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Modulation of immune recognition by Kaposi's sarcoma associated herpesvirus

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MODULATION OF IMMUNE RECOGNITION BY KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS

By

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DISSERTATION

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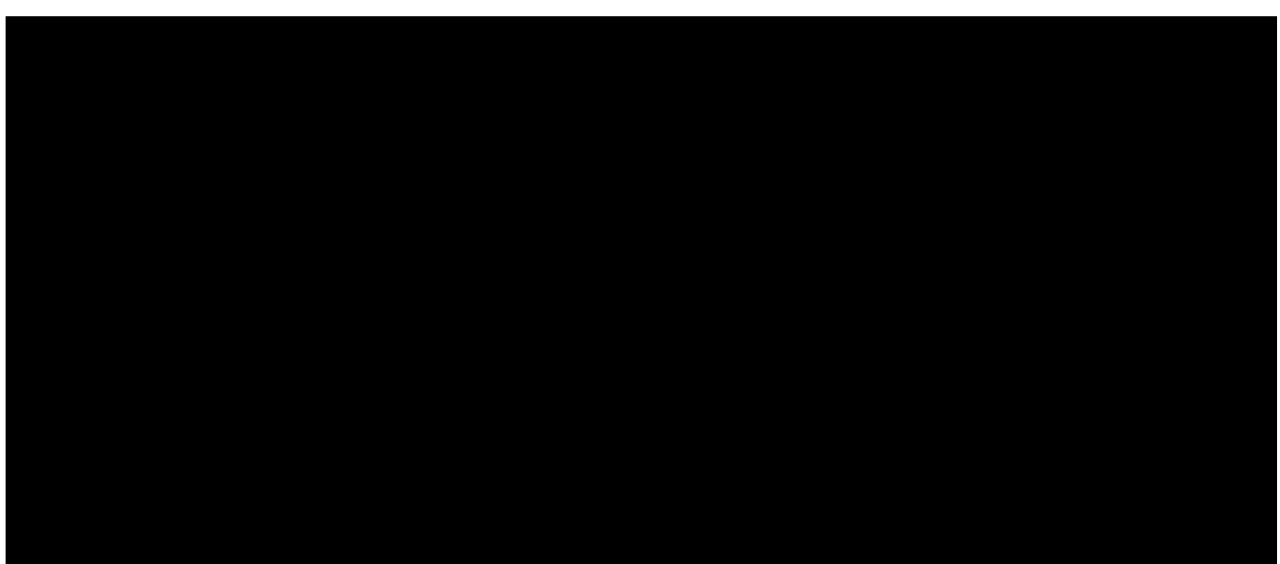

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MODULATION OF IMMUNE RECOGNITION BY KAPOSI'S SARCOMA ASSOCIATED HERPESVIRUS

ABSTRACT

David Jesse Sanchez

Proper functioning of the immune system is critical for the control and ultimate clearance of a viral infection. In particular, the ability for the immune system to recognize a viral infection is based on several pathogen recognition systems such as Major Histocompatability Complex Class I (MHC Class I) as well as the members of the non-classical MHC Class I, CD1 family. While MHC Class I is able to present pathogen specific epitotes to the adaptive branch of the immune system, the CD1 family is able to present lipids to innate T cells of the immune system. Kaposi's sarcoma associated herpesvirus is the causative agent of Kaposi's sarcoma as well as at least two lymphoproliferative disorders. The immune system control of this virus and the diseases it causes is highlighted in the case of AIDS-associated KS where a diminishment in the adaptive immune system by HIV allows for a heightened pathogenesis of the KSHV dependent disease. KSHV encodes two Modulators of Immune Recognition, MIR1 and MIR2. Both act to downregulate MHC Class I from the cell surface, thus making infected cells less prone to immune system recognition. In addition, MIR2 is able to downregulate B7.2 and ICAM-1, both important components in T helper cell activation and immunological synapse formation.

The mechanism of MIR mediated downregulation has only recently begun to be elucidated. Genetic mapping of MIR2 has been able to show that the target recognition domains of MIR2 reside within it transmembrane domains. In addition, truncation analysis of the MIR2 proteins shows that there are three central domains required for MIR2 function: the amino terminal zinc finger, the paired transmembrane domains, and the carboxy region juxtaposed to the transmembrane domains that we designate as the conserved region (CR).

