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Identification of the Kaposi's Sarcoma-associated Herpesvirus (KSHV) Surface Glycoprotein Targets of Human KSHV-specific Neutralizing Antibody Responses

Yasaman Mortazavi
University of Nebraska - Lincoln, yass.mortazavi@huskers.unl.edu

IDENTIFICATION OF THE KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) SURFACE GLYCOPROTEIN TARGETS OF HUMAN KSHV-SPECIFIC NEUTRALIZING ANTIBODY RESPONSES

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Yasaman Mortazavi

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IDENTIFICATION OF THE KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) SURFACE GLYCOPROTEIN TARGETS OF HUMAN KSHV-SPECIFIC NEUTRALIZING ANTIBODY RESPONSES

Yasaman Mortazavi, M.S.

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Advisor: Charles Wood

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the etiological agent of Kaposi’s sarcoma (KS), and is also associated with two B cell malignancies, primary effusion lymphoma and multicentric Castleman's disease. The distribution of KSHV varies globally with high prevalence in some areas of sub-Saharan Africa (SSA), where seroprevalence can be as high as 80%. It is estimated that nearly 44,000 new cases of KS emerge annually globally, with the highest incidents occurring in Africa, where KSHV is endemic. Currently, there is no prophylactic vaccine against KSHV, and efforts to develop prophylactic vaccines have been limited.

Elicitation of neutralizing antibodies (nAbs) often correlates with protection during viral infections in humans, but it is not clear whether nAbs against KSHV will also convey protection against infection. KSHV initiates infection via interaction with various receptors

on the surface of target cells, which is primarily mediated by multiple viral glycoproteins embedded in the viral envelope. These glycoproteins play important roles in virion attachment and entry into target cells, therefore they could be potential targets for KSHV-specific nAbs. Characterization of the human neutralizing antibody responses against KSHV envelope glycoproteins and identifying the targets of these antibodies, are needed for designing an effective vaccine against KSHV.

KSHV virions incorporate eight glycoproteins into their envelope: ORF8 (gB), ORF28, ORF68, ORF22 (gH), ORF47 (gL), ORF39 (gM), ORF53 (gN) and gpK8.1. Through testing the ability of each KSHV glycoprotein in recognizing/adsorbing KSHV-specific nAbs by this study, it was determined that multiple KSHV glycoproteins were able to bind to these antibodies in a majority of KSHV infected individuals. In addition, the ability of each KSHV glycoprotein in depleting the KSHV nAbs from KSHV positive plasma varied among individuals. Among all the KSHV envelope glycoproteins, gH/gL complex showed high adsorption/recognition levels of KSHV-specific nAbs in 80% of the participants analyzed. These findings suggest that nAbs responses are variable in KSHV infected individuals, and multiple KSHV envelope glycoproteins are the targets of KSHV-specific nAbs, with gH/gL complex being the most predominant target, which can serve as a potential target antigen for developing prophylactic vaccines against KSHV.

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Chapter 1

Literature Review

I. General biology of human herpesviruses

Herpesviruses belong to the herpesviridae family and are divided into three subfamilies of α herpesviruses, β herpesviruses, and γ herpesviruses based on their biological properties. Alpha herpesviruses have a broader host range than β herpesviruses, however, γ herpesviruses have even more restricted hosts than β herpesviruses (Whitley, 1996). The viral DNA of herpesviridae replicates in the host cell nucleus of a wide range of vertebrates including, humans, mice, horses, cattle, pigs, fish, and even in some invertebrates such as oysters (Davison, 2010). Herpesviruses are widespread among humans, and more than 90% of adults are infected with at least one herpesvirus which then can remain in a latent form lifelong (Whitley, 1996).

There are more than 130 known herpesviruses, of which 8 can infect humans: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, also known as HHV1 and HHV2); varicella-zoster virus (VZV); Epstein-Barr virus (EBV); human cytomegalovirus (HCMV or HHV-5); human herpesvirus 6A and 6B (HHV-6A and HHV-6B); human herpesvirus 7 (HHV-7); and Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8) (Brown and Newcomb, 2011; Whitley, 1996). A similar virion structure is shared among all herpesviruses, which is comprised of a core containing a relatively large, linear, double-stranded DNA genome encoding 70 to 200 genes. The viral genome is encapsidated in an icosahedral nucleocapsid composed of 162 capsomere

subunits, which itself is surrounded by a protein coat called tegument containing both viral proteins and viral mRNAs, and a lipid bilayer envelope obtained from the host cell which harbors a number of viral glycoproteins (Brown and Newcomb, 2011).

II. KSHV epidemiology

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), the most recently identified member of the herpesviridae, is the etiological agent of all types of Kaposi's sarcoma (KS) (endemic KS, epidemic KS, Classical KS and Iatrogenic KS) and also two lymphoproliferative diseases, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Chang et al., 1994; Sousa-Squiavinato et al., 2015). It is estimated that 44,000 new cases of Kaposi's sarcomas emerge each year, with the highest incidents occurring in Africa, where the virus is endemic (Bray et al., 2018; Torre et al., 2015). The seroprevalence of KSHV differs globally; with high rates in Africa and South America of between 30 to 80 percent, which is considered to be endemic, intermediate rates in the Mediterranean regions, 4 to 35 percent, and less than 10 percent in North America, Western Europe and Asia (Casper, 2011; Dedicoat and Newton, 2003; Gao et al., 1996; Rohner et al., 2014; Simpson et al., 1996; Whitby et al., 1998).

There are four clinical presentations of KS: Classical KS (CKS), a rare disease found in elderly men of Mediterranean and Eastern European decent; Endemic KS (EnKS), a more aggressive form of KS than classical KS found among HIV-1 negative individuals in Africa; Epidemic KS (EpKS), the most aggressive form of KS which is associated with HIV-1 co-infection; and Iatrogenic or posttransplant KS (IKS), which is associated with clinical procedures involving immunosuppressive therapies (Dollard et al., 2005; Moore and Chang, 1995, 1998). A vaccine for KSHV is not currently available but is needed, especially with decades of research, optimal treatments for KS remain poorly defined, and

clinical outcomes are particularly unfavorable in resource-limited settings (Gao et al., 1996).

KSHV transmission occurs largely via saliva, sexual contact, and in some rare cases through organ transplantation and blood transfusion (Alexander et al., 1998; Barozzi et al., 2003; Brayfield et al., 2004; Butler et al., 2011; Johnston et al., 2009; Mbulaiteye et al., 2004; Pauk et al., 2000). KSHV-associated malignancies are primarily present in people with acquired immunodeficiency syndrome (AIDS) and induced immunosuppression in organ transplant recipients (Bouvard et al., 2009; Parkin, 2006). KS is a major cause of sickness and death in adults in sub-Saharan Africa, where the prevalence of HIV/AIDS is still high, and a great threat to children as well (Feller et al., 2010; Mbulaiteye et al., 2006; Wabinga et al., 2000). Some early reports from sub-Saharan Africa revealed that maternal seropositivity for KSHV is a risk factor for infection among children, indicating a likelihood of mother to child transmission (Bourboulia et al., 1998). Despite a significant reduction in the incidence of KS cases since the introduction of antiretroviral therapy (ART), there are still a considerable number of new cases of KS arising each year worldwide (Bray et al., 2018; Torre et al., 2015). In addition, only about 50% of patients can accomplish a complete resolution of KS lesions with ART treatment, all justifying the need to develop a vaccine to project against KSHV infection. However, to date, there have not been significant efforts to develop a prophylactic vaccine against KSHV, with no reported preclinical or clinical vaccine trial in the last three decades (Nguyen et al., 2008; Wu et al., 2012).

III. KSHV life cycle

KSHV contains a large linear double-stranded DNA genome of approximately 160 Kb in size and encodes more than 100 open reading frames (ORFs), from which ORFs 4-75 are named by their homology with HSV ORFs (Dourmishev et al., 2003). Like all other herpesviruses, KSHV displays two distinct phases of life cycle; latent infection and lytic reactivation, which can be differentiated based on the viral genes that are being expressed, allowing the virus to transition between silent and active life cycle (Cai et al., 2010; Ye et al., 2011). During latency, which can persist for life, few viral genes are expressed in the infected cells. At this stage of infection, the viral genome circularizes in the nucleus and is maintained as a chromatinized nuclear episome, and no virion production is observed (Ballestas et al., 1999). Latency can be interrupted, and the virus can switch to lytic life cycle either spontaneously or via induced reactivation. As reactivation occurs, lytic genes are expressed and the assembly of new virions begins in the nucleus (Gradoville et al., 2000).

In KS lesions, only a small number of cells are going through lytic reactivation (i.e. lytically replicating), and most tumor cells are latently infected with KSHV. As a result, viral latent infection and latent genes are primarily essential for the development of KSHV induced malignancies (Giffin and Damania, 2014). The major viral genes expressed during KSHV latency are ORF71 (v-FLIP), ORF72 (v-Cyclin), ORF73 (latency-associated nuclear antigen, LANA) and K12/Kaposin (Kaposin A, B and C) (Dittmer et al., 1998; Kedes et al., 1997; Staskus et al., 1997).

LANA is expressed predominantly during latency and is the most abundant protein in latently infected cells. To maintain the viral genome in infected cells, LANA interacts with the host chromatin proteins to promote binding of the KSHV genome to host genome, thus avoiding viral genome loss during mitosis (Yan et al., 2019). Another major function of LANA is its interaction with the p53 protein. The p53 protein, generally referred to as a tumor suppression protein, is a transcriptional regulator of cell growth. The induction of p53 commonly leads to either cell cycle arrest or apoptosis. The loss of p53 is believed to correlate with cellular transformation and tumorigenesis. It has been shown that LANA interacts with p53 to suppress its transcriptional activity and prevents apoptosis. By promoting cell survival, LANA contributes directly to viral persistence and oncogenesis in KS (Friborg et al., 1999). LANA has also been reported to directly bind and inhibit Replication and Transcription Activator (RTA) promoter activities, therefore suppressing viral lytic gene expressions (Lan et al., 2004).

The viral cyclin is also expressed predominantly during the latent phase of KSHV viral cycle, and it is a homolog of cellular cyclin D2 which primarily regulates cell cycle and cell proliferation. (Jones et al., 2014; Li et al., 1997; Van Dross et al., 2005). The v-cyclin also phosphorylates the retinoblastoma (Rb) protein. The un-phosphorylated form of Rb binds to E2F, a transcriptional protein that leads to the transcription and translation of several S-phase genes to promote cell proliferation by binding to DNA, to prevent E2F binding to DNA leading to down regulate of S-phase proteins and cell proliferation (Harbour and Dean, 2000). Phosphorylation of Rb by v-cyclin will prevent Rb from

binding to E2F to enable it to promote the expression of S-phase genes and consequently cell proliferation (Harbour and Dean, 2000).

Another viral protein expressed during latent infection is the v-FLIP protein, a homolog to cellular FLIP. This protein is expressed from one of the three latency associated genes that are involved in the regulation of proliferation and apoptosis (Dittmer et al., 1998; Fakhari and Dittmer, 2002; Jenner et al., 2001). It has been suggested that v-FLIP can block Fas-mediated apoptosis by inhibiting procaspase 8 cleavage (Bertin et al., 1997). Additionally, v-FLIP inhibits viral reactivation and lytic replication by suppressing AP-1 pathway (Ye et al., 2008).

The latent state of KSHV can be disrupted to enable KSHV to enter a new phase of replication cycle, lytic reactivation. During lytic reactivation most viral genes are expressed to replicate the viral DNA using the cellular replication machinery, and new virions are generated and released from infected cells to spread the infection. The viral ORF50 (RTA) plays a major role in KSHV lytic reactivation from latency, and a mutant KSHV with RTA deletion was shown to be incapable of lytic reactivation (Lukac et al., 1999). A number of studies have demonstrated that RTA can transactivate a series of important viral lytic genes, including DBP (ORF6), gB (ORF8), DNA polymerase (ORF9), assembly protein (AP), PAN (non-coding RNA), thymidine kinase (TK), vIL-6 (K2), ORF37, ORF52, ORF56, ORF57, ORF59, ORF65, K8, K9 and K1 (Bu et al., 2008; Byun et al., 2002; Chen et al., 2009). RTA is also capable of auto-activating its own promoter (Deng et al., 2000).

The K8 encodes the K-bZIP (K8α) protein which belongs to the basic-leucine zipper (bZIP) family of transcription factors (Tang and Zheng, 2002). Studies have shown two different functions for this protein; suppressing RTA auto-activation to inhibit lytic gene expression under certain conditions, and regulating lytic life cycle by promoting viral DNA replication via interaction with *ori-Lyt* (Liao et al., 2003; Liu et al., 2018). Upon lytic reactivation, viral lytic genes encoding different viral structural proteins, including viral envelope glycoproteins are also being expressed. These proteins are necessary for the assembly and egress of new infectious virions.

IV. KSHV infection and host cell tropism

KSHV has a broad tropism and can infect various cell types including endothelial, epithelial, B cells, monocytes, macrophages, dendritic, keratinocytes, fibroblasts and neuronal cells (Chandran, 2010; Chandran and Hutt-Fletcher, 2007; Kikuta et al., 1997; Pica and Volpi, 2007; Tso et al., 2017). Similar to most DNA viruses, KSHV infects host cells using the fundamental cellular mechanism of membrane fusion mediated by the interaction of several viral glycoproteins with target cell receptors (Connolly et al., 2011).

The KSHV infection of target cells is a multistep complex process which involves a series of interactions between multiple viral envelope glycoproteins with multiple host cell surface receptors. Different viral glycoproteins and host cell receptors may be involved, and will vary dependent on the target cells. This process is initiated via host cell receptor recognition and binding followed by the virus entry into the cytosol. This is acquired either via direct fusion of the viral envelope with the host cell plasma membrane, or fusion of viral envelope with the endosomal membranes after the internalization of the virus by endocytic pathways, including micropinocytosis in some cell types, such as endothelial HMVEC-d and HUVEC cells. In contrast, clathrin-mediated endocytosis are involved in other cells types, such as fibroblast HFF cells (Akula et al., 2003; Kerur et al., 2010; Raghu et al., 2009; Rappocciolo et al., 2008). Then the viral capsid released in the cytoplasm is transported to the nuclear periphery, where disassembly of the capsid and the release of viral genome into the nucleus occurs (Chandran, 2010; Connolly et al., 2011).

