8, CEF and HIV-1 peptide pools using PBMC or PBMC that were depleted of Treg with a magnetic bead depletion system (F) over many years of study.


A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HHV8 copies/mL
Years pre-KS
Subject 4
(HIV-/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
PBMC
Treg depleted
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
Years pre-KS


A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HHV8 copies/mL
Years pre-KS
Subject 5
(HIV-/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
PBMC
Treg depleted
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
Years pre-KS

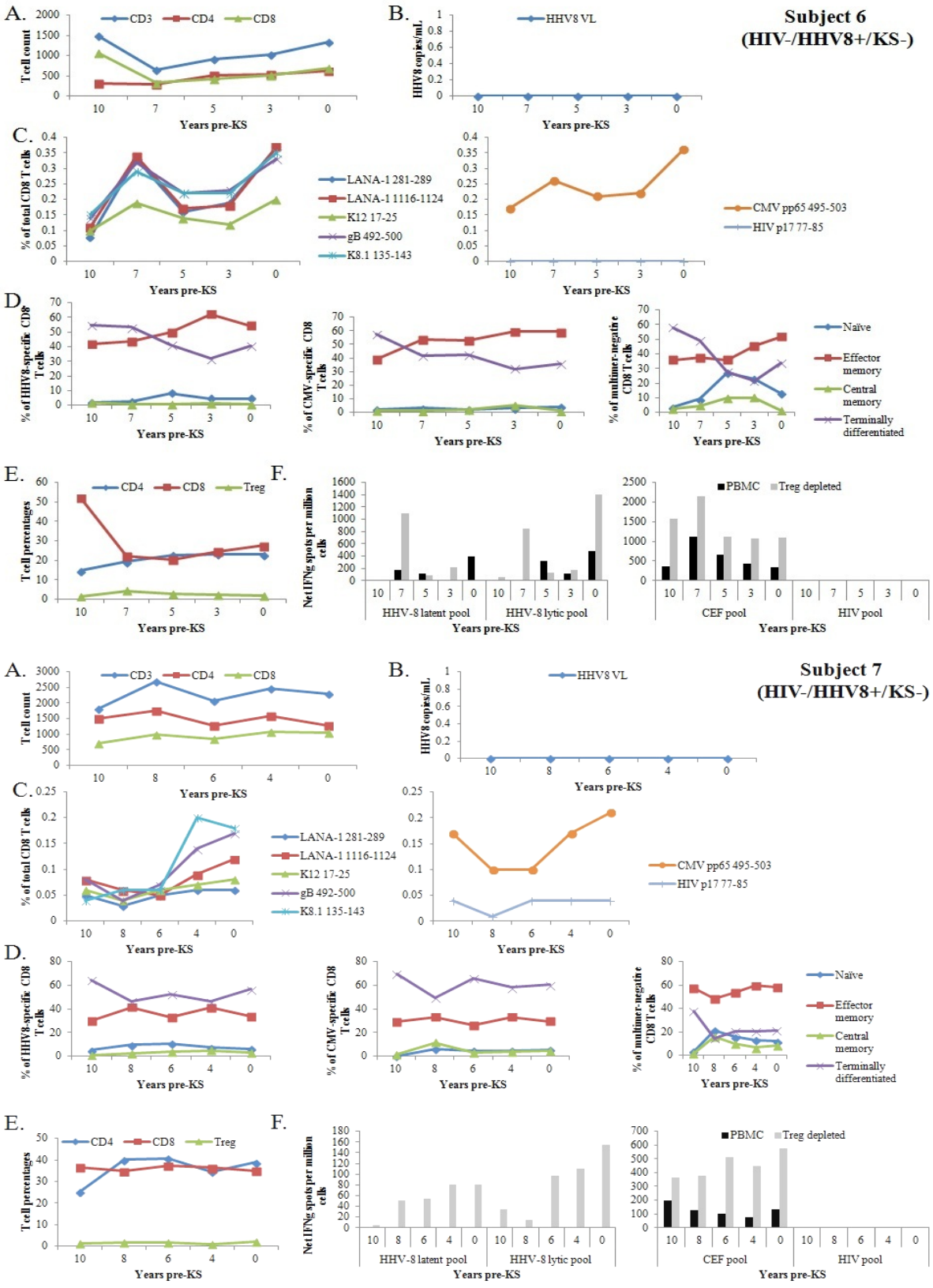
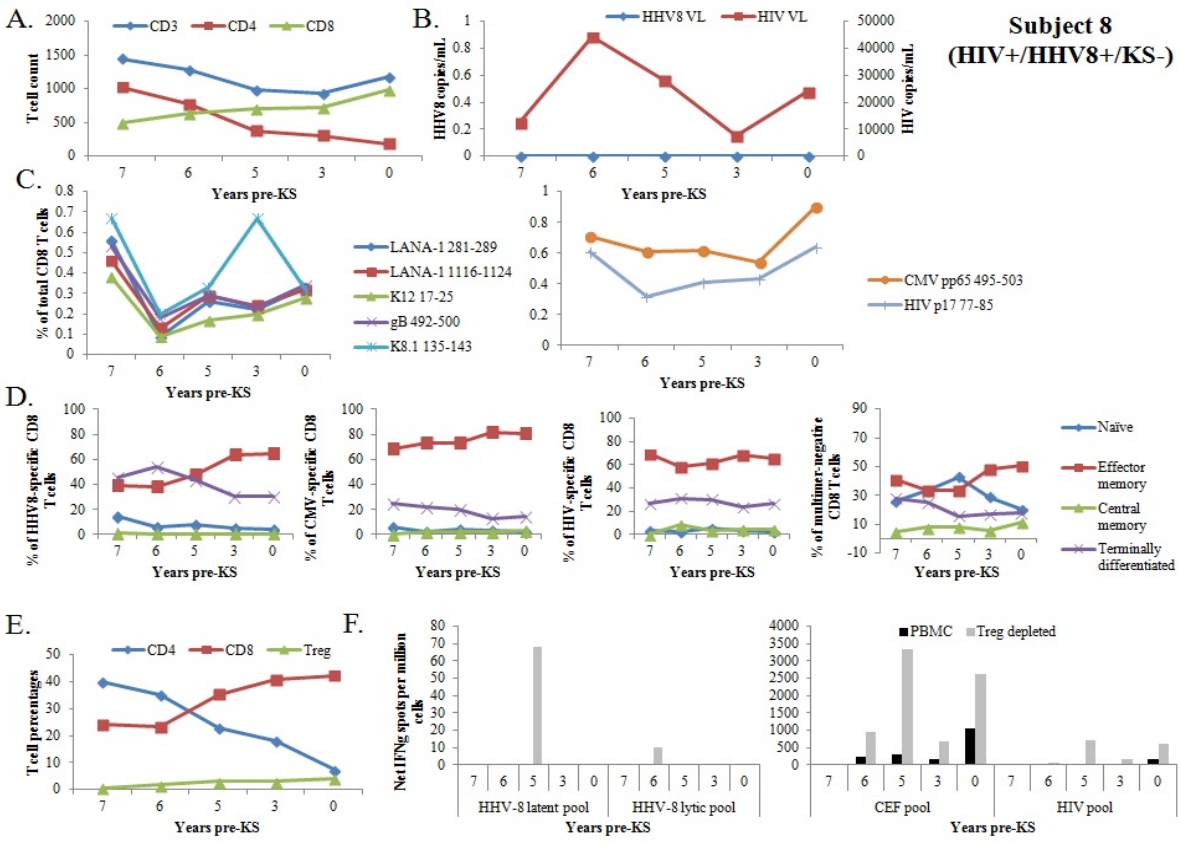
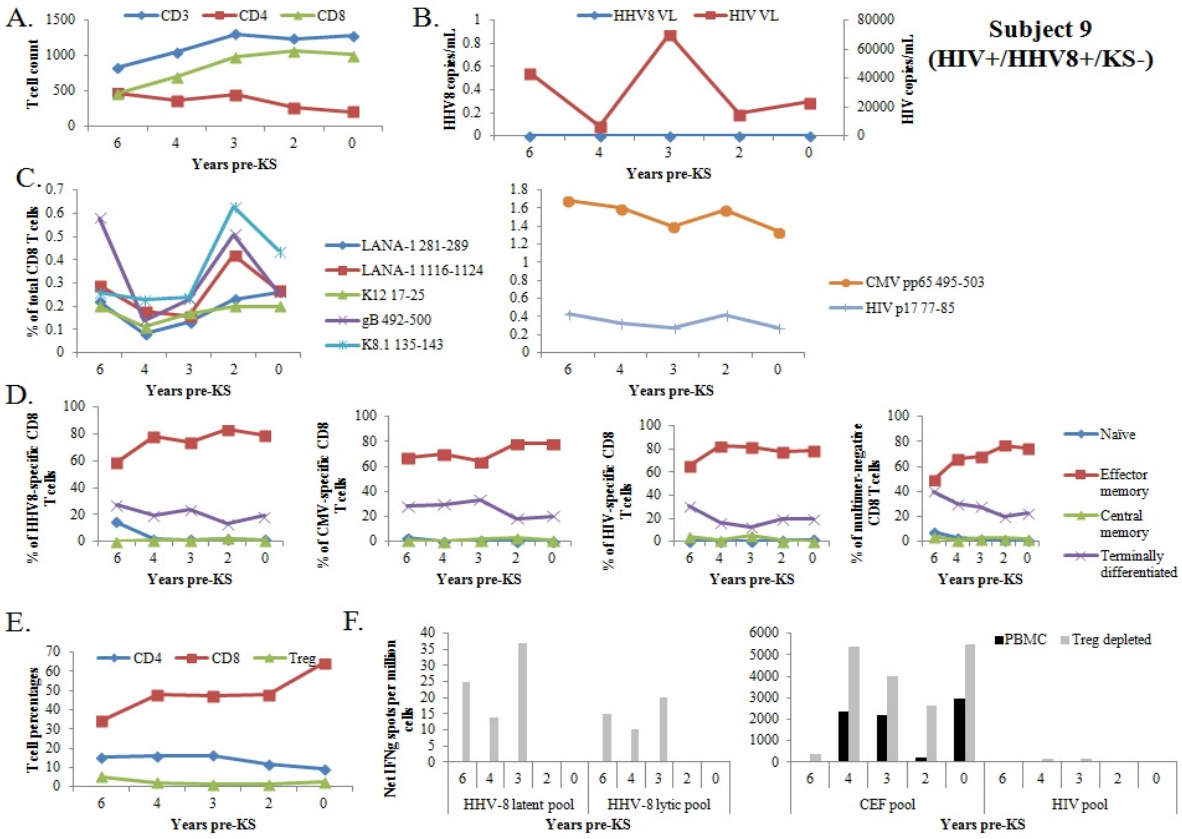