In addition to modulation of mediators of the adaptive response, the MIR proteins are also found to modulate the CD1 branch of the innate immune system. CD1 molecules are structurally related to MHC class I and present lipids to an invariant T cell, termed Natural Killer T cells (NKT) or CD1 restricted T cells. Either MIR protein is able to induce the reduction in surface levels of CD1d. Downregulation of CD1d is dependent on ubquitination on a singular cytoplasmic lysine of the CD1d chain, which leads to internalization by endocytosis. There the chains reside without enhanced turnover, in contrast to MHC Class I. This downregulation does not allow B cells to activate CD1d restricted T cells as effectively as control cells. In addition, we went on to look at the modulation of CD1d surface levels by KSHV and it encoded ORFs. Expression of a limited set of KSHV genes, including glycoproteins, induced the increase of surface levels of CD1d. In addition, the levels of CD1c are seen to increase by expression of the glycoproteins. Finally, the expression of a KSHV specific glycoprotein, K8.1A, lead to the decrease in cell surface CD1c, while a related splice variant, K8.1B did not. This work suggests the intricacies of the CD1 system in recognizing viral infection.

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Chapter 1

Introduction

INTRODUCTION

Recognition of an infection by the immune system is a critical step in the overall control of the infection. In addition to the physical barriers that prevent infection, the immune system is comprised of a robust set of pathogen recognition and detection systems that coordinate a response that often is able to either clear or control the infection. The immune system can be divided into two main branches, the innate and the adaptive immune systems. In general, the innate system responds rapidly within a few hours to general signs of infection while the adaptive response is able to recognize very specific peptide epitotes of an infectious agent and respond in a highly directed manner, though in the delayed timeframe of four to seven days after the start of infection. Coordinated activation and proper function of the array of systems of the two branches of the immune system is required for a robust immune response against an infectious agent.

Even in the face of the defenses the immune system provides, many pathogens are able to establish persistent or even life-long infections. This work presents studies toward understanding the mechanisms of proteins encoded by Kaposi's Sarcoma-associated herpesvirus that modulate the immune response and presents the implication for pathogenesis of the virus.

Kaposi's Sarcoma Associated Herpesvirus

Kaposi's Sarcoma-associated herpesvirus is now known to be the etiologic agent of Kaposi's sarcoma [1-3] and at least two other lymphoproliferative disorders, Primary Effusion Lymphoma (PEL) [4-6] and Multicentric Casteleman's Disease[7]. Kaposi's

sarcoma (KS) was initially described by Moritz Kaposi in 1872 [8]. For many years, this disease, characterized by the red nodules and lesions on the skin, was a rare tumor of middle aged or elderly men. In this classical form of the disease, mainly Mediterranean and Eastern European adult men were affected. As the twentieth century progressed, other forms of KS began to be described including endemic KS, which is primarily seen in the countries of equatorial Africa [9, 10], as well as iatrogenic KS which is seen in patients undergoing immunosuppressive therapy for organ transplantation [11]. But even with these other forms of KS, the overall incidence of KS was low until the beginning of the AIDS pandemic in the early 1980s when AIDS-associated KS began to become highly prevalent [12].

AIDS-associated KS is an aggressive form of KS that was seen in many AIDS patients before the advent of anti-retroviral therapy. In fact, the initial reports describing patients with AIDS were often presentations of Kaposi's sarcoma [12]. The correlation between AIDS and KS inspired a series of epidemiological studies that KS was likely to have an infectious etiology beyond HIV [13]. Several groups began to screen KS tissue biopsies for the causative agent and in 1994 Chang and Moore, then at Columbia University, used representational difference analysis (RDA) of KS tissue versus surrounding tissue to determine that KS tissue contained herpesvirus genomic DNA [14]. These segments allowed the sequencing of the entire viral genome, which was determined to be that of a novel herpesvirus, the eighth human herpesvirus (HHV-8) termed Kaposi's Sarcoma-Associated Herpesvirsus or KSHV.

KSHV is a gamma herpesvirus like Epstein-Barr Virus, but most closely related to the rhadinovirus subfamily which contains Herpesvirus Simiri and the recently described retroperitoneal fibromatosis-associated herpesvirus-*Macaca nemestrina* (RFHVMn) and -*Macaca mulatta* (RFHVMm) [15]. The genome contains the common herpesvirus replication ORFs as well as approximately 15 unique ORFs that were designated as K genes (K1 through K15). KSHV has been detected in the most of the spindle cells that make up the bulk of a KS lesion [16] and has been detected in all clinical forms of KS[17, 18]. As has been learned through studies on AIDS associated KS as well as iatrogenic KS, a functional immune system is critical in the controlled persistence of KSHV. Importantly, these cases have shown a strong role for the adaptive branch of the immune system, which encompasses many necessary recognition systems to control viral pathogens.