V. KSHV envelope glycoproteins

In addition to encoding envelop glycoproteins gB (ORF8), gH (ORF22), gL (ORF47), gM (ORF39) and gN (ORF53) which are conserved among herpesviruses, KSHV also encodes some unique envelope glycoproteins gpK8.1, ORF28 and ORF68 (Neipel et al., 1997; Russo et al., 1996; Zhu et al., 2005). The KSHV gB is synthesized as a 110-kDa protein precursor which undergoes further cleavage and processing. The virion envelope-associated form of gB consists of multimers of the cleaved 75-kDa and 54-kDa polypeptides that are di-sulfide linked which are associated with high levels of mannose and complex sugars (Akula et al., 2001a; Baghian et al., 2000; Wang et al., 2003). Similar to other herpesviruses, KSHV gH, 120-kDa in size, forms a non-covalent complex with KSHV gL. Studies have shown that gH can be transported to the membrane independently from gL, however, the interaction with gH is required for efficient transport of gL to the cell surface (Hahn et al., 2009; Naranatt et al., 2002). KSHV gN is required for functional processing of gM, and these two glycoproteins form heterodimers (Koyano et al., 2003). The KSHV gpk8.1 gene codes for two forms of proteins; the 228-amino acid (aa)-long gpK8.1A and the 167-aa-long gpk8.1B which is processed from gpK8.1A via an in-frame deletion of 61 amino acids. The gpk8.1A is the predominant component of the KSHV virion envelope (Raab et al., 1998; Zhu et al., 2005; Zhu et al., 1999). Currently, the roles played by ORF28 and ORF68 in KSHV infection are still not fully understood.

VI. KSHV host cell surface receptors for KSHV

KSHV infection is mediated through virus interaction with host cell receptors. A broad range of receptors are utilized by KSHV from which the major ones are heparin sulfate (HS); α3β1, αVβ3 and αVβ5 integrins; DC-SIGN (Dendritic cell specific intracellular adhesion molecule-3 (ICAM-3) grabbing non-integrin), xCT, CD98, and several members of Ephrin family receptor tyrosine kinases (Ephs) (Grosskopf et al., 2019; Naranatt et al., 2002). Treatment of KSHV with soluble HS has been showed to block virus biding to target cells including, endothelial (HMVEC-d), fibroblast (HFF), B-cell (BJAB), epithelial (HEK293) and monocyte (THP1) cells (Akula et al., 2001a, 2002; Birkmann et al., 2001; Hahn et al., 2009; Jarousse et al., 2008; Kabir-Salmani et al., 2008; Kerur et al., 2010; Naranatt et al., 2002; Veettil et al., 2008; Wang et al., 2003). Studies have suggested that pre-incubation of virus with soluble α3β1, αVβ3 and αVβ5, and also pre-treatment of cells with anti- α3β1, αVβ3 and αVβ5 antibodies prevents virus entry but has no effect on virus binding to HMVEC-d, HFF and THP1 cells (Akula et al., 2002; Garrigues et al., 2008; Kerur et al., 2010; Veettil et al., 2008). Treatment of cells with mannan (Linear polymer of mannose, the natural ligand of DC-SIGN) or with anti- DC-SIGN antibodies can inhibit virus binding and entry into B cells and THP-1 cells (Kerur et al., 2010; Rappocciolo et al., 2008; Rappocciolo et al., 2006). Pre-treatment of HMVEC-d cells with anti- xCT and CD98 antibodies have been shown to block virus gene expression, but has no effect on virus binding and entry (Akula et al., 2002). Treatment of cells with anti- EphA2R antibodies and incubation of virus with soluble EphA2 blocks virus entry but has no effect

on virus binding in HMVEC-d and HFF cells (Bhattacharyya et al., 2010; Hahn et al., 2012).

VII. Interaction between viral glycoproteins and host cells surface receptors

KSHV gB is believed to be the major viral envelope glycoprotein involved in the initiation of virus binding and entry into target cells. KSHV gB mediates virus binding and entry via its interactions with host cell surface molecules (receptors) heparin sulfate (HS) and entry receptors α3β1, αVβ3 and αVβ5 integrins in HMVEC-d and HFF cells (Akula et al., 2001a, 2002; Akula et al., 2001b; Veettil et al., 2008; Wang et al., 2003). KSHV gB also has the ability to bind to DC-SIGN molecule of B cells due to its high mannose structure (Hensler et al., 2014). In contrast to other human herpesvirus gB homologs, KSHV gB is the only glycoprotein that possesses an integrin-binding RGD (Arg-Gly-Asp) motif; it is located at amino acids 27 to 29 which is thought to be the minimal peptide region required for interaction with host cell surface integrins. Studies have shown that α3β1, αVβ3 and αVβ5 integrins play important roles in KSHV infection in HFF, HMVEC-d, HEK293 and monocytes. A reduction of infection is also observed with RGD peptides and antibodies against RGD-gB peptide (Akula et al., 2002; Chakraborty et al., 2011; Garrigues et al., 2008; Kerur et al., 2010; Veettil et al., 2008).

Similar to gB, KSHV gpK8.1 is another envelope glycoprotein that has the ability to interact and bind to host cell HS molecules (Birkmann et al., 2001; Wang et al., 2001). The non-covalently linked gH/gL complex has also been shown to interact with HS molecules, and pre-incubation of virus with anti- gH and gL antibodies can block KSHV entry, but not KSHV binding in HFF cells (Naranatt et al., 2002). More recent studies have demonstrated that KSHV gH/gL complex interacts with the KSHV entry receptors EphA2, EphA4, EphA5 and EphA7 which are important for virus entry and cell-to-cell

transmission (Grosskopf et al., 2019; Naranatt et al., 2002). The interaction of KSHV gM/gN heterodimer with host cell receptors is still unknown, however, they are primarily involved in virus penetration and egress (Koyano et al., 2003).

VIII. KSHV-specific neutralizing antibodies

Effective prophylactic viral vaccines stimulate an individual's humoral immune system to elicit neutralizing antibodies (nAbs) typically against surface glycoprotein(s) that mediate binding and entry of an infectious agent into the host cells (Nguyen et al., 2008; Ogembo et al., 2015). These nAbs can recognize, bind and neutralize the biological effects of the viral antigen. Studies based on influenza have demonstrated that nAbs elicited by vaccination are capable of preventing viral infection. These nAbs primarily target the viral membrane surface proteins hemagglutinin (HA) and neuraminidase (NA) (Soema et al., 2015; van Els et al., 2014). Studies on nAbs from our lab and others have shown that KSHV infected individuals do not readily develop nAbs in non-KS asymptomatic patients. In contrast we have found that most KS patients developed nAbs (Kumar et al., 2013). These findings suggest that KSHV-specific nAbs are incapable of preventing KS, but as in the case of influenza, they could potentially have the ability to prevent the viral infection by binding to the viral glycoproteins.

Since KSHV envelope glycoproteins are presented on the viral surface and are critical for viral entry into permissive cells, they are attractive vaccine antigen candidates for eliciting nAbs. Over the past few decades, several studies have been conducted to study the effect of KSHV envelope glycoproteins-specific nAbs on the neutralization of KSHV infectivity of target cells. One study found that incubation of KSHV with polyclonal rabbit anti-gB antibodies was able to neutralize the viral infection of HFF cells in a dose-dependent manner (50% to 70% inhibition) (Akula et al., 2001a). Other studies have shown that immunizing mice or rabbits with Newcastle disease virus-like particles (VLP)

expressing KSHV gpK8.1, gB and gH/gL, either alone or together, can elicit nAb responses against KSHV (Barasa et al., 2017; Mulama et al., 2019).

A number of studies have also demonstrated that anti- gH, gL, and gpK8.1 antibodies can neutralize KSHV infection without affecting the virus binding to its target cells (Naranatt et al., 2002; Wang et al., 2001; Wang et al., 2003). These observations suggest that these glycoproteins play essential roles in the entry process of the virus. Also, it is likely that the fusion machinery of KSHV comprises of gB, gH, gL and gpK8.1. Therefore, these KSHV envelope glycoproteins could potentially be the target(s) of KSHV-specific nAbs to prevent infection.

Given the critical roles of KSHV glycoproteins in viral entry, we hypothesized that KSHV glycoproteins play a pivotal role in the elicitation of KSHV-specific nAbs in infected individuals. To test this hypothesis, we expressed the KSHV glycoproteins in various expression systems and characterized the nAbs responses in infected individuals. We further studied the ability of various KSHV glycoproteins in the adsorption/removal of KSHV-specific nAbs from the plasma of KSHV infected individuals who have high nAbs titers, to identify the glycoproteins which are most commonly recognized. Our study will lead to the identification of the viral glycoprotein(s), which could serve as potential vaccine target(s) to induce a protective nAbs response.

Chapter 2

KSHV gB can be efficiently expressed in the adenoviral expression and can induce neutralizing antibodies in mice

Introduction

All herpesviruses initiate infection via binding to various receptors on the surface of target cells, which is primarily mediated by the viral glycoproteins embedded in the viral envelope (Subramanian et al., 2008). These glycoproteins play important roles in virus attachment to target cells, fusion of the viral envelope with either cytoplasmic or endosomal membranes, and virion morphogenesis and egress (Gao et al., 1996). Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), the most recently identified member of the herpesviridae, is the etiological agent of Kaposi's sarcoma (KS) and also two lymphoproliferative diseases, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Chang et al., 1994; Sousa-Squiavinato et al., 2015). Among all KSHV envelope glycoproteins, glycoprotein B (gB) is believed to be the essential protein for virus binding and entry into permissive cells, incorporated into the virion envelope.

KSHV gB initiates virus binding to host cells via its interactions with host cell surface heparin sulfate (HS), and entry receptors α3β1, αVβ3 and αVβ5 integrins (Akula et al., 2001a, 2002; Akula et al., 2001b; Veettil et al., 2008; Wang et al., 2003). Additionally, among all KSHV glycoproteins, only gB possesses the RGD (Arg-Gly-Asp) motif which is the minimal region of many extracellular matrix (ECM) proteins required for the

interactions with a subset of host cell surface integrins (Akula et al., 2002; Chakraborty et al., 2011; Garrigues et al., 2008; Kerur et al., 2010; Veettil et al., 2008). One study found that incubation of KSHV with polyclonal rabbit anti-gB antibodies was able to neutralize the viral infection of HFF cells in a dose-dependent manner (50% to 70% inhibition) (Akula et al., 2001a). Moreover, several studies have shown that mice or rabbits immunized with Newcastle disease virus-like particles (VLP) expressing gB can elicit nAbs responses against KSHV (Barasa et al., 2017; Mulama et al., 2019). Collectively, this suggests that gB plays a major role in KSHV infection of target cells and potentially is the primary target of KSHV-specific neutralizing antibodies (nAbs), present in the plasma of KSHV infected individuals.

In this study, we have focused on expressing KSHV gB and characterizing its role in elicitation of KSHV-specific nAbs. We hypothesized that KSHV envelope glycoprotein gB is the primary target of the KSHV-specific nAbs, and our immediate approach is to use a mammalian expression system that would provide us with adequate levels of gB for our immunization study. To test this hypothesis, we cloned the full length gB gene into a replication incompetent adenovirus type 5 (Ad5-gB) which has been shown to be a good delivery and expression system to obtain high expression levels of cloned genes. Next, we used the expression vector to inoculate mice to elicit antibodies against KSHV gB, and then tested the ability of these antibodies in neutralizing KSHV.

There are several advantages in using an adenovirus vector compared to other gene delivery systems. For example, unlike lentivirus, the recombinant adenovirus vector can avoid activation or inactivation of host genes by remaining epichromosomal. In addition,

generation of the adenovirus is relatively simple and would yield high virus titers; high expression levels of the target protein can also be achieved by increasing the multiplicity of infection (MOI) without significant negative effects on the host cells. Also, adenoviral vectors have advanced significantly over the years since the first attempts to use them in gene therapy, and are currently being tested clinically in several gene therapies, anticancer studies and as vaccine vector (Wold and Toth, 2013).

Results

Construction of the recombinant Ad-gB and its expression *in vitro*

The recombinant adenovirus encoding KSHV gB was generated by cloning the full length gB gene into a replication incompetent adenovirus type 5 vector. To facilitate the detection of gB, the sequence for the 3xFLAG epitope tag was fused to the carboxyl-terminus of the glycoprotein. The recombinant adenovirus was then evaluated by restriction enzyme, and rescued in 293 cells. Next, the expression of gB was assessed in Vero cells by infection with Ad-gB (Figure 1). At 72 to 120 hours post-infection, intense brown colored stained cells were demonstrated by immunohistochemical (IHC) staining using antibody against the 3xFLAG epitope tag, indicating high expression level of KSHV gB (Figure 2). Non-infected Vero cells served as a negative control and produced no evident chromagen deposition.

To further confirm gB expression in Vero cells, western blotting was performed on 120-hour Vero infected cells with Ad-gB against 3xFLAG epitope tag to determine the size of the gB expressed. In parallel Endo H treated lysate was also analyzed to confirm that the gB expressed is of the expected size of 95 kDa (Figure 3). With total cell lysate without Endo H treatment, a 140 kDa band was detected for gB which is expected to be larger than 95 kDa because it should be glycosylated. Upon Endo H treated, a 95 kDa band corresponding to the un-glycosylated gB in the treated lysate was detected, suggesting the correct size gB was made.

Examine the ability of Ad-gB to elicit KSHV nAbs *in vivo*

To evaluate the neutralizing ability of Ad-gB immunized plasma, 4 mice were inoculated with 10 x 10^{10} Ad-gB viral particles intramuscularly. Mice were boosted with 10 x 10^{10} Ad-gB viral particles on day 29 and bled on day 37. Plasma collected at Day 37 were used to conduct KSHV infection neutralization assays. Plasma from mice immunized with PBS served as the negative control and was used to normalize the percent neutralization. At 1:18 dilution of plasma a 45-65% neutralization of KSHV infection of 293T cells was observed among the plasma from four mice (Figure 4). This moderate neutralizing activity of the Ad-gB immunized plasma implicates that there are possibly other antigenic determinant(s) of KSHV-specific nAbs that needs to be identifies.

Material and methods

Cell cultures

Human embryonic kidney T (293T), HEK-293, Vero and BC-3 cells were obtained from ATCC (CRL-3217, CRL-1573, CCL-81and CRL-2277, respectively). HEK-293, HEK- 293T and Vero cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). BC-3 cells were maintained in RPMI with 20% FBS and 1% P/S. Vero.219 cells [a gift from Jeffrey Vieira (Vieira and O'Hearn, 2004)] stably expressing green fluorescent protein from the KSHV genome during latency were used to generate recombinant KSHV (rKSHV.219) and were maintained in DMEM with 10% FBS and 1% P/S and 6 µg/ml of puromycin. All cell cultures were maintained at 37°C in a 5% CO_2 incubator.