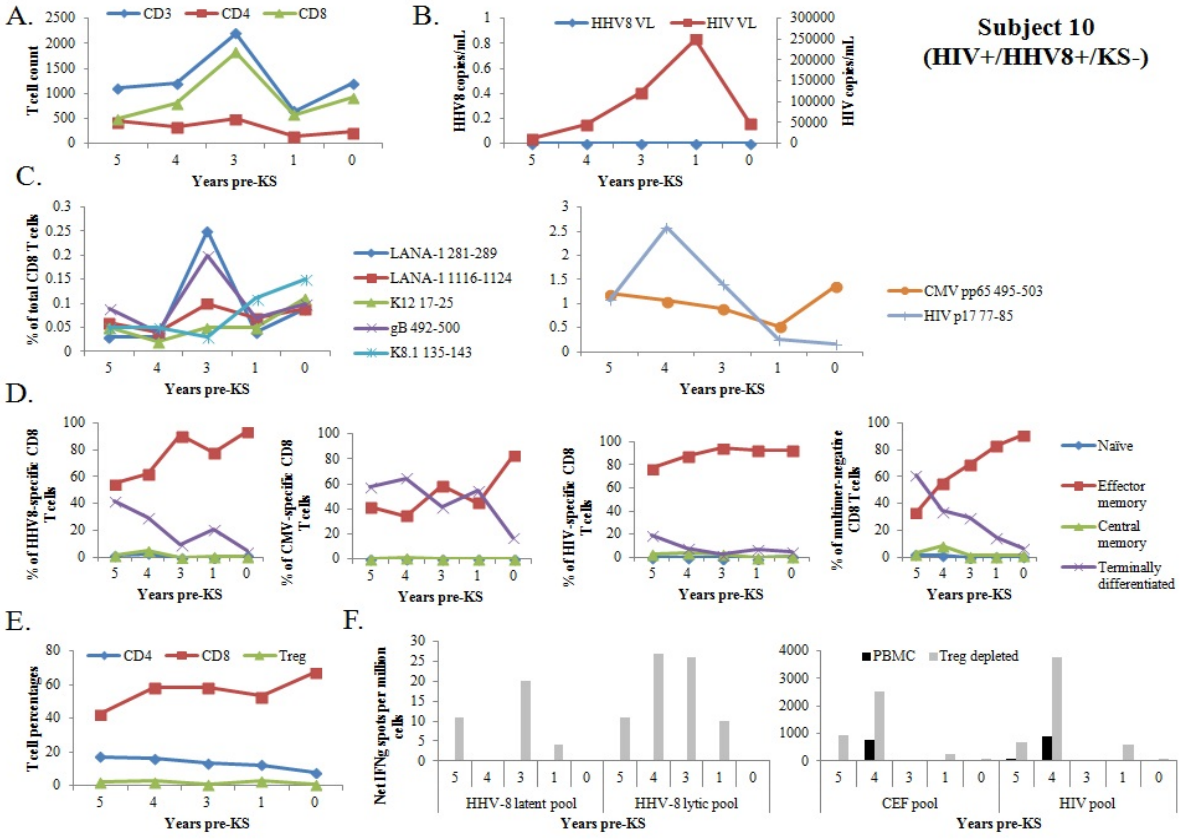
Figure 25. HIV-/HHV8+/KS- subjects

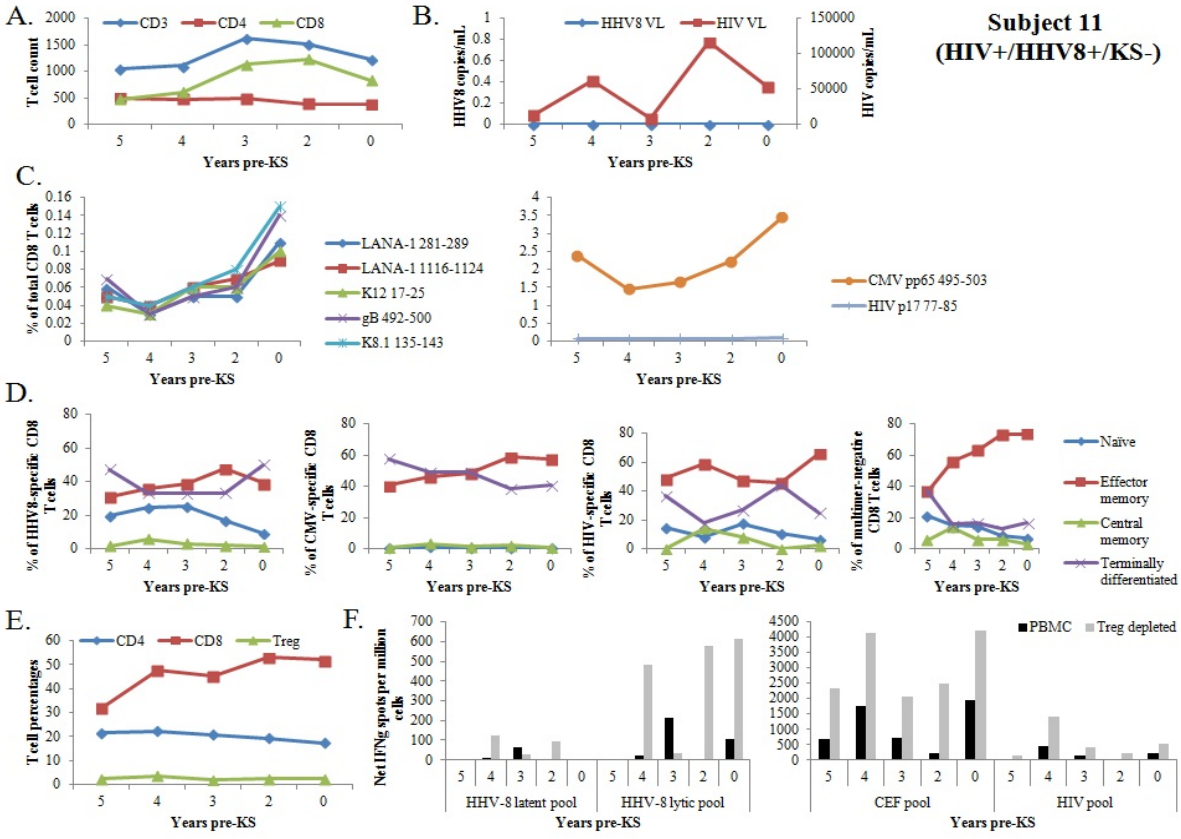
C.3 HIV+/HHV8+/KS-

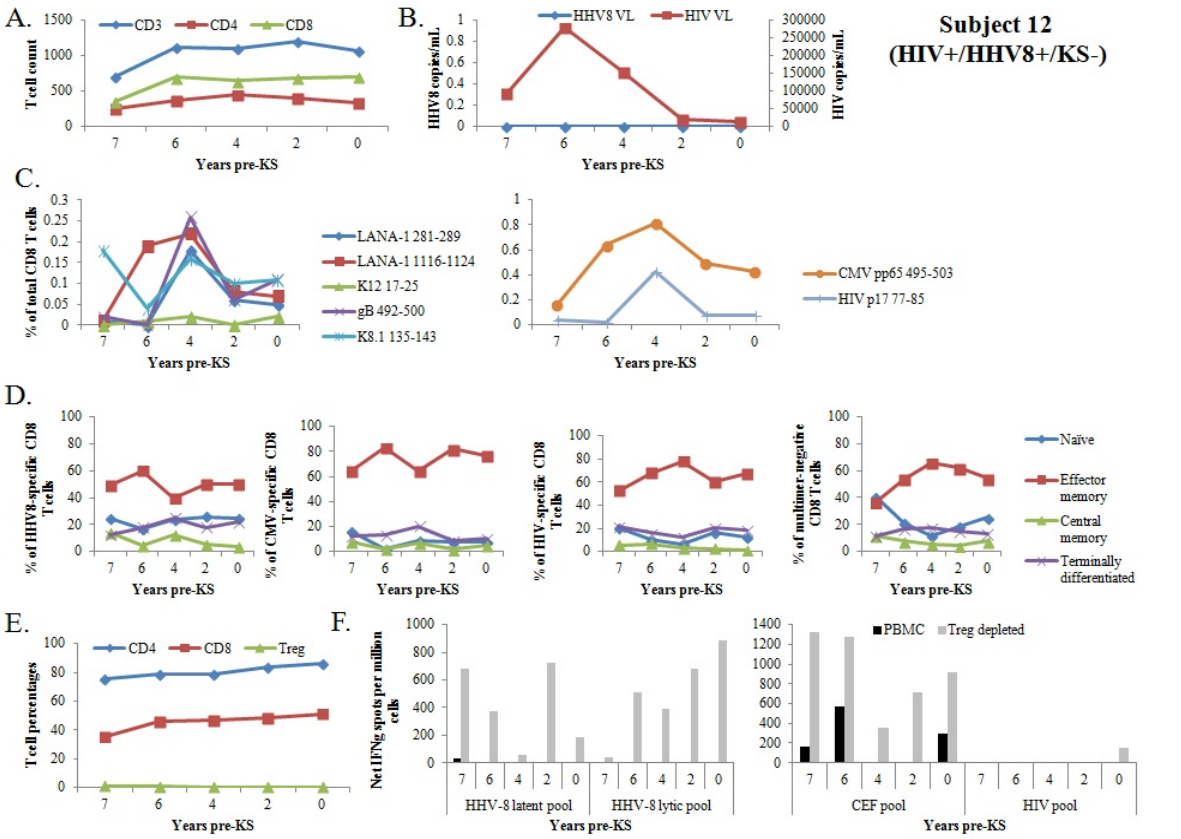
In 10 HIV+/HHV8+/KS- subjects (Figure 26), we measured blood T cell counts by flow cytometry (A), HHV-8 and HIV-1 plasma viral load by PCR (B), levels of HHV-8, CMV and HIV-1 epitope-specific $CD8^+$ T cells by dextramer staining (C) and their phenotype by flow cytometry (D), frequency of Treg by flow cytometry (E) and IFN-γ ELISPOT responses to HHV-8, CEF and HIV-1 peptide pools using PBMC or PBMC that were depleted of Treg with a magnetic bead depletion system (F) over many years of study.