Antigen Recognition by the Adaptive Response

Replication of a virus within a cell adds the viral proteins to the whole of the cellular proteome during the infection. There are several systems that are used by the adaptive branch of the immune response to monitor the cellular proteome. Through these systems, the immune response is able to monitor the production of both cellular and foreign proteins and mount a specific immune response against the antigen expressing cell. The Major Histocompatability Complex Class 1 (MHC-1) is a protein complex that presents small peptide, 8 to 11 amino acid in length. These peptides bound to the MHC-1 are monitored by CD8+ T cells, often referred to as Cytotoxic T Lymphocytes, CTLs. Via their rearranged T cell receptor (TCRs), CTLs specific to foreign peptides are selected for

during the T cell development and subsequently activated during an infection. The selected CTLs lead to destruction of cells that are producing the foreign protein by means of release of perforin and granzymes which can lead to cell death, as well as engagement of apoptosis inducing death receptors on the cell surface (such as Fas). CTL's also release cytokines like IFN-γ which may also modulate virus replication.

The peptides that are presented on MHC-1 are derived from the entirety of the cytosolic protein repertoire. Primarily, proteosomal degradation of defective ribosomal products (DRiPs) as well as other proteins within the cell leads to the production of a spectrum of peptides that range in size from several peptides long to larger peptides [19, 20]. The transporter associated with antigen processing (TAP) protein allows for the uptake of these peptides into the endoplasmic reticulum (ER) [21, 22]. There, the MHC-1 alpha chain is associated with the beta-2 microglobulin chain of the MHC-1 complex and a single peptide is loaded into the peptide binding groove of the MHC-1 alpha chain [23]. This loading is primarily based on the binding affinity of the peptide into the peptide binding groove. Once this trimeric (MHC-1 alpha, beta-2 microglobulin, and peptide) complex exits the ER, it enters the Golgi apparatus where the glycosolated chains of the MHC-1 alpha chain are further modified and finally arrives at the plasma membrane where the peptide is presented to the extracellular environment in the context of MHC-1[21, 23].

Virus replication depends on the production of viral proteins within an infected cell. In addition, because of the high level of protein synthesis associated with the replication of a

virus, there are many DRiPs that are produced during viral replication [20]. Since the MHC-1 pathway presents the proteome of each cell, virus infected cells will present the viral proteins in addition to the cellular proteins. Thus, the MHC-1 pathway is very important in the control of viral replication. Conversely, from the virus standpoint, the proper functioning of the MHC-1 pathway is detrimental to productive viral infections and is often a target of viral proteins. DNA viruses often make a concerted effort to stop presentation of peptides derived from their genomes.

Modulation of Antigen Processing by Herpesviruses

Herpesviruses produce a vast amount of proteins for their replication. Within this mix is a variety of proteins that produce immunologically active epitotes after their normal degradation [24]. Thus it is in the best interest of the virus to have evolved various and often synergistic strategies to stop the biosynthesis of functional MHC-1 [25]. The importance of control of herpesviral infection by the T cell response is highlighted by the more severe viral pathogenesis that is seen in AIDS patients including Herpes simplex virus[26, 27] and Cytomegaolvirus [28-30] in addition to the diseases caused by the gammaherpesviruses EBV and KSHV[31-33]. Though many viral families have encoded proteins that disrupt MHC-1 biosynthesis, among the best studied viral system of MHC-1 modulation are those of the herpesviruses [25]. Recently, the proteins that interfere with antigen presentation have been termed Viral Proteins Interfering with Antigen Presentation (VIPRs) to indicate their role in the viral response to the immune systems of the body[34].

Herpesviruses are often extremely efficient at establishing lifelong persistent infection and as such, they are self required to set a definitive balance between immune system that protects the host and the persistence of the infection. To strike a definitive balance of a long term infection, all herpesviruses seem to encode at least one if not more VIPRs [35]. The known herpesviral VIPRs seem to target very distinct points in the biosynthetic path of MHC Class I. Herpes Simplex Virus (HSV) ICP47 binds to the TAP protein preventing the import of peptides into the ER [36, 37]. Human Cytomegalovirus contains several VIPRs including US6 which stops TAP translocation of peptides from the lumunal side of the ER [38] and US2 and US11 which induce retrotranslocation of the MHC Class I chains into the cytosol which leads to subsequent proteosomal degradation[39, 40]. Mouse Cytomegalovirus also contains several VIPRs such as m152/gp40 which causes MHC Class I to be retained in the ER *cis*-Golgi complex (ERGIC) [41] and m4/gp34 which associate with MHC Class I on the plasma membrane that leads to blockage of CTL recognition of the cell[35, 42]. Taken together, these show that herpesviruses use varieties of proteins and also a variety of targets to solve. In fact the *in vivo* relevance of these genes has become more apparent in mouse models of viral infection in which knock out viruses that show that these seemingly functionally duplicated proteins, are required in concert to prevent destruction by the divergent aspects of the adaptive immune system [35].