Construction of the recombinant Ad-gB

The recombinant adenovirus encoding KSHV gB was generated by cloning PCR amplified full length sequence (forward [5'-GCTCGGATCCATGACTCCCAGGTCTAGATTGG-3'] and reverse [5'-GCAGAATTCCTAACTCCCCCGTTTCCGGACTG-3']) of gB gene from BC-3 genomic DNA using high fidelity enzyme (Q5 HF DNA polymerase) into a shuttle with 3xFLAG epitope tag sequence fused to the carboxyl-terminus of the gB genome. Then, the insert was PCR amplified (forward [5'-AAATGGGCGGTAGGCGTGTAC-3'] and reverse [5'-TAGGAAAGGACAGTGGGAGTGGC-3']) and cloned into a replication incompetent adenovirus vector type 5. Next, the plasmid was transformed into competent Stellar cells and screened

by restriction enzyme digestion. The plasmid was then linearized with PacI and transfected into HEK-293 cells to generate the recombinant adenovirus. Finally, the Ad-gB virus was purified using cesium chloride (CsCl) density gradient ultra-centrifugation as previously described (Privatt et al., 2019).

Immunohistochemistry (IHC)

10^4 cells/chamber of Vero cells were seeded into a 4-chamber slide. At 24-hours post seeding, the cells were infected with either 500, 1000 or 2000 particles of Ad-gB. IHC was performed at 3,5 and 7 days post-infection. The slides were washed and fixed in 4% paraformaldehyde for 30 minutes at room temperature. The fixed slides were then incubated in 0.3% H_2O_2 methanol solution for 30 minutes at room temperature, followed by washes and antigen retrieved in sodium citrate solution for 15 minutes at 98°C. After cooling to room temperature, the slides were washed and blocked with 10% normal goat serum for 30 minutes at room temperature and incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) at 1:300 in blocking solution at 4°C overnight. The next day, the slides were washed and incubated with Dako EnVision+ System-HRP anti-mouse polymer for 30 minutes at room temperature and color was developed with DAB solution (Dako). Counterstaining of the nucleus was performed using hematoxylin. The final stained slides were then air-dried, rinsed twice in xylene solution for 5 minutes each, and then coverslip was added with Cytoseal 60 (Thermo Scientific). The slides were examined with a Nikon Eclipse 50i microscope under 20X magnification. Three times 3 fields of pictures (i.e., 9 pictures) were taken at 20X magnification and digitally stitched.

Immunoblot

10^6 cells/well of Vero cells were seeded into a 6-well plate. At 24-hours post-seeding, cells were infected with 1000 viral particles of Ad-gB. After 120 hours, the proteins were extracted from total cell lysate using RIPA lysis buffer in the presence of protease inhibitor. Endo H treatment was also performed on extracted proteins according to the manufacturer's protocol (New England BioLabs). The extracted protein was then measured by Pierce BCA protein assay (Thermo Scientific) and equal amounts of protein was loaded and resolved on a 10% SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane. After blocking with 5% skim milk in 1X PBS with 0.5% Tween 20 for 2 hours at room temperature, the membranes were incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) at 1:100 or mouse monoclonal anti-GAPDH (6C5) IgG (Santa Cruz Biotechnology) at 1:2000 as primary antibodies overnight at 4°C. Next day, after washing, the membranes were incubated with the secondary donkey anti-mouse 800CW or 680CW antibodies (Li-Cor Biosciences) at 1:10000 for 2 hours at room temperature, and then washed repeatedly. The membranes were then visualized with Odyssey infrared imager (Li-Cor Biosciences).

Mice immunization with Ad-gB

Four male CD46 transgenic mice with a C57BL/6 background were immunized with 10 x 10^{10} Ad-gB viral particles on days 0 and 29 intramuscularly. The mice were bled on day 37, and then the plasma were tested for neutralizing activity against KSHV.

Neutralization assay against KSHV

To perform the neutralization assay, the heat inactivated mice plasma (56°C for 1 hour) were incubated with recombinant KSHV expressing GFP protein (rKSHV.219) at 1:50 dilution at 37°C for 1 hour. HEK-293T cells in 96-well plates (2.5 X 10^4 cells/well) were infected with virus-plasma mixture, centrifuged (400g for 20 minutes at room temperature) and then incubated at 37°C in a 5% CO_2 incubator for 72 hours. Neutralization activity of sera was then analyzed by flow cytometry at 72 hours post-infection as followed:

$$\%\, neutralization = 100 - \left[\left(\frac{S}{C}\right) X\ 100\right]$$

S = % GFP positive cells in wells with Ad-gB immunized mice plasma.

C = % GFP positive cells in wells with PBS immunized mice plasma.

Discussion

KSHV virions incorporate eight glycoproteins into their envelope, of which glycoprotein B is believed to be the major protein for attachment and entry into targets cells due to its interactions with cell surface heparin sulfate molecules and entry via RGD dependent binding to the integrins. Studies have shown than interruption of gB interactions with target cell surface molecules would significantly inhibit the infection of target cells (Akula et al., 2001a). In this study, we attempted to characterize the KSHV nAbs responses against KSHV gB and to determine if gB is potentially the primary target of KSHV-specific nAbs.

We showed that infection of Vero cells with Ad-gB resulted in high expression levels of gB glycoprotein *in vitro.* Immunization of mice with Ad-gB induced humoral immune responses, resulting in moderate, about 45-65%, neutralizing activity at 1:18 dilution of plasma which was not as high as we expected considering the necessity of gB in virus binding and entry into target cells. However, it was still comparable with a previous study which showed that immunizing mice with Newcastle disease virus-like particles (VLP) expressing gB can elicit nAbs responses against KSHV (Barasa et al., 2017). This indicates that gB can induce KSHV nAbs in immunized animals, and is potentially one of the targets of the KSHV-specific nAbs. Additionally, because the nAbs responses against gB were lower than expected, we speculate that other KSHV envelope glycoproteins are necessary for inducing a stronger neutralizing response against KSHV. To identify all the targets of the KSHV-specific nAbs, there is a need to characterize the KSHV-specific nAbs responses in KSHV infected individuals against all the eight KSHV envelope glycoproteins.

Figure legends

Figure 1. Gel electrophoresis of the digested recombinant adenovirus.

Two bands corresponding to the insert and the empty viral vector at the predicted sizes are shown. The digestion reaction was done using SwaI enzyme which was previously introduced into both vector and the insert.

Figure 2. Detection of gB expression via immunohistochemistry (IHC).

Vero cells were infected with Ad-gB, using different amounts of virus input and analyzed at different time points after infection. IHC was carried out using monoclonal mouse anti-FLAG antibody at 1:300 dilution. Brown color stained cells were visualized indicating high expression level of gB protein.

Figure 3. Detection of gB expression via immunoblot.

Vero cells were infected with Ad-gB, and western blot was performed on RIPA lysed cells, with or without treatment with Endo H using anti-FLAG antibody at 1:100 dilution. The gB band was detected by the secondary donkey anti-mouse 800CW antibody via green color. GAPDH was also investigated using mouse monoclonal anti-GAPDH (6C5) IgG (Santa Cruz Biotechnology) at 1:2000 as primary antibody and the secondary donkey anti-mouse 680CW antibody via red color.

Figure 4. Neutralization assay of rKSHV.219 with mice plasma.

Neutralization of rKSHV.219 was carried out using three dilutions (1:9, 1:18 and 1:50) of heat inactivated plasma from four mice. Plasma from mice immunized with PBS served as the negative control and was used to normalize the percent neutralization.

Figure 1

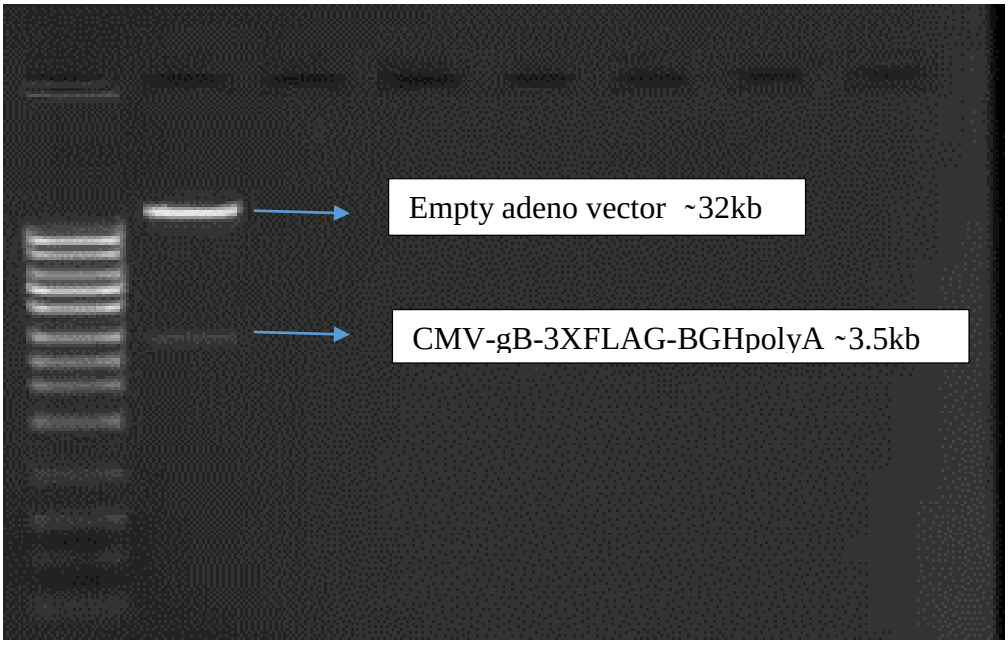


Figure 2

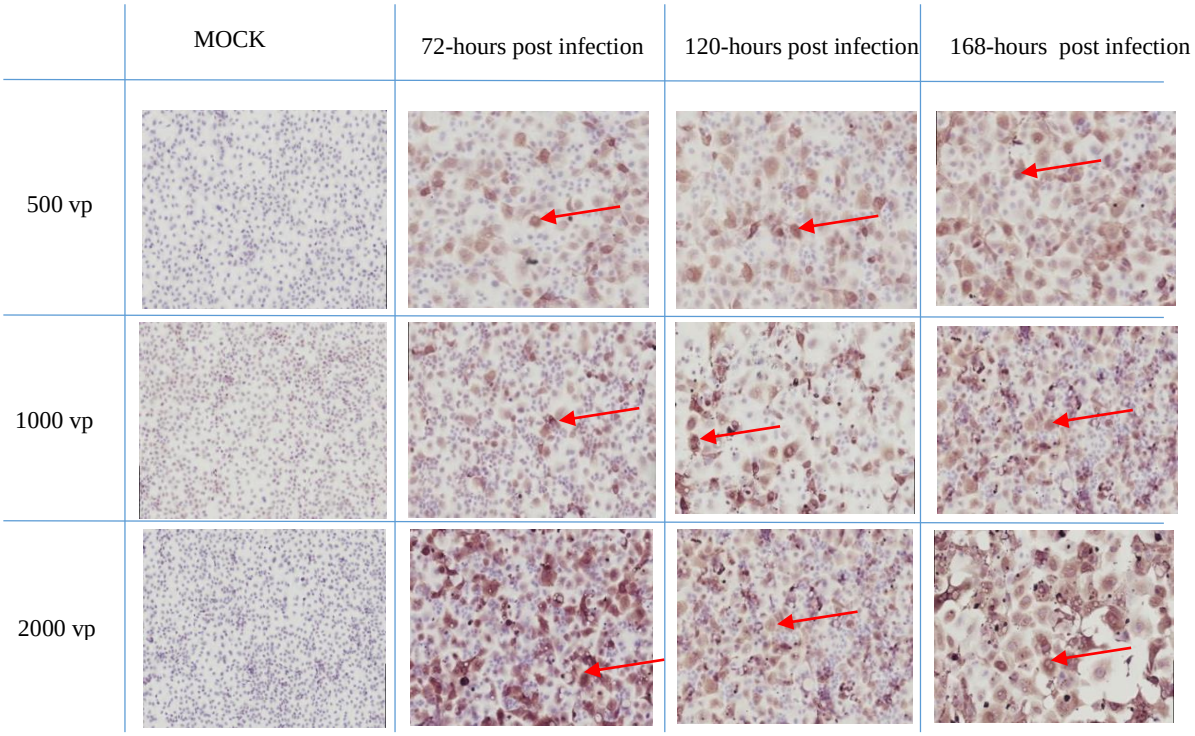

MOCK
72-hours post infection
120-hours post infection
168-hours post infection
500 vp
1000 vp
2000 vp