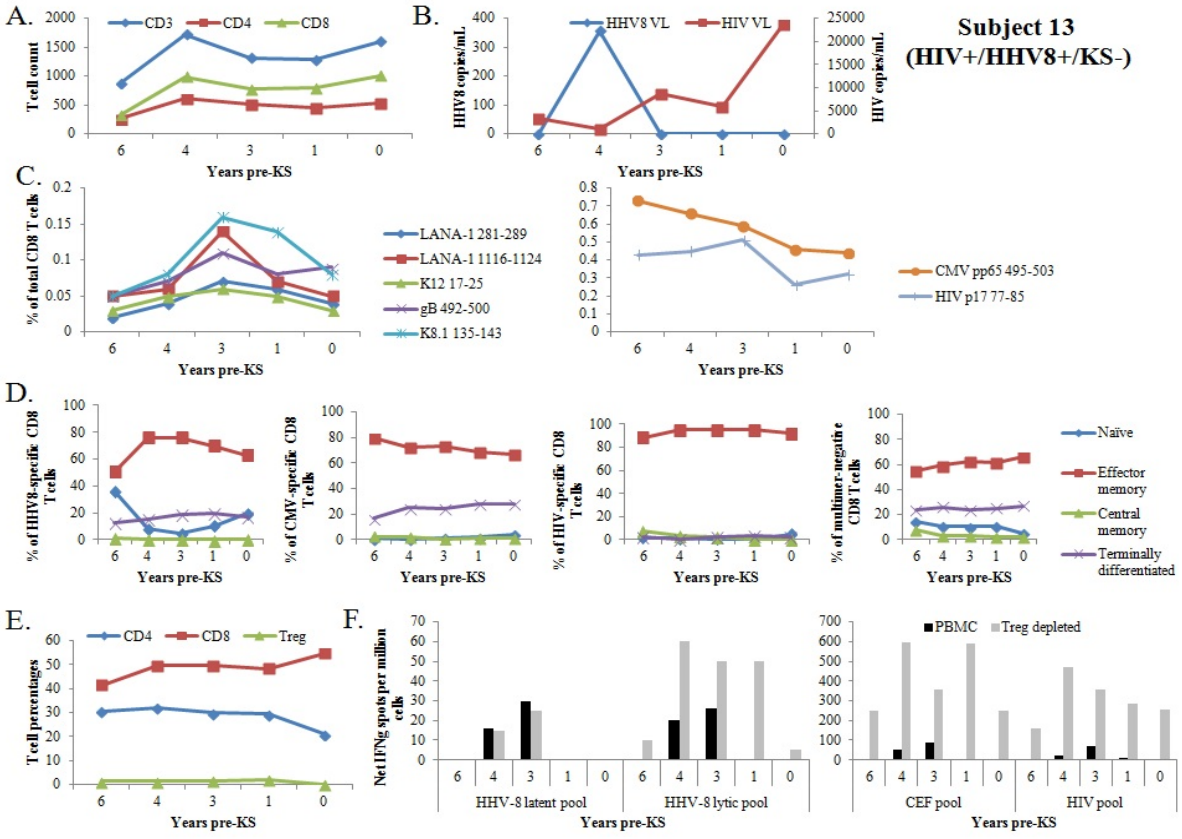


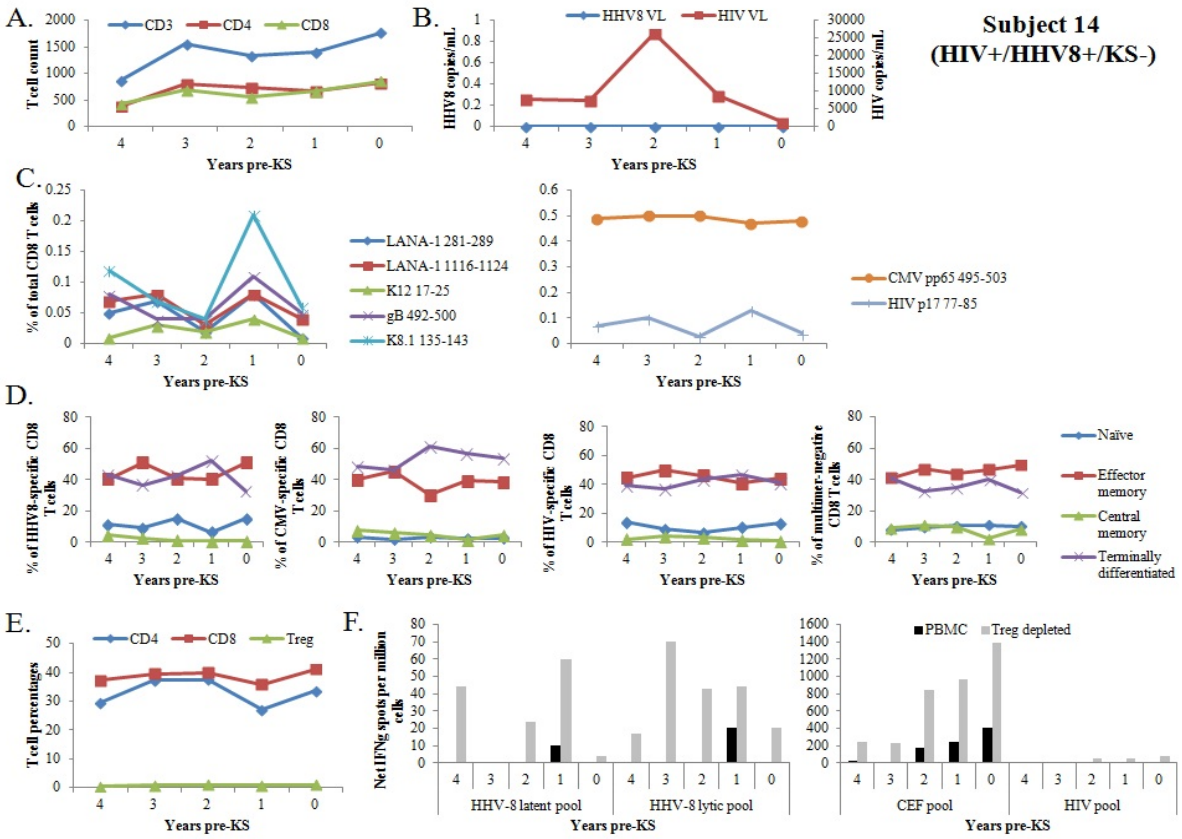

A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
Years pre-KS
Subject 9
(HIV+/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Years pre-KS
Naïve
Effector memory
Central memory
Terminally differentiated
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool
Years pre-KS

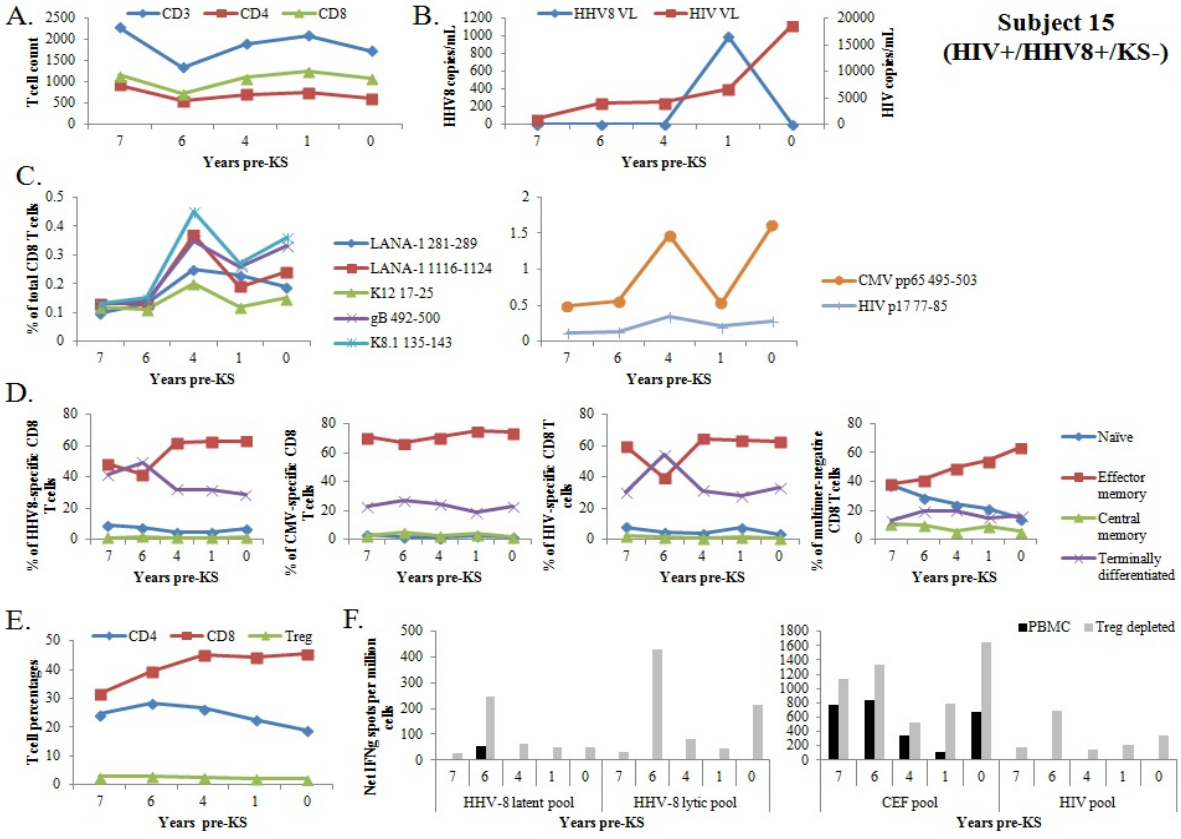

A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
Years pre-KS
Subject 10
(HIV+/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Years pre-KS
Naïve
Effector memory
Central memory
Terminally differentiated
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool
Years pre-KS