The Modulators of Immune Recognition

To search for KSHV encoded proteins that interacted with the antigen processing pathways of a cell, a systematic screen of the unique KSHV ORFs was conducted. It was proposed that the K genes, approximately 15 ORFs unique to KSHV, encoded the majority of the pathogenesis related proteins of KSHV including those that would affect immune recognition of infected cells. Using a set of replication defective retroviruses that each encoded an individual K genes, the K genes were screened for their ability to reduce the level of MHC Class I on the cell surface of a cell[43]. This screen showed that two genes, ORF K3 and ORF K5, could reduce the levels of MHC Class I when either genes was expressed. Several other groups, have confirmed these findings [44-46]. These gene products were found to induce the enhanced endocytosis of MHC Class I from the plasma membrane. MHC Class I chains proceeded throughout the secretory pathway seemingly unperturbed in the cells expressing the K3 or K5 genes, but were rapidly internalized upon reaching the plasma membrane. [43] This was supported by the fact that expression of a dominant negative form of Dynamin, a cellular GTPase that is involved in the formation of an endocytosed vesicle, could restore the surface levels of MHC Class I. The MHC Class I chains are then internalized and shunted to the lysosome for degradation.

The K3 and K5 ORFs were originally described as membrane bound zinc finger containing proteins. Indeed, both ORFs have the same overall predicted structure (Figure) in which an amino terminal zinc finger that is similar in organization to a RING finger, is followed by two transmembrane domains and a long Carboxy terminal tail[43].

The initial localization of these proteins was determined to be in the endoplasmic reticulum of the cell. This provides an interesting paradox in that the K3 and K5 proteins are localized to the ER while their main mode of action on the targeted chains of MHC Class I are at the plasma membrane. Still, a direct interaction between the K3 gene product and MHC Class 1 has been documented [47].

Further work by our group and others also demonstrated the expression of the K5 ORF lead to a decrease in surface levels of B7.2, the ligand for CD28, which is involved in co-stimulation, and Intracellular Adhesion Molecule 1, ICAM-1, the ligand for LFA-1 and involved in stable formation of immunological synapses[48]. Expression of the K5 ORF thus leads to an impaired ability for T helper cell activation. Another group described the downregulation of B7.2 by ORF K5, but attributed it as a defense against natural killer cell by using a natural killer cell line that expresses CD28 and is thus activated by B7.2 [49]. Work by our group showed that though that particular cell line expressed CD28, natural killer cells derived from human peripheral blood do not express CD28 and as such, ORF K5 expression did not protect cells from NK mediated killing. Rather, it actually made them more susceptible possibly due to the reduced levels of MHC Class I on the cell surface of ORF K5 expressing cells[48]. However, these results do not exclude the previous findings and in fact hint that modulation of NK receptors by KSHV is still a critical feature that should be studied further since reduction of surface levels of MHC-1 by ORF K5 and presumably ORF K3 may make cells more susceptible to NK cell killing

Taken together these studies lead to the naming of the gene products of ORF K3 and K5 as the Modulators of Immune Recognition, MIR proteins. ORF K3 and ORF K5 were thus designated MIR1 and MIR2 respectively.

MIRs: From Function to Family

As the MIR proteins were being functionally being defined, a homologous protein in MHV-68 was also defined as an immune modulator [46]. This protein was initially named MK3 to designate that it was a mouse version of KSHV's K3 protein. In fact, the protein is highly homologous to the MIR proteins and is able to downregulate surface MHC Class I levels. A fundamental difference stood out though, while MIR proteins are able to enhanced endocytosis of targets and their subsequent degradation by the lysosome, MK3 induced ER associated degradation (ERAD) and proteosomal degradation of the targets in the cytosol [50].