Figure 3

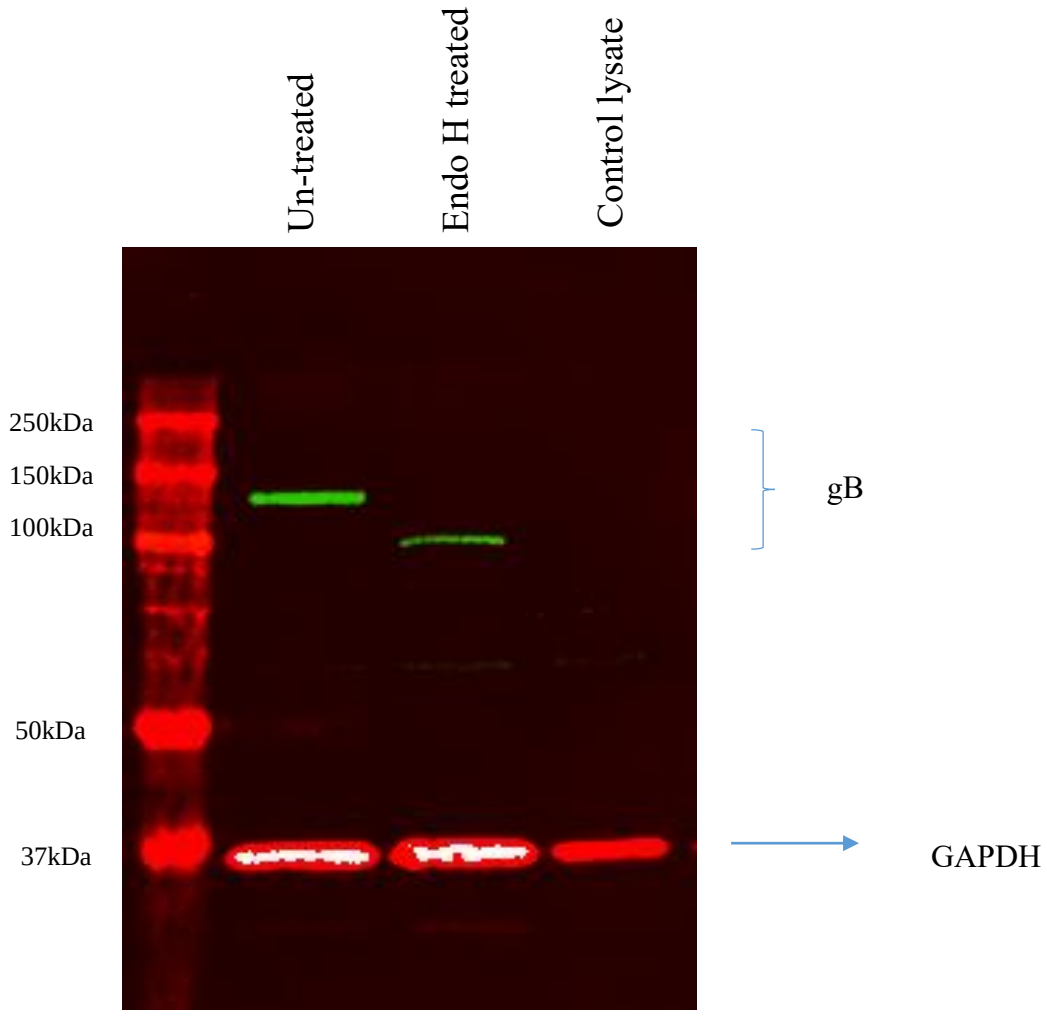
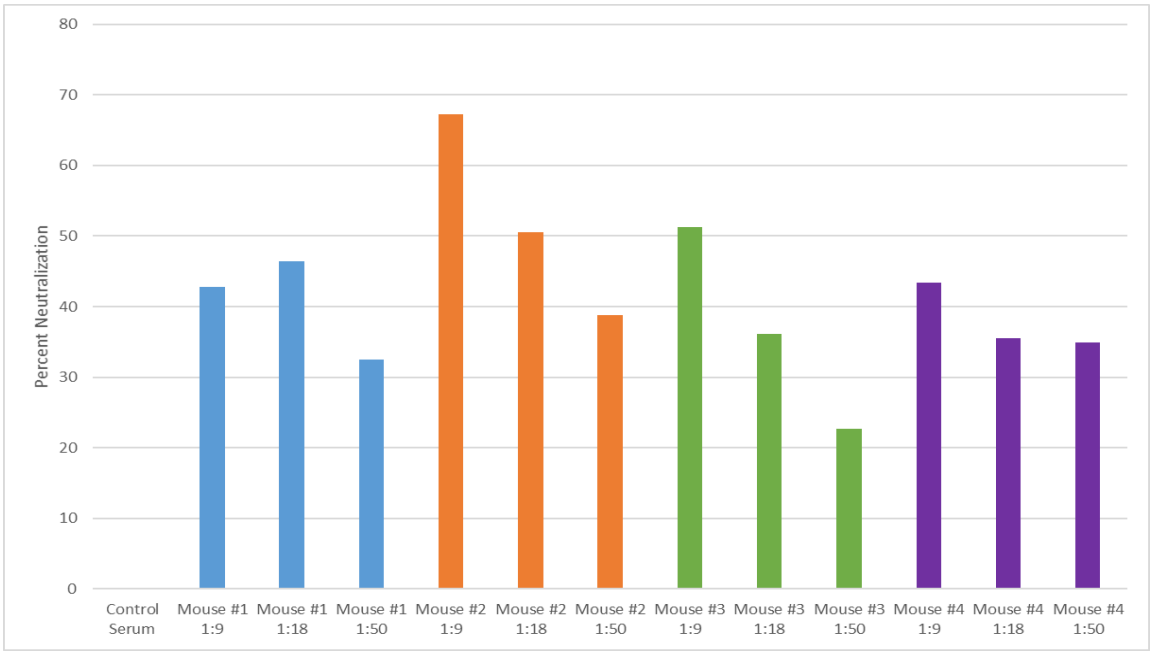

Un-treated
Endo H treated
Control lysate
250kDa
150kDa
100kDa
50kDa
37kDa
gB
GAPDH

Figure 4


80
70
60
50
40
30
20
10
0
Percent Neutralization
Control Serum
Mouse #1 1:9
Mouse #1 1:18
Mouse #1 1:50
Mouse #2 1:9
Mouse #2 1:18
Mouse #2 1:50
Mouse #3 1:9
Mouse #3 1:18
Mouse #3 1:50
Mouse #4 1:9
Mouse #4 1:18
Mouse #4 1:50

Chapter 3

Kaposi's Sarcoma-associated Herpesvirus (KSHV) gH/gL Complex is the Predominant Neutralizing Antigenic Determinant in KSHV infected individuals

Yasaman Mortazavi[1], Salum J. Lidenge[1,2,3], Tara Tran[1], John T. West[1], Charles Wood[1,*] and For Yue Tso[1,*]

[1]Nebraska Center for Virology and School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE, USA

[2]Ocean Road Cancer Institute, Dar es Salaam, Tanzania

[3]Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania

*Address correspondence to Charles Wood, cwood1@unl.edu; For Yue Tso, ftso2@unl.edu

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS), one of the most prevalent cancers of people living with HIV/AIDS in sub-Sahara Africa. The seroprevalence for KSHV is high in the region, and no prophylactic vaccine against the virus is available. In this study, we characterized the antigenic targets of KSHV-specific neutralizing antibodies (nAbs) in asymptomatic KSHV infected individuals and KS patients with high nAbs titers. We quantified the extent to which various KSHV envelope glycoproteins (gB, ORF28, ORF68, gH, gL, gM, gN and gpK8.1) adsorbed/removed KSHV-specific nAbs from the plasma of infected individuals. Our study revealed that plasma from a majority of KSHV neutralizers recognize multiple viral glycoproteins. Moreover, the breath of nAbs responses against these viral glycoproteins varies among endemic KS, epidemic KS and asymptomatic KSHV infected individuals. Importantly, among the KSHV glycoproteins, the gH/gL complex but neither gH or gL alone, showed the highest adsorption of KSHV-specific nAbs. This activity was detected in 80% of the KSHV infected individuals regardless of their KS status. The findings suggest that the gH/gL complex is the predominant antigenic determinant of KSHV-specific nAbs. Therefore, gH/gL is a potential target for development of KSHV prophylactic vaccines.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), is the causative agent of all forms of Kaposi sarcoma (KS) and two additional lymphoproliferative diseases, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Chang et al., 1994; Sousa-Squiavinato et al., 2015). The distribution of KSHV varies globally. In some areas such as sub-Saharan Africa (SSA), KSHV seroprevalence can be as high as 80%; whereas in the US and Europe the prevalence is 3-20% (Blumenthal et al., 2019; Torre et al., 2015). The prevalence of KS increases significantly with HIV-1 infection, making it one of the leading cancers among people living with HIV/AIDS in SSA (Brower, 2011; Mesri et al., 2010). Globally, it is estimated that nearly 44,000 new cases of KS emerge annually, with the highest incidence occurring in Africa, where KSHV is endemic (Bray et al., 2018). The two main clinical manifestations of KS are Endemic KS (EnKS) and Epidemic KS (EpKS). EnKS is found among HIV-1 negative individuals, whereas EpKS is associated with HIV-1 co-infection (Moore and Chang, 1995, 1998). Additionally, in specific ethnic groups of elderly men of Mediterranean or Eastern European descents there exists Classical KS (CKS), and Iatrogenic KS (IKS) is associated with drug-induced immune suppression (Dollard et al., 2005). The recurrent rate for KS is high even with chemo or radiotherapy (Krown, 2011). Therefore, the most cost-effective strategy to reduce KS incidence is to prevent KSHV infection through vaccination, and understanding the antigenic determinant of KSHV-specific nAbs will contribute to informative vaccine research for KSHV.

Although KSHV-specific nAbs have been reported in KS patients and KSHV-infected individuals, their role in KSHV pathogenesis is not known (Kumar et al., 2013; Wakeham et al., 2015). More importantly, it is unclear whether having KSHV-specific nAbs prior to exposure to KSHV could be beneficial in preventing its infection and, therefore could serve as an adaptive correlate of protection against KS development. Based on lessons learned from vaccination against other viruses, such as influenza, nAbs elicited through vaccination primarily target viral membrane surface proteins (Soema et al., 2015; van Els et al., 2014). It has also been shown that immunization with KSHV envelope glycoproteins gB, gpK8.1 and gH/gL can elicit nAbs responses in animal models (Barasa et al., 2017; Mulama et al., 2019). However, whether these glycoproteins are the targets for nAbs elicited through infection, as in KSHV infected individuals, and whether these glycoproteins can induce nAbs in immunized humans, remain to be determined.

Herpesviruses initiate infection via binding to various receptors on the surface of target cells. This process is primarily mediated by multiple viral glycoproteins embedded in the viral envelope (Subramanian et al., 2008). These glycoproteins play important roles in virus attachment to target cells, fusion of the viral envelope with either cytoplasmic or endosomal membranes, as well as the virion morphogenesis and egress (Gao et al., 1996). KSHV incorporates eight glycoproteins into its virion envelope: ORF8 (gB), ORF28, ORF68, ORF22 (gH), ORF47 (gL), ORF39 (gM), ORF53 (gN) and gpK8.1 (Zhu et al., 2005). Studies have shown that distinct envelope glycoproteins are involved in KSHV infection of different cell types with gpK8.1, gB, and gH/gL as the major players (Chakraborty et al., 2012; Chandran, 2010; Mohl et al., 2019). The current model for

KSHV infection of most cell types suggests that virion binding is mainly initiated via gB or gpK8.1 interactions with the host cell heparin sulfate (HS) or integrin proteins, which then trigger gH/gL engagement with the ephrin receptor tyrosine kinases leading to fusion (Akula et al., 2002; Birkmann et al., 2001; Garrigues et al., 2008; Kerur et al., 2010; Naranatt et al., 2002; Veettil et al., 2008; Wang et al., 2001). Given the critical roles of KSHV glycoproteins in viral entry, we hypothesized that KSHV glycoproteins could play a pivotal role in the elicitation of KSHV-specific nAbs in infected individuals. To test this hypothesis, we studied the ability of various KSHV glycoproteins in the adsorption/removal of KSHV-specific nAbs from the plasma of KSHV infected individuals who have high nAbs titers. Glycoprotein targeted by the nAbs could be identified by a reduction in the nAbs response after depletion with a specific glycoprotein.

In this unique approach, we have characterized the variable specificity of nAbs responses in KSHV infected individuals, demonstrating that multiple KSHV glycoproteins are the targets of KSHV-specific nAbs. There are no differences in their responses in individuals with either EnKS, EpKS or asymptomatic individuals. The results reveal that the highest nAbs depletion occurred in the presence of the gH/gL complex, and this activity was evident in the majority of KSHV infected individuals. Thus, suggesting that gH/gL complex is the most prominent target of KSHV-specific nAbs, and therefore the most plausible antigenic target for prophylactic vaccine development against KSHV.

Results

Study Cohort

The study cohort was comprised of 25 individuals from Zambia and Tanzania: 21 KS patients and 4 asymptomatic KSHV seropositive participants. Among the KS patients, there were 15 EpKS and 6 EnKS individuals, while the non-KS participants were consisted of one HIV-1^- and 3 HIV-1^+ individuals (Table 1). The age of the study subjects ranged from 24 to 68 years old, with a median of 27 years for EnKS, 41 years for EpKS and 39 years for non-KS participants. All the EnKS cases were male, while 73.3% of the EpKS cases were male, and all the non-KS cases were female (Table 1). The duration of symptomatic KS ranged from 2 months to 108 months, with a median of 12 months for EnKS and 24 months for EpKS. Majority of the EpKS patients were on anti-retroviral treatment and had HIV-1 plasma viral load below detection limits. The 1/ IC_{50} which indicates the dilution of plasma that accomplish 50% inhibition of KSHV infection was performed using neutralization assays to determine the KSHV-specific nAbs titers in the plasma. The KSHV sero-status and total anti-KSHV antibody titers for all participants were determined by immunofluorescence assay (IFA). Both the total anti-KSHV antibody and KSHV-specific nAbs titers were variable among KSHV infected individuals. For the available samples, the presence of KSHV virions in plasma was analyzed by PCR for the KSHV ORF26 gene. All participants in this cohort are high neutralizers as defined by our flow cytometry-based neutralization assay (supplementary figure 2).

KSHV envelope glycoprotein expression in 293T cells

Plasmids encoding KSHV envelope glycoproteins, gB, ORF28, ORF68, gH, gL, gM, gN and gpK8.1, were generated by cloning the full length sequence of each gene into the pcDNA3.1 mammalian expression vector (Figure 1A). To facilitate the detection of these glycoproteins, the sequence for the 3xFLAG epitope tag was fused to the carboxyl-terminus of each glycoprotein construct, the expression of which was evaluated after transfection of 293T cells. At 72-hours post-transfection, expression of all KSHV glycoproteins were detected in the whole cell lysates of their respective transfected cells using immunoblotting against the 3xFLAG epitope tag (Figure 1B). However, since the input plasmids transfected into the cells was based on total DNA amount, rather than equal copy number, there are some variation in expression among different glycoproteins.

Expression of KSHV glycoproteins in transfected 293T lysates does not necessarily demonstrate whether these proteins were on the cell surface in significant quantities. To verify their cell surface expression, the cellular membrane fraction was extracted from 293T cells at 72-hours post-transfection with commercially available membrane extraction reagents. The cell surface expression of the different KSHV glycoproteins was then confirmed by immunoblotting against the 3xFLAG epitope tag in the extracted plasma membrane fractions (Figure 1C). The purity of the extracted membrane fraction was validated by the significantly lower amount of GAPDH protein in comparison to the cytosolic fraction on immunoblot (Data not shown). For most of the KSHV glycoproteins, their corresponding bands, as reported by other groups, were detected by immunoblot in

both the total cell lysates and membrane fractions (Barasa et al., 2017; Chandran, 2010; Dollery et al., 2019; Zhu et al., 2005). However, the molecular weight of the gM protein in our study is higher than its theoretical size of 45 kDa. To determine the cause of this unexpectedly high molecular weight of gM protein, the gM expression plasmid was sequenced, but no mutation was found. Furthermore, post-translational glycosylation was also ruled out with Endo H treatment, where the molecular weight of gM remained unchanged after treatment (Data not shown). Further investigation on how post-translational modifications such as phosphorylation affected the size of gM protein will be warranted.

To further confirm the expression and transfection for each glycoprotein and to demonstrate that the majority of the transfected cells indeed express the glycoproteins, IHC staining against the 3xFLAG epitope tag was performed on the transfected cell cultures *in situ* at 72-hours post-transfection. Since previous studies suggested that gH and gL form a noncovalent complex that is necessary for efficient transport of gL to the cell surface, co-transfection of gH/gL was also investigated (Hahn et al., 2009). Likewise, due to the previous reports that gN is required for functional processing of gM and together they form heterodimers, co-transfection of gN/gM was included as well (Koyano et al., 2003). In agreement with the immunoblot results, the IHC demonstrated intense brown color stained cells, indicating high protein expression for all the KSHV glycoproteins in transfected cells (Figure 2A). Importantly, majority of the cells in the transfected culture expressed their respective KSHV glycoprotein, demonstrating high transfection efficiency of each KSHV

glycoprotein encoding plasmid. Non-transfected and empty-vector transfected 293T controls produced no evident chromagen deposition.