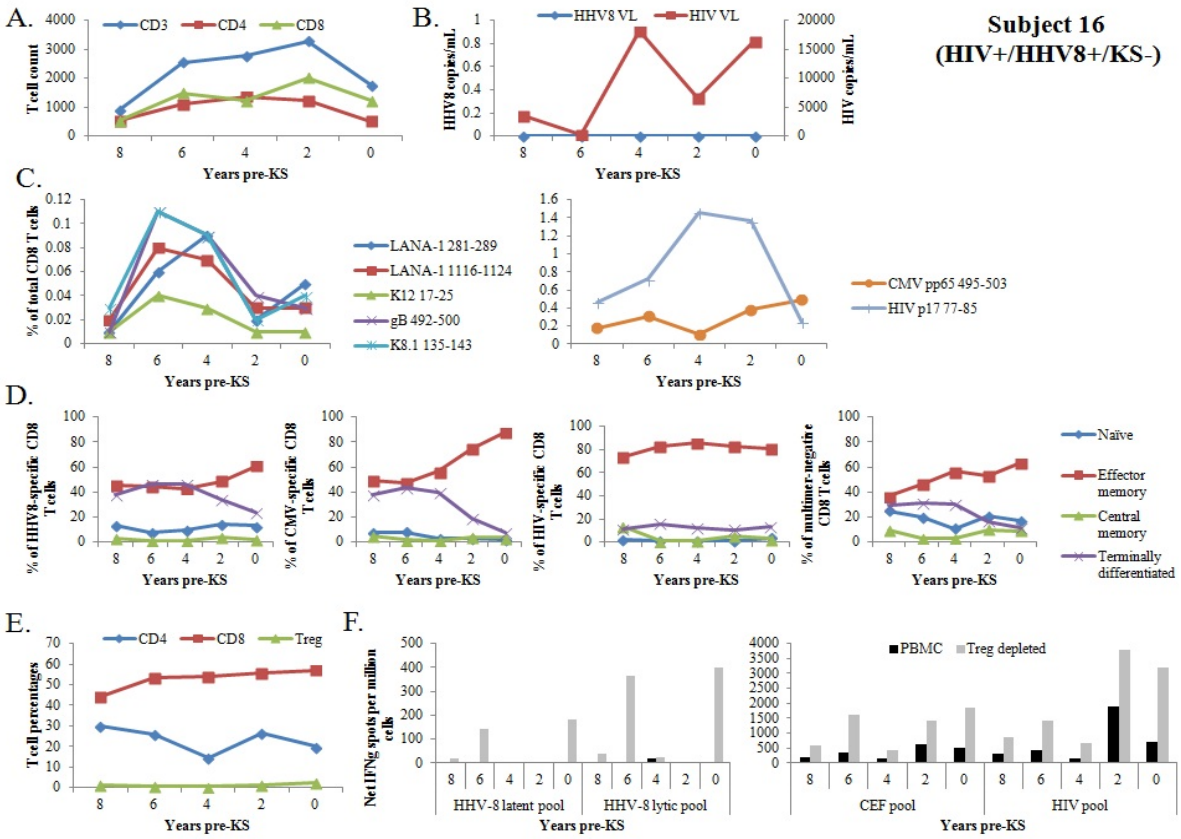

Subject 11
(HIV+/HHV8+/KS-)
A.
T cell count
CD3
CD4
CD8
Years pre-KS
B.
HHV8 copies/mL
HIV copies/mL
HHV8 VL
HIV VL
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
E.
T cell percentages
CD4
CD8
Treg
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
PBMC
Treg depleted


Subject 12
(HIV+/HHV8+/KS-)
A.
T cell count
CD3
CD4
CD8
Years pre-KS
B.
HHV8 copies/mL
HIV copies/mL
HHV8 VL
HIV VL
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
E.
T cell percentages
CD4
CD8
Treg
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
PBMC
Treg depleted


A.
T cell count
CD3
CD4
CD8
Years pre-KS
B.
HHV8 copies/mL
HIV copies/mL
HHV8 VL
HIV VL
Years pre-KS
Subject 13
(HIV+/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
T cell percentages
CD4
CD8
Treg
Years pre-KS
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool
Years pre-KS


A.
T cell count
CD3
CD4
CD8
Years pre-KS
B.
HHV8 copies/mL