Studies of the MIR proteins went on to further determine the exact cell biological mechanism that they use to reduce surface expression of their targets. An important finding came when it was shown that the MIR Zinc finger [51]as well as the zinc finger of MK3 [50] could function as an E3 ubiquitin ligase. Mutational analysis as well as genetic analysis show that the zinc fingers of either MIR2 or MK3 were able to lead to the ubiquitination of the targets of MIR2 or MK3 [50, 51].

For the MIR proteins this became especially important as ubiquitin itself had just undergone a review of its importance in cell biology as not only a tag for degradation but

also one for vesicular trafficking [52-54]. Of recent interest has been the involvement of Tumor susceptibility gene 101 (TSG101) in the targeted trafficking [55]. TSG101 is a component of a multi-subunit complex termed the ESCRT-1 (Endosomal complex required for transport) complex which is composed of several proteins that are required for the proper recognition and trafficking of target proteins into the endosomes [56]. Knockdown of TSG101 by siRNA was used to show that TSG101 was critical for the MIR1-mediated degradation of MHC Class I and subsequently restored surface levels of the MHC Class I [47].

In addition to the insights gained from the mechanistic studies into the MIRs, an interesting set of discoveries was beginning to take shape. At first, the MIR proteins seemed to be very unique and not identifiable in any BLAST query. The discovery of the MK3 homologue of MHV-68 was readily explainable by divergence within the viral family. What was not readily explainable was the identification of several MIR homologues within the poxvirus family, a completely different viral family. This Myxomavirus protein, named Myxoma virus leukemia associated protein or M153R, has the ability to downregulate several proteins involved in the immune response including MHC Class I, CD4 and Fas (CD95) [57, 58]. In addition, several other genes in other pox family viruses have been found to encode proteins that have a similar zinc finger as well as two transmembrane regions on the carboxy end of the zinc finger. These include ORF C7 in swinepoxvirus, gb153R of rabbit shop fibroma virus and other proteins in Yaba-like lumpy skin disease and yaba-like virus [59].

With the large number of proteins that were being found in divergent viral families, it became evident that there might be a host derived source for these completely divergent viruses to have acquired such similar proteins. Bioinformatic approaches allowed for the identification of several proteins that contained the general protein organization of a Zinc finger followed by an even number of transmembrane domains (L. Coscoy and T. Rose, unpublished data). In addition, functional analysis of the first human homologue, termed c-MIR, showed that expression of the protein lead to specific downregulation of B7.2 and not MHC Class I [60]and (L.Coscoy and D. Ganem, unpublished data). In addition, other family members have now been shown to downregulate MHC Class I [61]. These proteins all seem to induce ubiquitination like the viral MIR proteins and additional seem to induce enhanced endocytosis similar to the KSHV MIR proteins. The exact mechanistic details for these proteins are being worked out, but importantly, understanding the physiologic role of these proteins in an immune response may provide many insights in how the immune response can be self limiting or modulate itself.

Innate Recognition Systems of the Immune System

While the adaptive immune system is setup to slowly recognize exact peptide epitotes of a pathogen, the innate system is setup to rapidly recognize very general signs of infection. The innate branch of the immune system detects varying signals from cellular stress to general signs of pathogen replication like double stranded RNA of RNA virus replication [62]. The cellular components of the innate immune system include macrophages, dendritic cell, in addition to many other cells such as Natural Killer (NK) cells and lineages of the adaptive immune system. They are setup to detect and respond to various

infectious signals and release chemicals that control the infection, directly destroy the infectious agent [63]. In addition, the chemical messengers that are released by the innate immune system often orchestrate the cues that are given to the adaptive immune system to destroy the foreign pathogen in a more efficient manner especially with regards to polarizing the T helper subsets that are allowed to expand.

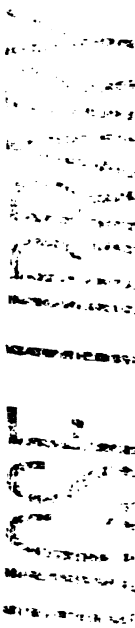
The CD1 Family and Its Role In Viral Infections

The CD1 family is composed of a set of membrane bound proteins that are structurally similar to MHC Class I. These proteins were initially functionally described as upregulated proteins in tumor cells that could be lysed by T cells that were both CD4 and CD8 negative[64]. This molecule turned out to be CD1a, related to the other molecules, CD1b, CD1c, and CD1e [65]. Each of these molecules is similar to MHC Class I, but different in that in place of the charged, shallow, peptide binding groove, a deep hydrophobic groove exits [66]. This groove is theoretically able to bind long hydrophobic tails such as those of lipid tails and has been shown in a variety of system to bind molecules that have long tails, both lipid and recently, peptidolipid [67] [68, 69]. These molecules have come to be thought of as important detectors of bacterial pathogens and as such are key in the control of different bacterial pathogens.