Additionally, to demonstrate KSHV glycoproteins are indeed expressed on the transfected cell surface and are recognized by KSHV antibodies present in KSHV infected plasma, IFA was performed on non-permeabilized 293T cells at 72-hours post-transfection using a pooled plasma from all the study participants (n = 25) as the primary detection antibody. The cell surface expression of all the KSHV glycoproteins were confirmed by the presence of green colored cells (Figure 2B). Among the glycoproteins, the signal for ORF28, ORF68, gL alone and gM alone were relatively weak, which could be due to low levels of antibodies against these proteins in the pooled plasma. Whereas, the relatively stronger green color signals in the gH/gL or gM/gN co-transfected cells, in comparison to their respective single plasmid transfected cells, indicated the interaction between gH/gL or gM/gN as reported by others which may explain why the complexes are better recognized by KSHV infected plasma (Hahn et al., 2009; Koyano et al., 2003). No signal was detected in the non-transfected and empty vector transfected 293T cells.

Antigenic determinant of KSHV-specific nAbs in the plasma of KSHV infected individuals

To determine which KSHV glycoprotein(s) are the target for KSHV-specific nAbs in the plasma of non-KS individuals and KS patients, 293T cells expressing various KSHV glycoproteins were incubated with the patient plasma (n = 25) to adsorb nAbs against specific glycoproteins. The residual neutralizing capability of the depleted plasma was then

quantified in a flow-cytometry based neutralization assay. If nAbs against a specific KSHV glycoprotein were prevalent in a plasma, depletion by the 293T cells expressing that particular glycoprotein would result in reduction in the ability of the depleted plasma to neutralize KSHV infection. These neutralization titers were then normalized with the percent neutralization of the identical plasma absorbed with empty vector transfected cells and reflected as a relative percent nAbs absorbed.

Among the 15 EpKS cases, 80% of the patients' plasma contain nAbs that can recognize 8 out of 10 variations of the KSHV glycoproteins examined (Figure 3A). Only patients 21001, 21052 and TIL020 have nAbs that recognize less than 50% of the KSHV glycoproteins. Despite this breadth of KSHV glycoprotein recognition, the magnitude of neutralization attributable to each glycoprotein varied widely among individuals. For instance, ORF28 recognized nAbs responses at higher levels in patient 21017 than patient 3122. Similar patterns of nAbs breadth and magnitude were detected in the 6 EnKS and 4 non-KS cases (Figure 3B and C, respectively). Interestingly, among the entire cohort, only the gH/gL complex was consistently recognized by the majority of patients nAbs at high levels. In comparison to other KSHV glycoproteins, the near 80% response to gH/gL was significantly higher than that against all other glycoproteins or complexes (p-values ranged $p < 0.001$ to $p < 0.0001$) (Figure 4). Additionally, there is no correlation between the gH/gL-specific nAbs with the total anti-KSHV and total KSHV-specific nAbs responses, despite 80% of our study participants had demonstrated high level of nAbs against the gH/gL complex (Supplementary Figure 3A and B). Further no correlation was found between the nAbs responses against gH/gL complex and age, sex or symptomatic KS duration (among KS cases) in the cohort (supplementary Fig. 3C to E).

Similar nAb responses towards KSHV glycoproteins regardless of KS status

Our lab has previously shown that the magnitude of total KSHV nAbs responses are indistinguishable between EpKS and EnKS, but are significantly higher in KS patients than non-KS individuals (Lidenge et al., 2019). To determine if these observations extend to the KSHV glycoprotein-specific nAbs responses, the relative percent nAbs depleted by each KSHV glycoprotein was compared between the EpKS, EnKS and non-KS groups. Unlike total KSHV nAbs responses, the KSHV glycoprotein-specific nAbs responses were not statistically different between KS and non-KS groups (Figure 5). Thus, although the development of KS increased the magnitude of total nAbs in symptomatic patients, it had no detectable effect on the breadth and glycoprotein specificity in that increased response. Additionally, co-infection with HIV-1 seems to have no significant effect on the nAbs responses.

Discussion

Neutralizing antibodies are a powerful defense against many viral infections in animals and humans (Iwasaki, 2016). The nAbs against infections caused by viruses, such as influenza and flavivirus, have been shown to prevent viral infection, thus it suggests that a similar approach may also be feasible in preventing KSHV infection (Han and Marasco, 2011; Sui et al., 2009; Whitehead et al., 2007; Wrammert et al., 2008). Although, KSHV-specific nAbs did not prevent the development of KS, whether the presence of these nAbs prior to exposure to KSHV would prevent infection is still unclear (Kumar et al., 2013).

Since KSHV envelope glycoproteins are incorporated onto the viral surface and are critical for viral entry into permissive cells, they are the candidate antigens for eliciting nAbs through vaccination. In recent years, several studies have shown that immunizing mice or rabbits with Newcastle disease virus-like particles (VLP) expressing KSHV gpK8.1, gB and gH/gL, either alone or together, can elicit nAbs responses against KSHV (Barasa et al., 2017; Mulama et al., 2019). However, whether these glycoprotein-targeted responses are consistent with those in KSHV infected individuals with strong neutralizing responses have not been investigated. Addressing this question could provide insights for the design of prophylactic vaccines against KSHV.

Our study is the first to define the specificities of nAbs responses, within plasma of KSHV infected individuals, against KSHV envelope glycoproteins. In addition, ours is the first study to investigate whether differentials in magnitude or breadth of nAbs specificities exist between EnKS, EpKS and non-KS individuals. To ensure that our results were not

affected by the different KSHV glycoproteins expression, equal copy number of each glycoprotein encoding plasmid were transfected into equal number of cells, which was then used for the adsorption of nAbs from sample plasma with optimized number of cells and temperature (Supplementary figure 1B and 1C). A good example to support our assessment that the protein expression did not affect the adsorption result, will be gpK8.1, which was highly expressed in transfected cells based on the immunoblot, IHC and IFA data, but only small amount of nAbs were adsorbed by it. Using our optimized assays, we found that the neutralizing component of plasma in a majority of the study participants targeted multiple KSHV envelope glycoproteins. Although these glycoproteins can elicit nAbs in infected individuals, the magnitude of the neutralization response varies widely among individuals with some glycoproteins eliciting much stronger response in one individual than another. This strongly indicates that nAbs responses elicited by any KSHV envelope glycoprotein are highly individualized.

Among all the KSHV glycoproteins, only the gH/gL complex elicited nAbs responses that are consistent and comparable among most individuals. 80% of study participants showed high levels of nAbs against the gH/gL complex. Given this consistently strong nAbs reaction against gH/gL from different individuals, we speculate that gH and gL may undergo conformational changes when complexed, which then expose yet-to-be determined epitopes that are highly immunogenic, resulting in its high recognition by over 80% of the infected individuals. Interestingly, several individuals such as patients 21062 and TIL002, had nAbs that recognized both gH/gL and gM/gN, while other individuals also have similar nAbs among different glycoproteins. One possible explanation for this

observation could be that some nAbs are targeting the carbohydrate moieties, which might be common among different KSHV envelope glycoproteins. For example, studies have shown that KSHV gB is associated with high levels of mannose which could be targeted by KSHV-specific nAbs, but only in some individuals (Baghian et al., 2000). Another possibility is the presence of nAbs that recognize certain yet-to-be determined conformational epitopes that may only be exposed when the KSHV glycoproteins interact with one another. This is feasible if these glycoproteins are in close proximities with one another on the virion surface. Unfortunately, there is very little information on the KSHV glycoprotein homo- or hetero-oligomeric structures and certainly none for those on the KSHV virion, therefore, more studies will be needed to confirm our observation.

Although our previous finding indicated that the total KSHV nAbs responses are similar in EnKS and EpKS, and that these responses are higher compared to non-KS individuals, we found no evidence for such differences in terms of nAbs specificities against different KSHV glycoproteins (Lidenge et al., 2019). Thus, it would appear that the diversity of KSHV glycoproteins-specific nAbs responses is independent of KS disease progression, whereas the magnitude of the total KSHV nAbs response increases with disease progression, and both are independent of HIV-1 co-infection. We also did not find any correlation between KSHV glycoprotein-specific nAbs responses and the presence of KSHV virions in the plasma at the time of sampling. This may indicate that there is a tissue reservoir that is providing the necessary antigens to elicit humoral responses or these are anamnestic responses from previous episodes of higher plasma viral load.

In summary, our data suggest that the KSHV glycoproteins gH/gL complex is the most prominent target for elicitation of nAbs in KSHV infected individuals regardless of KS status. Thus, the KSHV glycoproteins gH/gL complex should be included as a potential antigen in future KSHV prophylactic vaccine design.

Materials and methods

Ethics statement

Written informed consent was obtained from all study participants from Tanzania and Zambia for sample collection and testing. The study was conducted in accordance with the Declaration of Helsinki and approved by the review boards of Tanzania National Institute for Medical Research, Ocean Road Cancer Institute, University of Zambia Biomedical Research Ethics Committee and the University of Nebraska-Lincoln (Tanzania CRITIC: IRB Number: 20141014709FB, and Zambia ZAMDAPP: IRB Number: 20170817442FB).

Cell cultures

Human embryonic kidney T (293T) and BC-3 cells were obtained from ATCC (CRL-3217 and CRL-2277, respectively). 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). BC-3 cells were maintained in RPMI with 20% FBS and 1% P/S. Vero.219 cells [a gift from Jeffrey Vieira (Vieira and O'Hearn, 2004)] stably expressing green fluorescent protein from the KSHV genome during latency were used to generate recombinant KSHV (rKSHV.219) and were maintained in DMEM with 10% FBS and 1% P/S and 6 µg/ml of puromycin. All cell cultures were maintained at 37°C in a 5% CO_2 incubator.

KSHV serological assays

The KSHV sero-status and total anti-KSHV antibody titers were determined by immunofluorescence assay (IFA) on activated BC-3 cells as previously described (Minhas et al., 2008). To determine the total KSHV-specific nAbs titer, neutralization assays were carried out in triplicate as previously described (Kumar et al., 2013). Briefly, heat-inactivated plasma (56°C for 1 hour) from study participant was incubated with rKSHV.219 virus at 1:50 dilution at 37°C for 1 hour. 293T cells seeded in 96-well plates (2.5 X 10^4 cells/well) were infected with the virus-plasma mixture, centrifuged (400 X g for 20 minutes) and then incubated for 72-hours at 37°C. Neutralization activity of the plasma, relative to a KSHV negative plasma, was then quantified by flow cytometry at 72-hours post-infection as followed:

$$\%\ neutralization = 100 - \left[\left(\frac{S}{C}\right) X\ 100\right]$$

S = % GFP positive cells in wells with KSHV positive plasma.

C = % GFP positive cells in wells with KSHV negative plasma control.

Plasma samples that were positive for KSHV nAbs at the 1:50 dilution were further titrated by 2-fold dilutions from 1:50 to 1:3200 to define the IC_{50} (50% inhibitory concentration).

HIV-1 serology and plasma viral load quantification by real-time PCR

The HIV-1 diagnosis was made according to Alere Determine HIV-1/2 Ag/Ab Combo test in Zambia and Tanzania HIV Rapid Test Algorithm. The HIV-1 serology results were

verified using HIV-1–2.0 First Response kit (Premier Medical Corporation Ltd). To quantify HIV-1 plasma viral load, the viral RNA was extracted from plasma following the QIAamp viral RNA extraction protocol (Qiagen) and measured using the RNA Ultra-Sense One-Step quantitative real-time PCR (qPCR) system (Applied Biosystems) as previously published (Tso et al., 2018).

KSHV virion in plasma

Plasma of study participants (400 µl) were centrifuged at 8000 X *g* at room temperature for 10 minutes to remove residual cells. Then 15 µl of DNase-I (Qiagen) was added to each sample and incubated for 2-hours at room temperature to digest cell-free genomic DNA. The samples were then incubated at 65°C for 20 minutes to inactivate DNase-I, and viral DNA was extracted by the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen). The presence of KSHV virion in plasma was then determined by nested-PCR of the extracted viral DNA using primers for the open reading frame 26 (ORF26) amplicon (forward [5'-AGCCGAAAGATTCCACCAT-3'] and reverse [5'-TCCGTGTTGTCTACGTCCAG-3'] in the first round, and forward [5'-CGAATCCAACGGATTTGACCTC-3'] and reverse [5'-CCCATAAATGACACATTGGTGGTA-3'] in the second round reaction) under conditions previously described (Lidenge et al., 2019). Contamination of the extracted viral DNA by cell-free genomic DNA was ruled out through a negative PCR result for the human β-actin gene.

KSHV envelope glycoprotein constructs

To construct the KSHV gB, gpK8.1, gH/gL, gM, gN, ORF28 and ORF68 3XFLAG tagged plasmids, the full length sequence of individual glycoproteins was PCR-amplified from BC-3 genomic DNA and cloned into the pcDNA3.1 mammalian expression vector (Invitrogen) with a 3xFLAG fused to the carboxyl-terminus of each glycoprotein. The resulted plasmids were confirmed with restriction enzyme digestions and sequencing.

Immunoblot

1 x 10^6/well of 293T cells were seeded into 6-well plates. At 24-hours post-seeding, 2 µg of each KSHV glycoprotein expressing plasmids were transfected into the 293T cells using FuGENE 6 transfection reagent (Promega). After 72-hours, the cellular membrane fraction of the transfected cells was extracted by lysing the cells, in the presence of proteinase inhibitor cocktail (Thermo Scientific), with Mem-PER eukaryotic membrane protein extraction reagent according to the manufacturer's protocol (Thermo Scientific). The total cell lysis of the transfected cells was obtained by the lysing the cells in RIPA lysis buffer with proteinase inhibitor cocktail (Thermo Scientific). The extracted protein was then measured by Pierce BCA protein assay (Thermo Scientific) and equal amount of protein for each glycoprotein was loaded and resolved in a 4-15% gradient SDS-PAGE (Bio-Rad). The proteins were then transferred onto a nitrocellulose membrane and blocked with 5% skim milk in 1X PBS with 0.5% Tween 20 for 2-hours at room temperature. The membranes were then incubated with either mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) at 1:100 or mouse monoclonal anti-GAPDH (6C5) IgG (Santa Cruz Biotechnology) at 1:2000 overnight at 4°C. Next day, after washing, the membranes were

incubated with donkey anti-mouse 800CW or 680CW antibodies (Li-Cor Biosciences) at 1:10000 for 2-hours at room temperature, and then washed repeatedly. The membranes were then visualized with Odyssey infrared imager (Li-Cor Biosciences).