HIV copies/mL
HHV8 VL
HIV VL
Years pre-KS
Subject 14
(HIV+/HHV8+/KS-)
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
T cell percentages
CD4
CD8
Treg
Years pre-KS
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool
Years pre-KS


Subject 15
(HIV+/HHV8+/KS-)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
Years pre-KS
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
PBMC
Treg depleted
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
Years pre-KS


Subject 16
(HIV+/HHV8+/KS-)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
Years pre-KS
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
Years pre-KS
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
Years pre-KS
E.
CD4
CD8
Treg
T cell percentages
Years pre-KS
F.
Net IFNg spots per million cells
PBMC
Treg depleted
HHV-8 latent pool
HHV-8 lytic pool
CEF pool
HIV pool
Years pre-KS

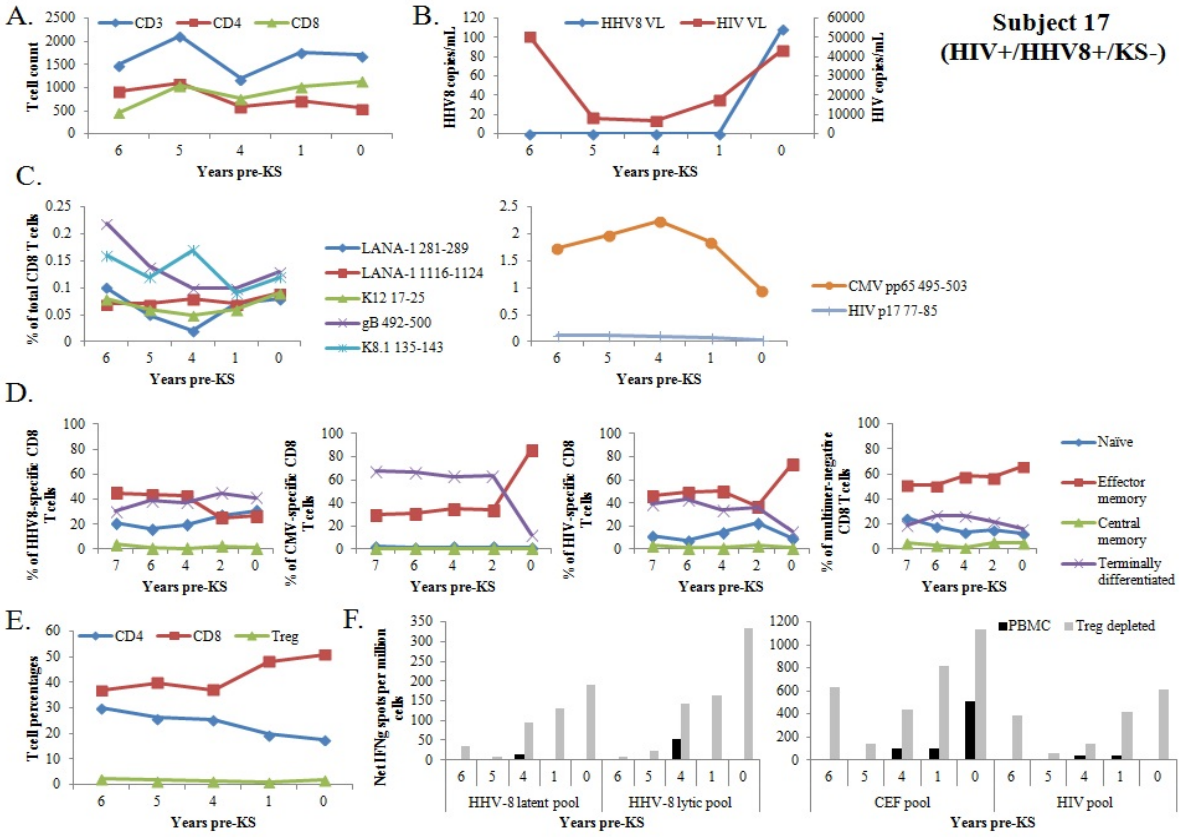
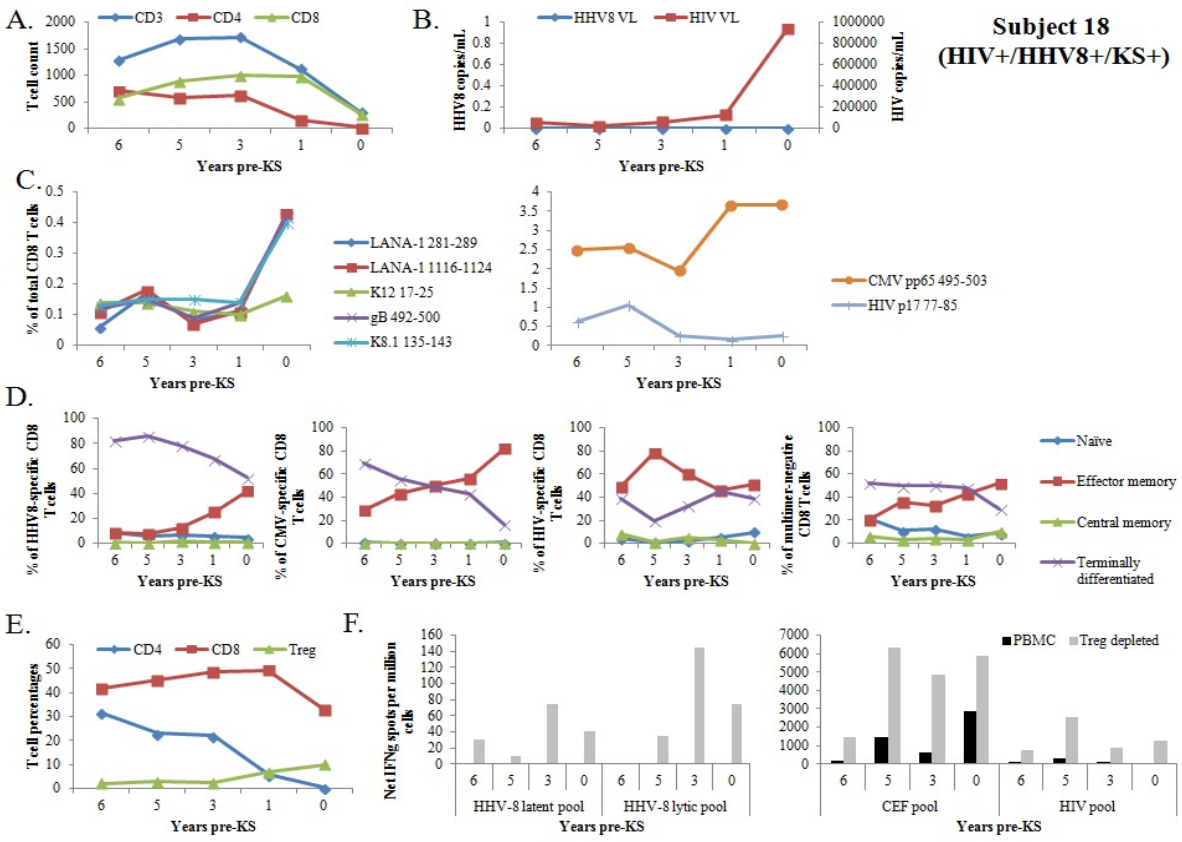