A second group of CD1 molecules was discovered which is composed on the one member termed CD1d. CD1d is restricted in tissue distribution including dendritic cells, marginal zone B , hepatocytes and enterocytes [70, 71]. Just like other CD1 molecules, CD1d is nonpolymorphic but as of yet, the exact antigen it presents is unclear. CD1d

restricted T cells comprise a subset of T cells that are often referred to as Natural Killer T (NKT) Cell which express an invariant alpha chain of the TCR[70, 72, 73]. Activation of this cell type by the glycolipid alpha-galactoceramide, a marine sponge derived lipid, induces the production of cytokines such as IFN-γ and IL-4 [74]. Other, nonclassical NKT subsets also exist.

Recently, CD1d has increasingly become implicated in the control of viral infections. The main evidence for a role in viral infections and pathogenesis came from studies in a mouse model of Hepatitis Virus B infection[75]. Here the expression of the Hepatitis B Viral genome in hepatocytes does not lead to acute hepatic injury to the cells, unless CD1d restricted T Cells, which are often termed Natural killer T Cells, were adoptively transferred into the animal. In addition, expression of the HBV envelope proteins induces a strong upregulation of the surface CD1d levels (J. Baron and D. Ganem, Unpublished Data).



Further evidence for a role of C D1d in the control of viral infection came with the use of knockout mice and experimental infection of these mice. When mice that were missing the CD1d allele were experimentally inoculated with Respiratory Syncitial Virus or Herpes Simplex Virus, they produced higher titers of virus than wildtype mice.

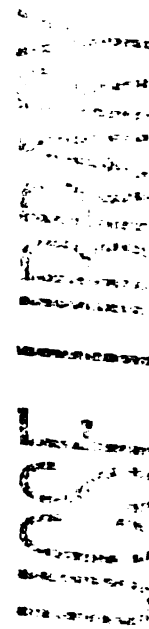
However, the exact link between CD1d and viral infection has remained elusive, though these results seem to imply some that CD1d may act a trigger to enhance anti-viral immunity [76] [77, 78]. CD1d seems to reside in late endosomal compartments of the

cell and may act as a sensor for cell stress in this system or to acquire antigens for presentation [79] [80]. A model of the mode of action of CD1d restricted T cells in anti-bacterial immunity has become to be understood recently. Recent studies seem to implicate upregulated CD1d levels as a stress derived receptor that acts in concert with IL-12 released from the site on an infection to potentiate CD1d restricted T cells to be more cytolytic toward infected cells[24] [81]. In addition, recent evidence in a viral system seems to support this model in encephalomyocarditis virus infections [82]. As of yet, there have been no reports of a VIPR that affects the CD1d pathway.

Overview of the Thesis

The work in this thesis was conducted to understand the mechanism of the MIR proteins as well as extend the understanding of their modulation of the immune response by looking at their contribution to modulation of the innate immune system. While this chapter introduced the general aspects of the immune system that KSHV modulates through the MIR proteins, Chapter 2 documents the studies we undertook to elucidate the functional organization of the MIR2 protein. This chapter was originally published as: *Functional organization of MIR2, a novel viral regulator of selective endocytosis.* David Jesse Sanchez, Laurent Coscoy and Don Ganem. Journal of Biological Chemistry. February 22, 2002. Volume 277, Issue 8 p6124-30. Chapter 3 focuses on modulation of the CD1d system by the MIR proteins and the implications of this modulation for viral defense by the immune system. Chapter 4 recounts our early, and still-incomplete studies

of other viral modulators of CD1d, and also examined the regulation of CD1c display by selected viral proteins.



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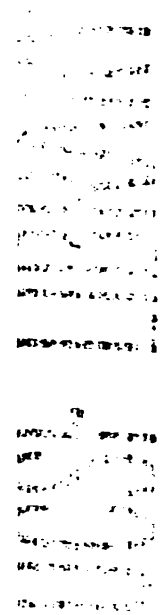
Chapter 2

Functional organization of MIR2, a novel viral regulator of selective endocytosis