Immunohistochemistry (IHC)

1x 10^4/chamber of 293T cells were seeded into 4-well chamber slides. At 24-hours post-seeding, equal copy number of KSHV glycoprotein expressing plasmids were transfected into 293T cells using FuGENE 6 transfection reagent (Promega). After 72-hours, the slides were washed and fixed in 4% paraformaldehyde for 30 min at room temperature. The fixed slides were then incubated in 0.3% H_2O_2 methanol solution for 30 minutes at room temperature, followed by washes and antigen retrieved in sodium citrate solution for 15 minutes at 98°C. After cooling to room temperature, the slides were washed and blocked with 10% normal goat serum for 30 minutes at room temperature and incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma-Aldrich) at 1:300 in blocking solution at 4°C overnight. The next day, the slides were washed and incubated with Dako EnVision+ System-HRP anti-mouse polymer for 30 minutes at room temperature and color was developed with DAB solution (Dako). Counterstaining of the nucleus was performed using hematoxylin. The final stained slides were then air-dried, rinsed twice in xylene solution for 5 minutes each and coverslip was added with Cytoseal 60 (Thermo Scientific). The slides were examined with a Nikon Eclipse 50i microscope under 20X magnification. Three times 3 fields of pictures (i.e., 9 pictures) were taken at 20X magnification and digitally stitched using NIS-Elements imaging software (Nikon).

Immunofluorescence assay for protein expression

5 x 10^4/chamber of 293T cells were seeded into 8-well chamber slides. At 24-hours post-seeding, equal copy numbers of KSHV glycoprotein expressing plasmids were transfected into 293T cells using FuGENE 6 transfection reagent (Promega). After 72-hours the slides were washed and fixed in 4% paraformaldehyde for 30 minutes at room temperature. The fixed slides were incubated with a heat inactivated (56°C for 1 hour) pooled plasma from all the study subjects at 1:40 at 4°C overnight. Next day, the slides were washed and incubated with mouse monoclonal anti-human antibody (ATCC, CRL-1786) at 1:4 at 37°C for 1 hour. Then the slides were washed and incubated with CY2 AffiniPure donkey anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch) at 1:200 at 37°C for 1 hour. Next, the slides were washed and stained with 0.001% Evans blue for 5 minutes at room temperature. After staining, the slides were washed and coverslip with mounting media. The slides were examined with a Nikon Eclipse 50i microscope under 20X magnification.

rKSHV.219 Production

A total of 18 x 10^6 Vero.219 cells per flask were seeded into T-225 flasks. At 24-hours post-seeding, the culture medium was removed and 3300 viral particles of adenovirus type 5 encoding KSHV RTA (Ad5-RTA) (gift from Dr. Jean Gustin) were incubated with the cells in the presence of 5M valproic acid without (w/o) puromycin. Next day, the cells were washed and fresh medium w/o valproic acid and puromycin was added. After 48-hour the supernatant was collected and was cleared by a 10 minutes spin at 2000 rpm. Then, the supernatant was collected and concentrated through a 20% sucrose cushion by 2-hours spin

at 28000 rpm. The virus pellet was re-suspended in medium and tittered on 293T using flow cytometry for measuring GFP positive cells to determine the amounts of virus needed to achieve 40-50% GFP positive cells in the neutralization assays (supplementary figure 1A).

KSHV glycoproteins transfected 293T cells absorption of nAbs from plasma

Thirteen µl of the heat inactivated plasma (56°C for 1 hour) was incubated with 1 x 10^6 293T cells (transfected with equal copy number of KSHV envelope glycoprotein plasmids either individually or in combination) in 578 µl volumes of medium (DMEM containing 10% FBS, 1% P/S and 1% protease inhibitor cocktail) at 4°C overnight. The number of cells and temperature used for absorption were optimized based on the gH/gL complex (Supplementary figures 1B and C). After incubation with the KSHV glycoproteins expressing 293T cells, the plasma-cell mixture was spun down at 6000 X g for 5 minutes to remove the cell pellets, and the supernatant was collected. Then neutralization assays were performed in triplicate, the supernatant was incubated with 59 µl of rKSHV.219 at 37°C for 1 hour. 293T cells in 96-well plates (2.5 x 10^4 cells/well) were then infected with virus-plasma mixture, centrifuged (400 X g for 20 minutes at room temperature) and incubated for 72-hours. Neutralization was quantified by flow cytometry at 72-hours post-infection. The percentage of neutralization was defined as followed:

$$\%\, neutralization = 100 - \left[\left(\frac{S}{C}\right) X\ 100\right]$$

S = % GFP positive cells in wells with KSHV glycoproteins absorbed sample plasma.

C = % GFP positive cells in wells with pooled plasma from HIV-1 and KSHV double-negative donors (n = 10).

The percentage of nAbs absorption was defined as followed:

$$Relative\ \%\ nAbs\ adsorption = \left[\frac{\mathrm{B} - \mathrm{N}}{\mathrm{B}}\right] X\ 100$$

N = % neutralization of KSHV glycoproteins absorbed sample plasma.

B = % neutralization of sample plasma absorbed with empty vector transfected 293T cells.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software Inc). The comparison of nAbs responses to gH/gL complex with other KSHV glycoproteins among all the participants was performed using the Mann Whitney test. The comparison of nAbs responses to each KSHV glycoprotein in the EnKS, EpKS and non-KS groups was performed using the Mann Whitney test. Correlation between the extent of nAbs absorbed by gH/gL and total anti-KSHV and nAbs responses was performed using the nonparametric Spearman correlation analysis.

Acknowledgments

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Figure Legends

Figure 1. KSHV envelope glycoprotein constructs and their expression in 293T cells by immunoblot. (A) Schematic representation of pcDNA3.1 mammalian expression vector encoding KSHV glycoproteins fused with 3xFLAG at the C-terminus of each glycoprotein (not drawn to scale). (B) KSHV glycoproteins expression was demonstrated in the whole cell lyses of transfected 293T cells by immunoblot against the 3xFLAG tag. (C) KSHV glycoproteins expression on the cell surface was demonstrated in the extracted membrane fractions by immunoblot against the 3xFLAG tag.

Figure 2. KSHV envelope glycoproteins expression in 293T cells by IHC and IFA. KSHV glycoproteins expression was confirmed in 293T cells at 72-hours post-transfection by IHC against the 3xFLAG tag. (B) The cell surface expression of KSHV glycoproteins was confirmed by IFA on non-permeabilized 293T cells at 72-hours post-transfection with pooled plasma from all study participants. Pictures were taken at 20X magnification.

Figure 3. Antigenic determinant of KSHV-specific nAbs in the plasma of KSHV infected individuals. Heat inactivated plasma of the study participants were absorbed with KSHV glycoproteins expressing 293T cells and neutralization assays were carried out as described in material and methods. The relative percent of nAbs absorbed was shown for (A) EpKS cases, (B) EnKS cases and (C) Non-KS (asymptomatic KSHV infected) cases.

Figure 4. Comparison of nAbs responses among various KSHV envelope glycoproteins. The gH/gL complex-specific nAbs response was compared with other KSHV glycoproteins among all the participants using the Mann Whitney test (*** $p<0.001$, **** $p<0.0001$).

Figure 5. Comparison of nAbs responses to each KSHV glycoprotein in the EnKS, EpKS and non-KS groups. The nAbs responses against each KSHV glycoproteins was compared between the EnKS, EpKS and non-KS individuals using the Mann Whitney test.

Supplementary Figure 1. Titration of rKSHV.219, optimization conditions for adsorption of plasma with KHSV glycoprotein expressing 293T cells. (A) rKSHV.219 titration in 293T cells. 293T cells were seeded at a density of 2.5 x 10^6 in triplicates into a 96-well plate. The cells were then infected with 10, 20, 30, 40, 60 or 80 µl of concentrated virus in a total volume of 200 µl for 72 hours at 37°C. Infected cells (GFP+ cells) were quantified using FACS by acquiring a total of 10,000 events. (B) Optimization of binding temperature during absorption of nAbs with gH/gL complex expressing 293T cells. (C) Optimization of the number of gH/gL complex expressing 293T cells during absorption with nAbs.

Supplementary Figure 2. Total KSHV-specific nAbs of the study participants. Neutralization assays were conducted by incubating rKSHV.219 with plasma at 1:50 dilutions. The level of neutralization was determined after 72 hours using flow cytometry to quantify the number of GFP+ cells acquiring a total of 10000 events.

Supplementary Figure 3. Comparison of the magnitude of nAbs responses to KSHV gH/gL complex with; total anti-KSHV antibodies and with total KSHV-specific nAbs responses, age, sex and symptomatic KS duration. The correlation between the extent of nAbs absorbed by gH/gL and (A) total anti-KSHV and (B) nAbs responses, (C) age, (D)

sex and (E) symptomatic KS duration was performed using nonparametric Spearman correlation analysis (ns $P>0.05$).

Figures

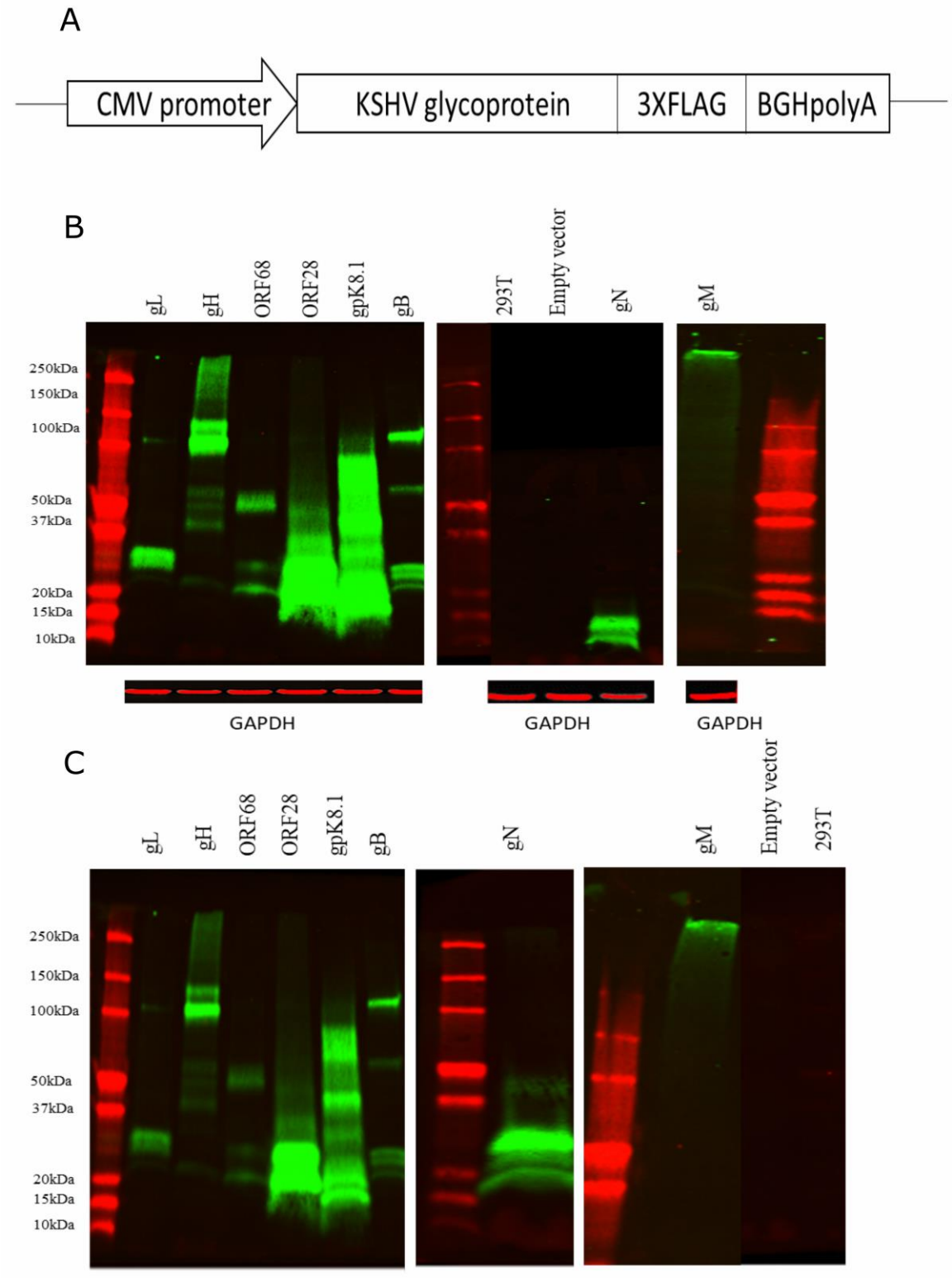
Table 1. Characteristics of the study cohort

	Patient ID	Sex	Age	KS months	HIV-1 months	HIV-1 plasma viral load [copies/ml]	ARV months	1/ IC_{50}	IFA	Plasma ORF26 PCR
Endemic KS (KSHV+,HIV-1-)	3096	M	27	3	NA	NA	NA	395.1	20480	-
	3111	M	24	7	NA	NA	NA	438.9	5120	-
	3135	M	25	12	NA	NA	NA	1672.4	5120	-
	3137	M	51	NI	NA	NA	NA	292.2	5120	-
	3139	M	27	14	NA	NA	NA	281.8	40960	+
	21182	M	57	108	NA	NA	NA	115.7	10240	NI
Epidemic KS (KSHV+,HIV-1+)	3060	F	44	8	12	BDL	9	1259.4	40960	+
	3080	F	21	3	5	BDL	1	199.9	1280	-
	3122	M	34	3	24	BDL	24	2779.9	40960	+
	21001	F	36	18	168	BDL	10	605.3	2560	+
	21009	M	36	60	120	BDL	9	232.2	1280	-
	21017	M	38	24	36	BDL	36	845.7	1280	+
	21021	M	41	60	NI	1.16E+05	NI	1606.7	1280	+
	21028	M	30	24	24	BDL	24	1167.5	5120	-
	21052	F	37	84	NI	BDL	48	1971.7	2560	-
	21053	M	44	9	5	BDL	4	890.4	2560	-
	21062	M	68	24	NI	BDL	NI	715.8	5120	+
	TIL002	M	68	3	48	2201	36	285.2	5120	NI
	TIL007	M	46	72	72	33	72	407.1	5120	NI
	TIL018	M	55	48	120	BDL	120	1600.5	5120	NI
	TIL020	M	51	2	7	BDL	7	454.4	10240	NI
Non-KS (KSHV+, HIV-1-)	3067	F	60	NA	NA	NA	NA	224.6	5120	NI
Non-KS (KSHV+, HIV-1+)	21598	F	45	NA	36	NI	NI	197.1	2560	NI
	594	F	33	NA	18	NI	12	175.4	2560	NI
	649	F	25	NA	6	NI	4	126.1	640	NI

'+' denotes KSHV virions present in plasma, and '-' denotes KSHV virions not present in plasma.

Abbreviations: KSHV, Kaposi;s sarcoma-associated herpesvirus; KS, Kaposi sarcoma; HIV-1, human immunodeficiency virus type 1; ARV, Antiretrovirals; IFA, Immunofluorescence assay; NA, not applicable; NI, no information; BDL, below detection limit.
IFA numbers represent total anti-KSHV antibody titer (reciprocal endpoint plasma dilution); 1/ IC_{50} numbers represent the dilution of plasma that has a 50% neutralizing activity.