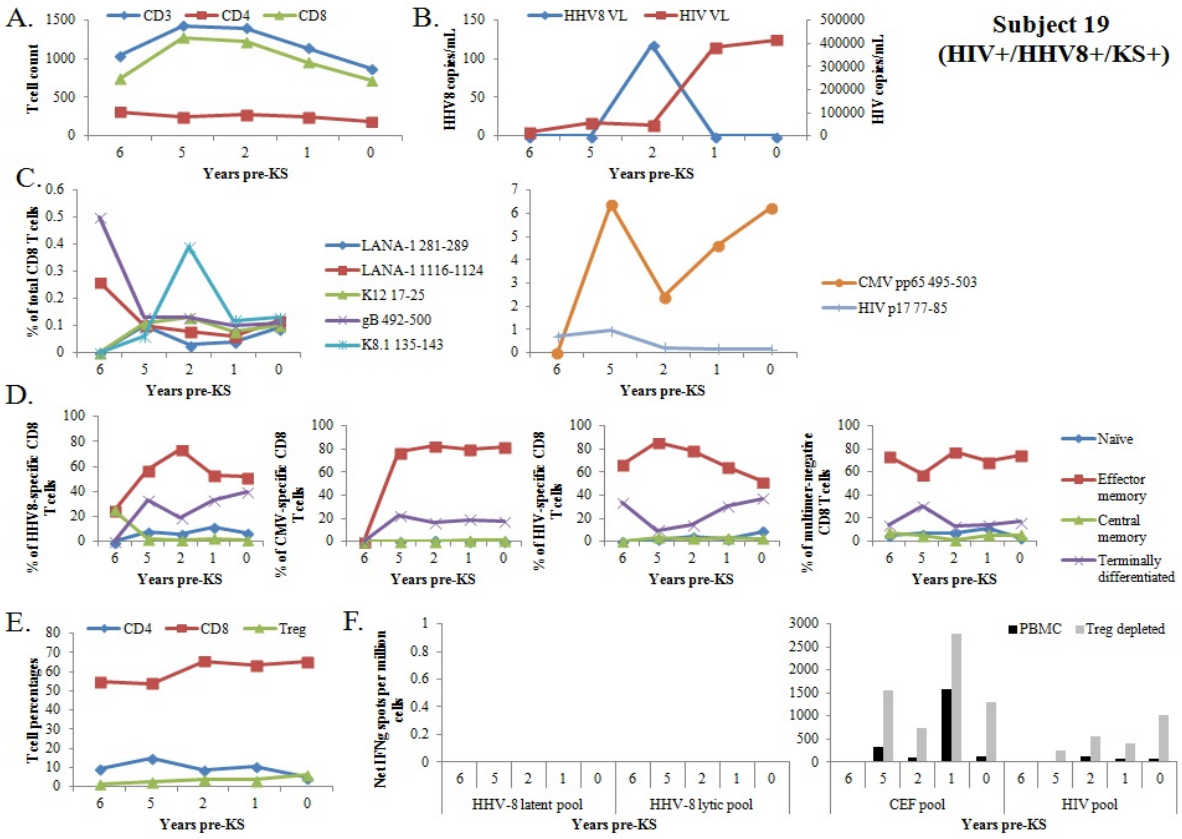
Figure 26. HIV+/HHV8+/KS- subjects

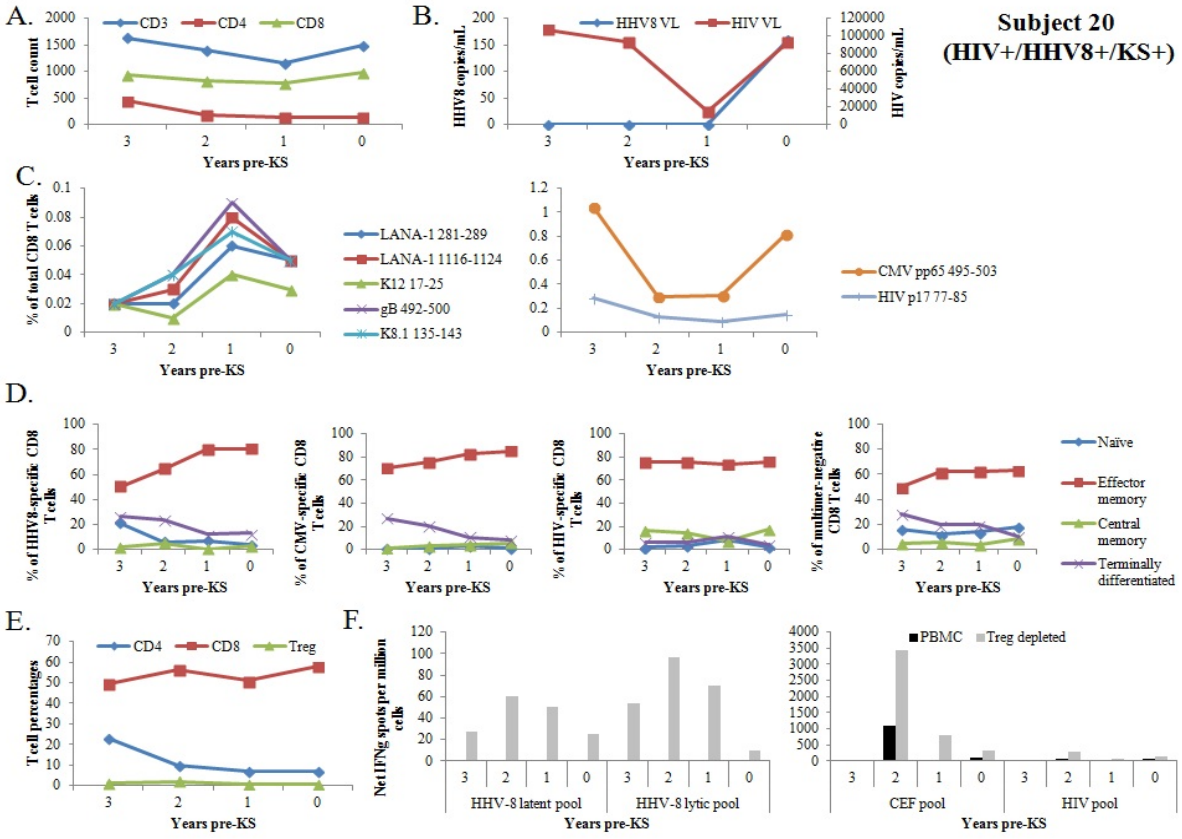
C.4 HIV+/HHV8+/KS+

In five HIV+/HHV8+/KS- subjects (Figure 27), we measured blood T cell counts by flow cytometry (A), HHV-8 and HIV-1 plasma viral load by PCR (B), levels of HHV-8, CMV and HIV-1 epitope-specific $CD8^+$ T cells by dextramer staining (C) and their phenotype by flow cytometry (D), frequency of Treg by flow cytometry (E) and IFN-γ ELISPOT responses to

HHV-8, CEF and HIV-1 peptide pools using PBMC or PBMC that were depleted of Treg with a magnetic bead depletion system (F) over many years prior to the development of KS.




Subject 19
(HIV+/HHV8+/KS+)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
E.
T cell percentages
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool


Subject 20
(HIV+/HHV8+/KS+)
A.
CD3
CD4
CD8
T cell count
Years pre-KS
B.
HHV8 VL
HIV VL
HHV8 copies/mL
HIV copies/mL
C.
% of total CD8 T cells
LANA-1 281-289
LANA-1 1116-1124
K12 17-25
gB 492-500
K8.1 135-143
CMV pp65 495-503
HIV p17 77-85
D.
% of HHV8-specific CD8 T cells
% of CMV-specific CD8 T cells
% of HIV-specific CD8 T cells
% of multimer-negative CD8 T cells
Naïve
Effector memory
Central memory
Terminally differentiated
E.
T cell percentages
F.
Net IFNg spots per million cells
HHV-8 latent pool
HHV-8 lytic pool
PBMC
Treg depleted
CEF pool
HIV pool

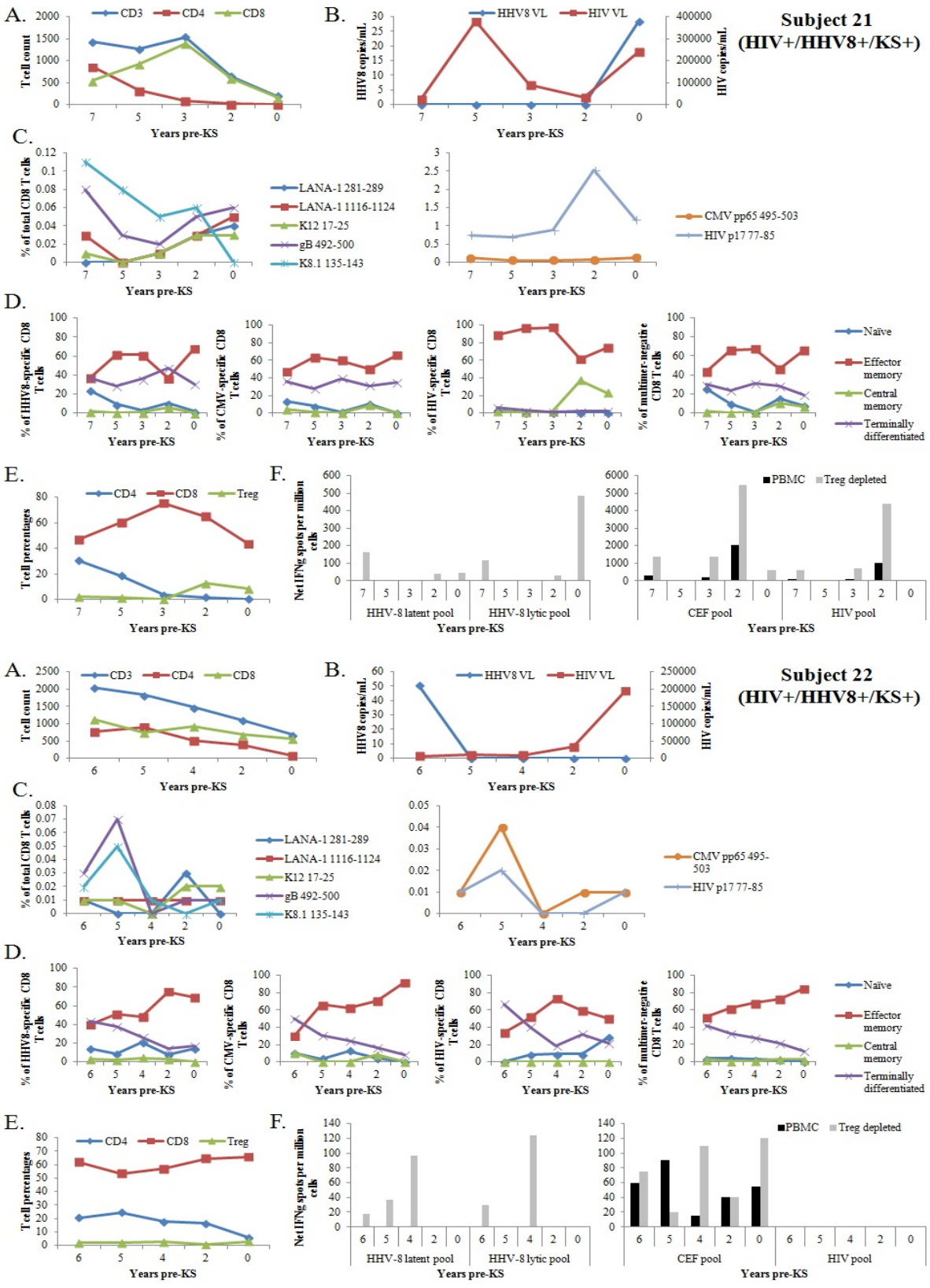


Figure 27. HIV+/HHV8+/KS+ subjects

APPENDIX D. IMMUNE MEDIATORS IN SERUM AND B CELLS DURING KS