SUMMARY

Kaposi's sarcoma-associated herpesvirus (KSHV) encodes two related proteins, MIR1 and MIR2, that lead to reduction of the cell surface levels of MHC class I and other polypeptides involved in immune recognition. MIR1 and MIR2 do not affect the assembly or transport of their target proteins through the secretory pathway; rather, they act to enhance the selective endocytosis of target chains from the cell surface. Sequence inspection reveals that the MIR proteins contain an N-terminal zinc finger of the PHD subfamily, two transmembrane (TM) domains, and a C-terminal conserved region (CR). Here we examine the transmembrane topology and functional organization of MIR2. Both the PHD domain and the CR are disposed cytosolically and are essential for MIR-mediated endocytosis. MIR proteins form homo-oligomers; this activity is independent of the PHD and CR elements and maps instead to the TM regions. Analysis of chimeras between MIR 1 and MIR 2 reveals that the TM regions also mediate target selectivity. Mutations that ablate the PHD or CR regions generate dominant negative phenotypes for MHC-I endocytosis. These findings suggest a domain organization for the MIR proteins, with the TM regions involved in target selection and the cytosolic PHD and CR domains involved in the possible recruitment of cellular machinery that directly or indirectly

INTRODUCTION

Herpesviruses are a family of large DNA viruses that are able to induce a persistent (usually lifelong) infection. To facilitate the production of such long-term infections, herpesviruses have evolved multiple strategies to evade immune detection. Most commonly, this is achieved by interrupting the synthesis, assembly or function of MHC class I (MHC-I) molecules, key proteins involved in the recognition of infected cells by cytotoxic T lymphocytes (CTLs) [1, 2]. Kaposi's sarcoma associated herpesvirus (KSHV; also known as human herpesvirus 8) is the etiologic agent of Kaposi's sarcoma (KS) and several other AIDS-related proliferative disorders [3, 4]. We [5] and others [6-8] have shown that KSHV possesses two genes, K3 and K5, that encode protein products termed MIR 1 and MIR2, respectively (for Modulator of Immune Recognition). MIR1 and MIR2 are homologous proteins that are localized predominantly in the endoplasmic reticulum (ER) and lead to reduction of the levels of MHC class I (MHC-I) chains present at the cell surface [5]. Despite their predominantly ER localization, MIR1 and MIR2 do not affect the assembly, glycosylation or transport of MHC-I chains in that organelle. Rather, they act to enhance the endocytosis of MHC-I chains from the cell surface, with the endocytosed chains subsequently targeted to the lysosome for

proteolytic destruction [5].

This enhanced endocytosis of MHC-I does not reflect a generalized induction of endocytosis, since many other surface proteins known to undergo internalization, including the transferrin receptor and MHC class II molecules, are unaffected by the MIR proteins [5]. However, MHC-I is not the sole target of the MIR protein family. Two other molecules involved in immune recognition can be downregulated by MIR2 expression: B7.2, a cell surface protein involved in helper T cell activation (costimulation), and ICAM-1 (intracellular adhesion protein-1), an adhesion protein important for the formation of the immunological synapse [9, 10]. Interestingly, neither of these two proteins is targeted by MIR1, indicating that there has been functional specialization of the MIRs during KSHV evolution.

Although the overall fate of the target surface immunoregulatory proteins is clear, our understanding of the molecular mechanisms by which MIR1 and MIR2 target these chains and mediate their endocytosis remains fragmentary. Analysis of the primary amino acid sequence of the MIR proteins shows that they contain a zinc finger domain of the PHD (plant homedomain) subfamily at their amino terminus, along with two transmembrane (TM) domains near the center of the proteins (see Figure 1A). Distal to

TM domain II is a short sequence (termed CR for conserved region) that is also highly conserved between MIR1 and MIR2. PHD-family zinc fingers, also known as LAP zinc fingers [11], have been implicated in protein-protein interactions [11-13] and are structurally similar to RING fingers [14]. Mutations in the Zn finger region abrogate MIR function [10], but beyond this little is known of structure-function relationships in the protein.

To gain a better understanding of which regions of the MIR proteins are important for function, we have examined the transmembrane topology and functional organization of MIR2. These studies show that the transmembrane domains of the protein play a key role in determining the target specificity of the protein. The PHD and CR elements are also critical for function, and mutations targeted to these regions result in dominant negative inhibitors of MIR2 action. These findings suggest a domain organization for the MIR proteins, with its TM regions involved in target selection and the cytosolic PHD and CR domains involved in the possible recruitment of cellular machinery that could directly or indirectly regulate internalization of target molecules.