Figure 1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gH
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ORF28
gpK8.1
gB
293T
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gN
gM
250kDa
150kDa
100kDa
50kDa
37kDa
20kDa
15kDa
10kDa
GAPDH
GAPDH
GAPDH
C
gL
gH
ORF68
ORF28
gpK8.1
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gM
Empty vector
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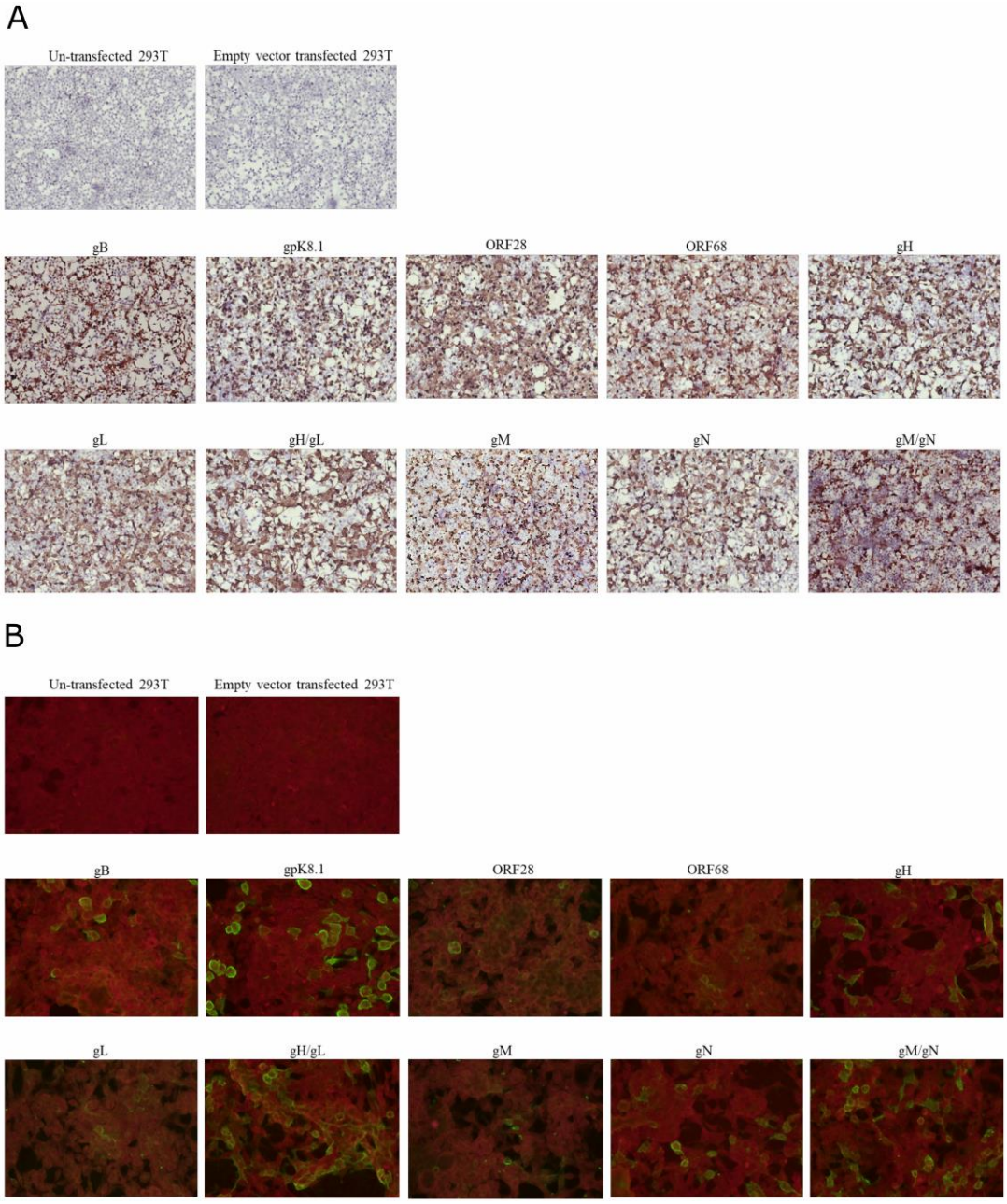
150kDa
100kDa
50kDa
37kDa
20kDa
15kDa
10kDa

Figure 2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Un-transfected 293T
Empty vector transfected 293T
gB
gpK8.1
ORF28
ORF68
gH
gL
gH/gL
gM
gN
gM/gN
B
Un-transfected 293T
Empty vector transfected 293T
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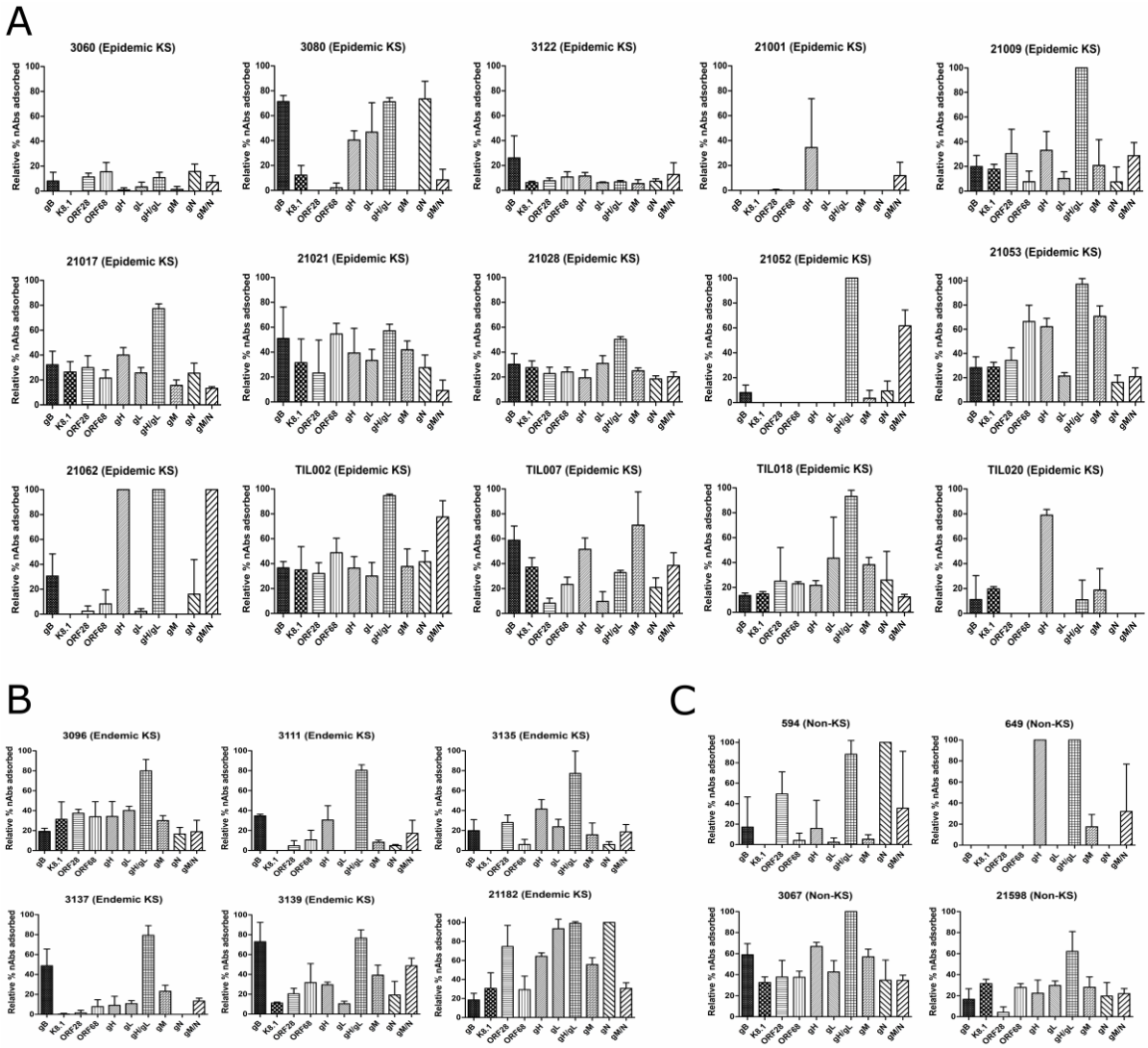
ORF28
ORF68
gH
gL
gH/gL
gM
gN
gM/gN

Figure 3


A
3060 (Epidemic KS)
3080 (Epidemic KS)
3122 (Epidemic KS)
21001 (Epidemic KS)
21009 (Epidemic KS)
21017 (Epidemic KS)
21021 (Epidemic KS)
21028 (Epidemic KS)
21052 (Epidemic KS)
21053 (Epidemic KS)
21062 (Epidemic KS)
TIL002 (Epidemic KS)
TIL007 (Epidemic KS)
TIL018 (Epidemic KS)
TIL020 (Epidemic KS)
Relative % nAbs adsorbed
gB
K8.1
ORF28
ORF68
gH
gL
gH/gL
gM
gN
gM/N
B
3096 (Endemic KS)
3111 (Endemic KS)
3135 (Endemic KS)
3137 (Endemic KS)
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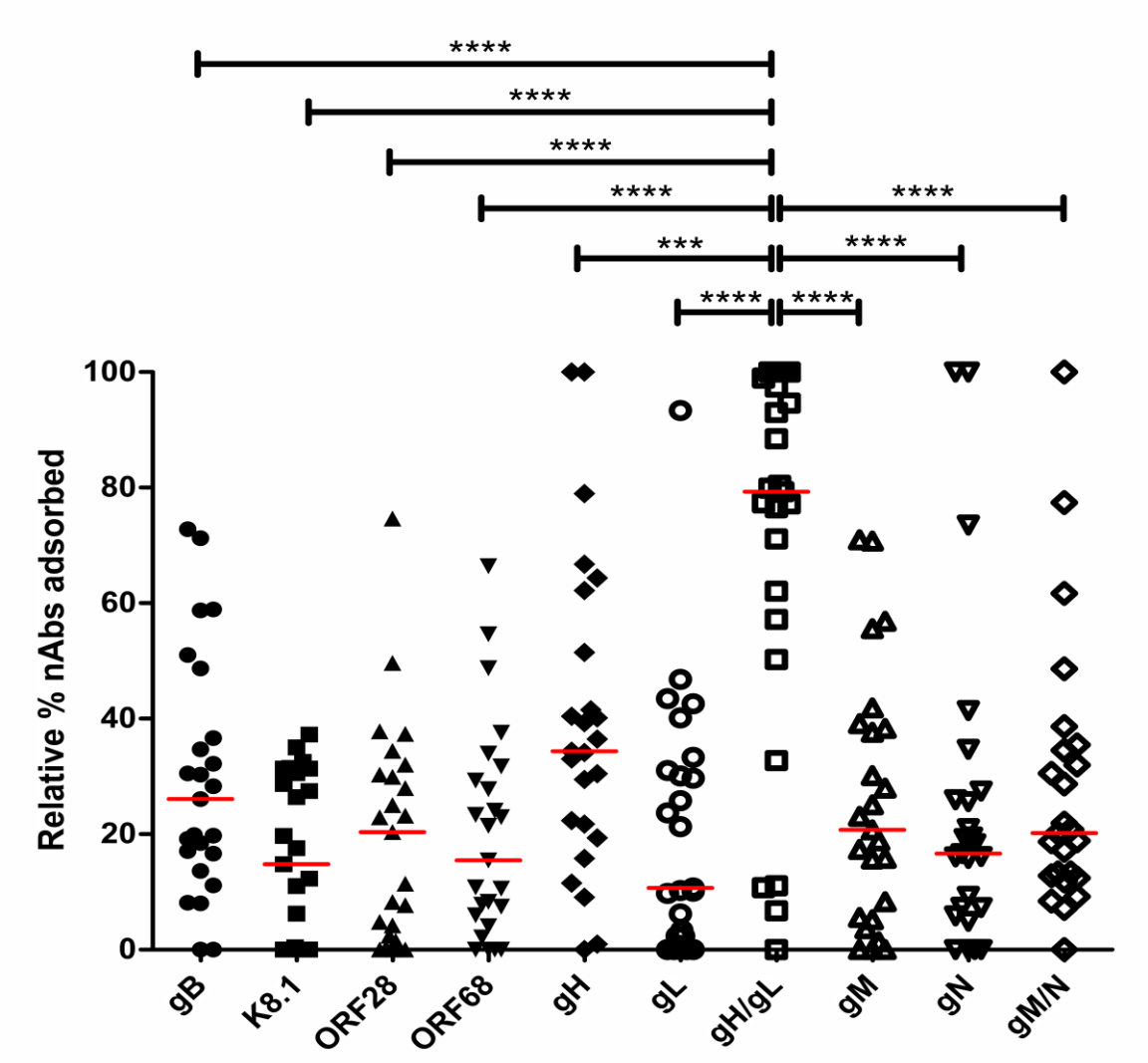
C
594 (Non-KS)
649 (Non-KS)
3067 (Non-KS)
21598 (Non-KS)