To further investigate the role that immune mediators play in HHV-8 disease development, we examined several mediators in additional MACS participants who were co-infected with HIV-1 and HHV-8, and who did (cases) or did not (controls) develop KS. Three visits were chosen, with one visit within a year of KS development in the cases and the corresponding visit in the controls, a second visit 2-5 and a third visit 6-10 years prior to KS. Lower numbers of $CD4^+$ T cells, and higher numbers of $CD8^+$ and $CD3^+$ T cells were present in KS cases compared to controls 2-5 years before KS development (i.e., diagnosis) (Figure 28A). Plasma viral loads for HIV-1 ($p=0.007$) and HHV-8 were higher within the year prior to KS in cases (Figure 28B). We noted a similar pattern of lower serum levels of IL-8, MIP-1α, MIP-1β and VEGF at 6-10 years pre-KS in cases, with a progressive increase in levels of these immune mediators in cases 2-5 years pre-KS and within 1 year of KS (Figure 28C). IL-8 and MIP-1α levels were nearly 2-fold higher and significantly increased ($p=0.01$ and 0.008, respectively) in KS cases within the year prior to KS. Levels of MIP-1β and VEGF increased over time in the KS cases and decreased in the controls, with higher levels evident in the cases the year prior to KS. TNF-α concentrations were higher at all time points in the KS cases compared to the controls. Both TNF-α and VEGF approached significance ($p=0.07$) in KS cases. IL-6 levels were slightly higher in the controls than cases over the total 6-10 years of follow-up. Collectively, these data show that levels of IL-8, TNF-α, MIP-1α, MIP-1β and VEGF, but not IL-6, are increased within a year of KS diagnosis.