Figure 4


****
****
****
****
****
***
****
****
****
Relative % nAbs adsorbed
100
80
60
40
20
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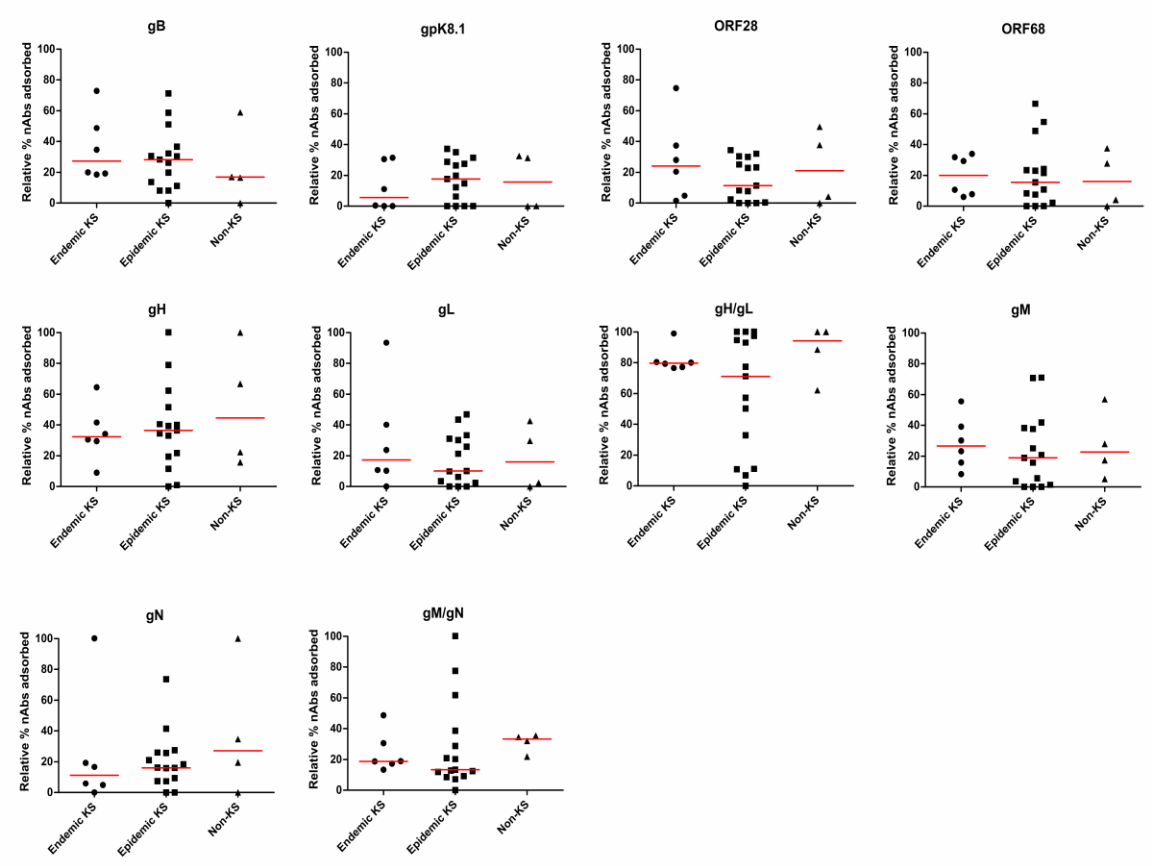
K8.1
ORF28
ORF68
gH
gL
gH/gL
gM
gN
gM/N

Figure 5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Relative % nAbs adsorbed
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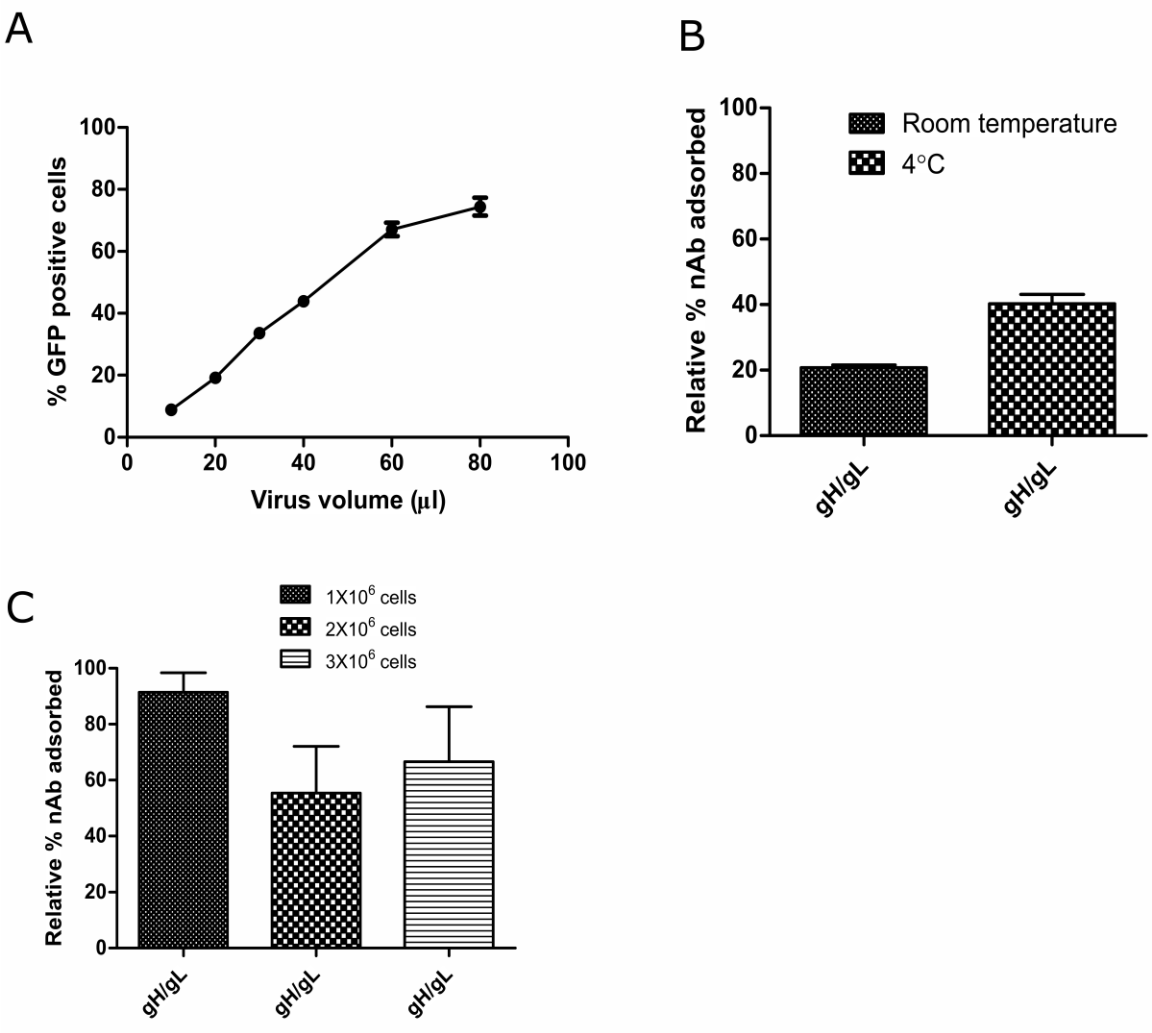
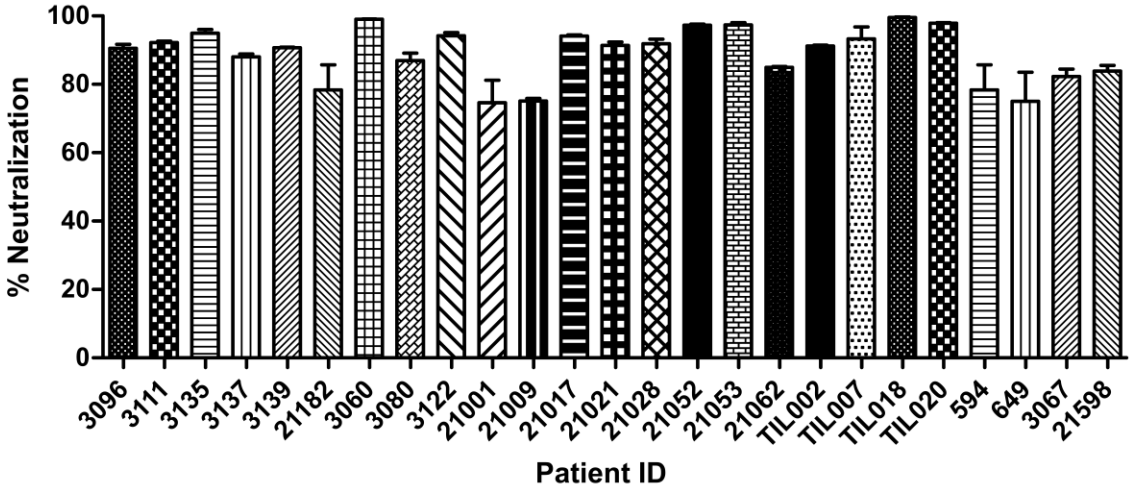
20
40
60
80
100
Endemic KS
Epidemic KS
Non-KS

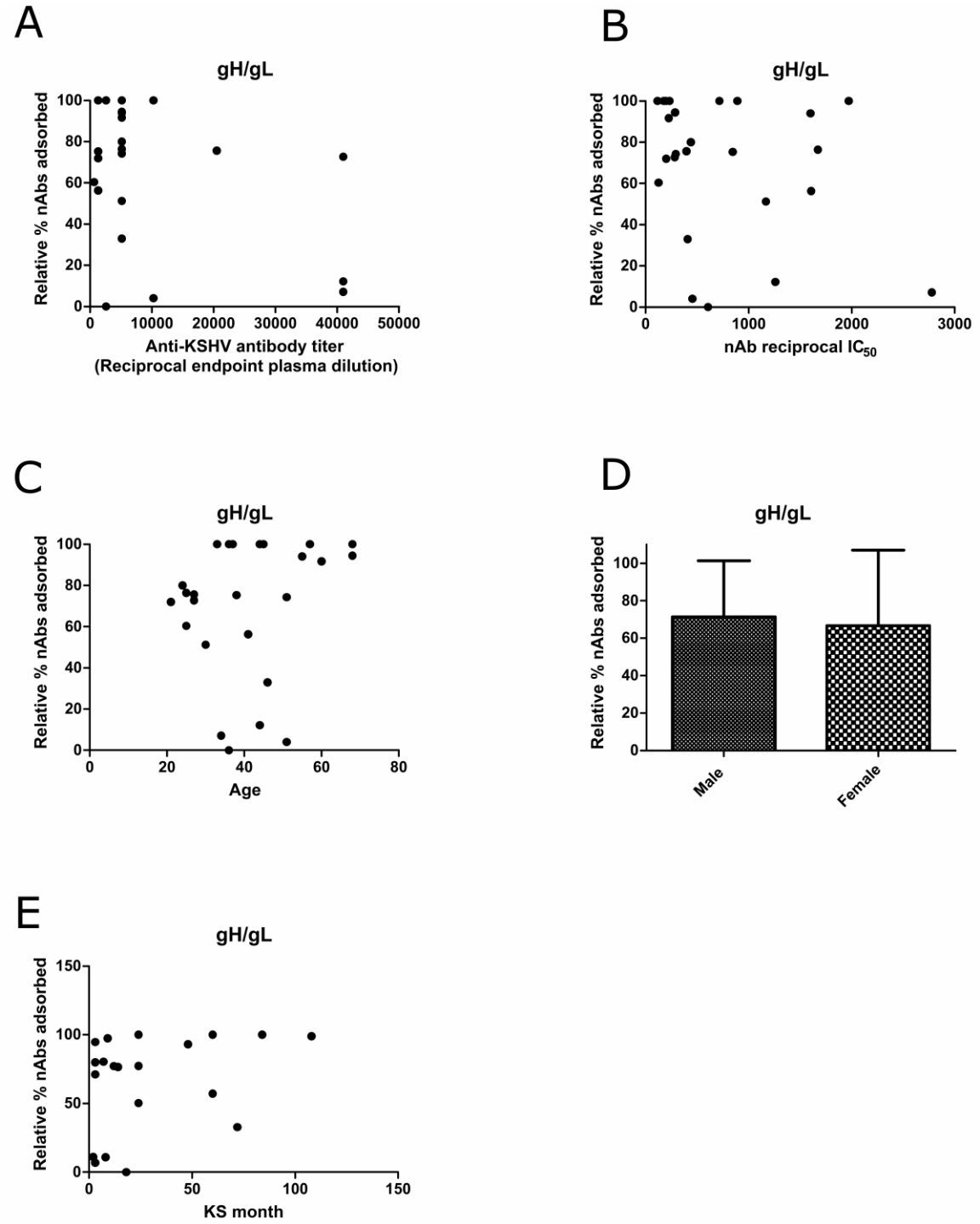
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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Discussion and concluding remarks

Neutralizing antibodies can be very effective in controlling some viral infections in humans (Iwasaki, 2016). Studies have shown that the nAbs against KSHV are not protective against KS since most KS patients have high titers of these antibodies, however, it is still not clear whether having KSHV-specific nAbs prior to exposure to KSHV could be beneficial in preventing its infection, and therefore could serve as an adaptive correlate of protection against KS development. (Kumar et al., 2013). In addition, the targets of the human KSHV-specific nAbs and whether these antibodies can be induced by a KSHV vaccine needs to be determined. Addressing these questions could provide insights for the design of an effective prophylactic vaccines against KSHV and KS development.

Studies on KSHV gB have demonstrated that this glycoprotein plays a major role in KSHV binding and entry into targets cells, hence, making it an attractive antigenic target to elicit nAbs against KSHV (Akula et al., 2001a, 2002; Akula et al., 2001b; Veettil et al., 2008; Wang et al., 2003). Immunization of mice with Ad-gB induced humoral immune responses, resulting in moderate, about 45-65%, neutralizing activity by the immunized plasma at 1:18 dilution. Because only moderate levels of neutralization were observed with Ad-gB immunized mice plasma, other KSHV envelope glycoproteins can be involved in eliciting stronger nAbs responses.

Characterization of the human KSHV-specific nAb responses against all the KSHV envelope glycoproteins revealed adsorption of KSHV-specific nAbs by different KSHV envelope glycoproteins, suggesting that multiple KSHV envelope glycoproteins are the targets for KSHV-specific nAbs in KSHV infected individuals. However, the extent of

adsorption by each KSHV envelope glycoprotein varied among the KSHV infected individuals, indicating that the neutralizing responses against any KSHV envelope glycoprotein varies in magnitude among KSHV infected individuals. Among all the KSHV glycoproteins, only the gH/gL complex elicited nAbs responses that are consistent and comparable among most individuals. About 80% of the participants showed high levels of nAbs adsorption against gH/gL glycoprotein. This implies that gH/gL is the most prominent target of KSHV-specific nAbs in humans. Consequently, having nAbs against gH/gL glycoprotein could possibly prevent the infection by KSHV. Hence, gH/gL glycoprotein is a potential antigen target for developing prophylactic vaccines against KSHV.

In an effort to compare KSHV immune responses between endemic KS and epidemic KS patients, it was recently shown that the total KSHV nAb responses are similar in endemic KS and epidemic KS, but higher than those in non-KS individuals (Lidenge et al., 2019). By comparing neutralizing antibody responses against KSHV envelope glycoproteins in endemic KS, epidemic KS and non-KS individuals, it was concluded that there is no significant difference in neutralizing responses against any KSHV envelope glycoprotein between these groups. These results suggest that the neutralizing responses against KSHV envelope glycoproteins varies among KSHV infected individuals, and there is no correlation with HIV-1 co-infection and KS disease progression.

Previous studies have focused exclusively on immunizing mice and rabbits with various recombinant KSHV glycoproteins to identify those that induced nAbs in the animals, but failed to reflect whether they would induce similar nAbs in humans. To understand what KSHV antigens trigger the induction of human nAb responses, this study

was conducted to characterize the KSHV-specific nAb responses in KSHV individuals to identify the potential targets of their responses upon natural KSHV infection. In summary, our results demonstrate that KSHV-specific nAb responses in humans vary among KSHV infected individuals, and multiple KHSV envelope glycoproteins can be the targets of these nAbs, with gH/gL glycoprotein being the most predominant target.

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