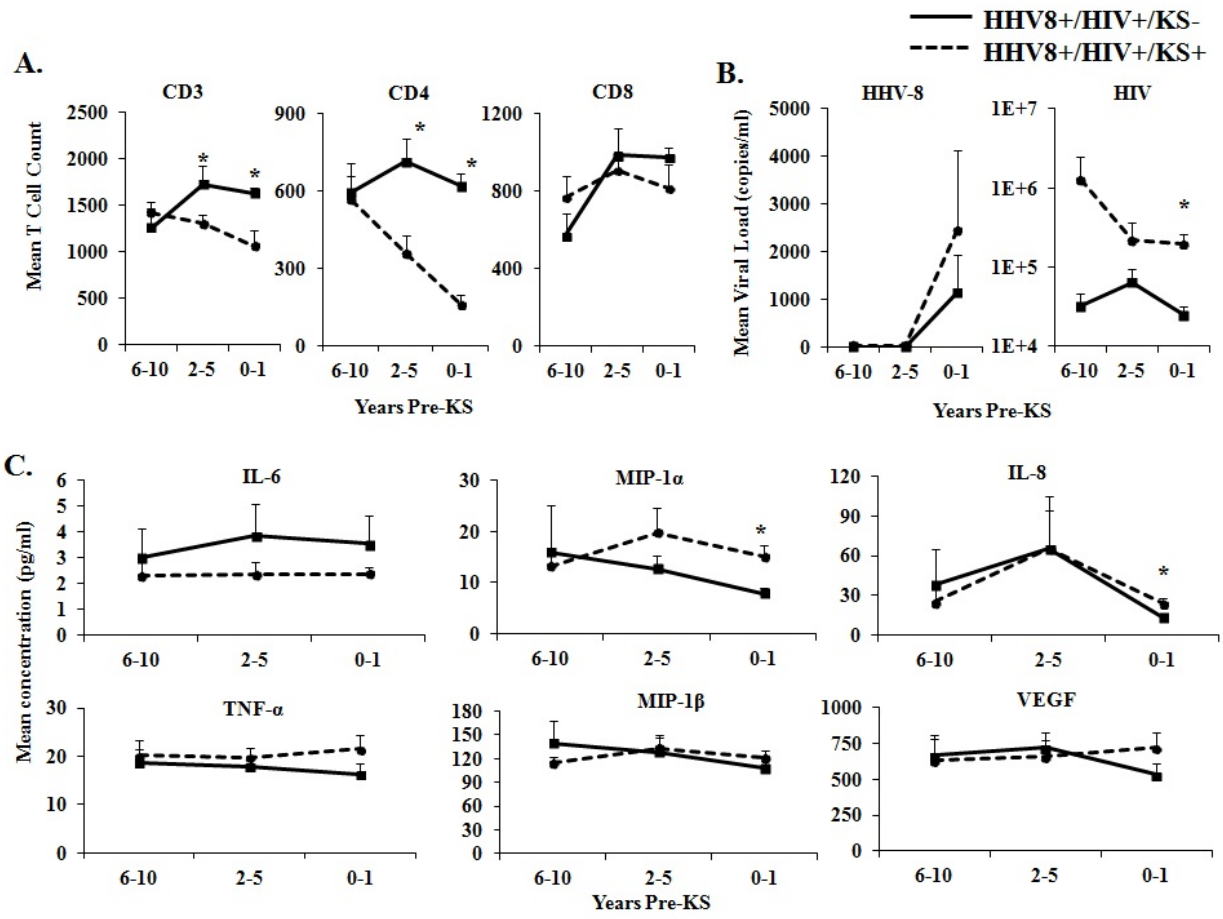


Figure 28. T cell counts, viral loads and serum biomarkers in MACS participants

HIV-positive, HHV-8-positive, KS-negative (controls) and HIV-positive, HHV-8-positive, KS-positive (cases) subjects were selected from the Pittsburgh MACS. CD3, CD4 and CD8 T cell counts at each visit were determined by flow cytometry (A, mean±SE., N=10 at years 2-10 prior, N=15 at years 0-1 prior, per group). Plasma HHV-8 and HIV-1 viral loads at each visit were determined by PCR (B, mean±SE., N=10 at years 2-10 prior, N=15 at years 0-1 prior, per group). For each visit, frozen serum samples were thawed and concentrations of TNF-α, IL-8, MIP-1α, IL-6, MIP-1β and VEGF were determined using an electrochemiluminescence MSD assay (C, mean±SE., N=10 at years 2-10 prior, N=15 at years 0-1 prior, per group). (*$p<0.05$ by t-test)

As B cells are a main target of HHV-8, we also determined whether B lymphocytes were producing these biomarkers. B cells were purified from PBMC of two KS cases and two non-KS controls and assayed for viral DNA and immune mediator mRNA at 0-1 years prior to KS development. As determined by PCR, both non-KS controls and one KS case had undetectable levels of HHV-8 in their $CD19^+$ B cells, while one KS case had a viral load of 183,306 copies/500,000 cells. RNA was extracted and used in a PCR assay to determine mRNA expression of immune mediators. Expression of IL-6, MIP-1α, IL-8 and VEGF mRNA was elevated in KS cases compared to controls, with 3.7, 1.9, 1.3 and 1.2-fold increases, respectively (Figure 29). MIP-1β levels were similar among cases and controls, while TNF-α was enhanced in KS controls. These data indicate that HHV-8 infected B cells produce more proinflammatory cytokines, chemokines and growth factors in HIV-1 infected individuals who are developing KS compared to those without KS.

This appendix was modified from: Human Herpesvirus 8 Lytic Infection Induces Polyfunctional Memory and Naive B Lymphocyte Reactivity, *submitted for publication*, E. Knowlton, G. Rappocciolo, L. Lepone, S. Nadgir, A. Bulotta, S. Berendam, J. Li, T. Reinhart, F. Jenkins and C. Rinaldo.

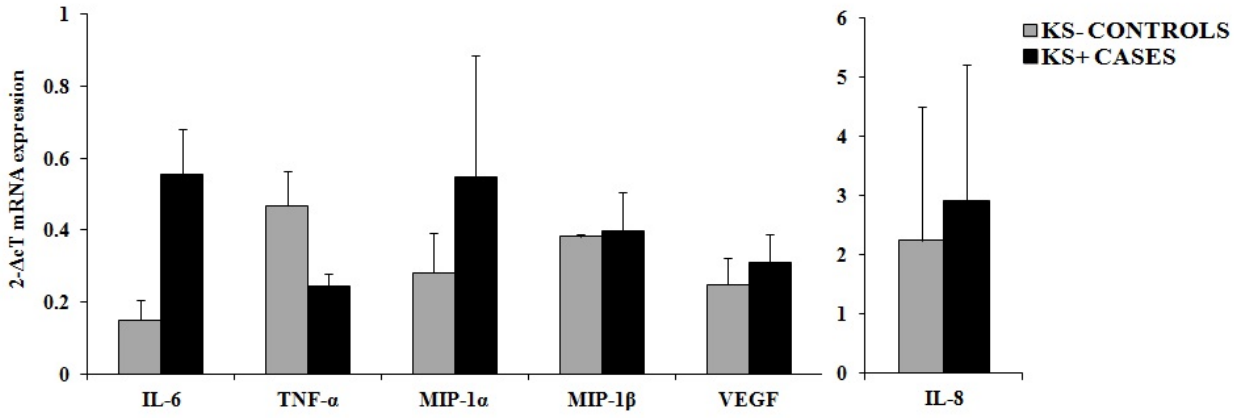


Figure 29. mRNA expression in B cells from KS cases and controls

$CD19^{+}$ cells were isolated from PBMC of two HIV-positive, HHV-8-positive, KS-negative (controls) and two HIV-positive, HHV-8-positive, KS-positive (cases) subjects from the Pittsburgh MACS at 0-1 years prior to KS development. RNA was extracted and used in a real-time PCR assay to determine mRNA expression of immune mediators (mean±SE., N=2 per group).

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