EXPERIMENTAL PROCEDURES

Cell Culture CV-1, Phoenix and 293T cells were grown in DMEM (DME-H21) and BJAB lymphoma cells were grown in RPMI medium 1640. All media was supplemented with 10% (vol/vol) FCS and penicillin/streptomycin. Transfections of CV-1 and Phoenix cells were performed using Fugene6 (Roche) according to the manufacturer's suggested protocol. BJAB cells were transfected by electroporation of 20 μg of plasmid DNA into 10^7 cells in 0.5 ml of serum-free medium [15]. BJAB Cells were transfected with 250mV voltage and 960μF of restistance. The transfection efficiency of BJAB cells was routinely 30-50% under these conditions.

Antibodies For immunofluorescence analysis, mouse anti-Flag (Sigma) was used at a concentration or 20 μg/ml and rabbit anti-HA (Sigma) was used at a dilution of 1:50. Sheep anti-mouse IgG conjugated to FITC (Sigma) and donkey anti-rabbit IgG conjugated to Texas Red (Santa Cruz Biotechnology) were used at a concentration of 4 μg/ml. For FACS analysis, mouse anti-HLA Class I (APC conjugated or FITC conjugated; Caltag Laboratories) and mouse anti-B7-2 (PE conjugated; Caltag Laboratories) were used at a dilution of 1:300. Western blotting used mouse anti-Flag

M2 (Sigma) at a dilution of 1:400 or rabbit anti-HA (Sigma) at a dilution of 1:256 or mouse anti-GFP. Secondary antibodies for Western blots were HRP conjugated goat anti-mouse Ig and donkey anti-rabbit (Santa Cruz Biotech.) that were both used at dilutions of 1:1500.

Plasmid Construction All constructs were made by PCR amplification of MIR1, MIR2, or EGFP. Chimeric fusions between EGFP and MIR2 truncation or MIR1 and MIR2 were made by amplification of overlapping PCR fragments. All expression constructs were introduced into the *BamHI* and *NotI* site of pCR3.1 (Invitrogen). The amino Flag-tagged/ internal HA-tagged version (See Figure 2A) of the MIR2 gene was subcloned from pCR3.1-Flag-MIR2-HA vector into pBMN-ZIN (kindly provided by G. Nolan, Stanford University). This retroviral vector permits the expression of pCR3.1-Flag-MIR2-HA and the neomycin resistance gene from a single bicistronic mRNA.

Retroviral infection. On transfection with the retroviral vectors (pBMN), the Phoenix packaging cell line produces replication-defective viral particles that can be used to stably transduce CV-1 cells. Phoenix cells were transfected and the virus-containing supernatant

was harvested 48 hours after the transfection, filtered through a 0.45-μm filter and diluted with Polybrene (4 μg/ml final dilution). CV-1 cells (in 6 well dishes) were infected by spin infection (800×*g* for 2 hours at 20°C) using 2 ml of viral supernatant. Selection of transduced CV-1 was started 36 hours after infection by adding 1.5 mg/ml of G418.

Selective Permeabilization Based Immunofluorescence Cells grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature and washed again with PBS. For selective permeabilization of the plasma membrane, cells were incubated in 5 μg/ml of digitonin (Sigma) in digitonin buffer (0.3M sucrose, 2.5mM $MgCl_2$, 0.1M KCl, 1mM EDTA, 10mM Pipes at pH6.8) for 5 minutes on ice. Complete permeabilization was achieved by incubating cells in 0.2% Saponin (Sigma) in PBS + 1% BSA for 40 seconds at room temperature as previously described [16]. All subsequent incubations of the completely permeabilized samples were carried out in the presence of 0.2% Saponin.

Cells were then washed with PBS and incubated in PBS + 1% BSA to block nonspecific binding. Cells were then incubated with primary antibodies diluted in PBS + 1% BSA, washed with PBS and incubated in secondary antibodies diluted in PBS + 1%

BSA. After extensive washing, the cells were mounted onto glass slides with VectaShield (Vector Laboratories) and analyzed by using a Leica fluorescent microscope using appropriate filters.

As a negative control, cells not exposed to either detergent displayed only background levels of staining with Flag or HA antibodies (not shown). When cells were exposed to either digitonin or saponin under the above conditions, over 90% of cells examined were stained by anti-Flag antibody, indicating efficient detergent action.

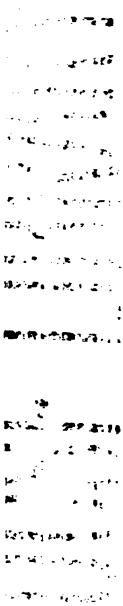
Coimmunoprecipitation 10 cm dishes of 293T cells were transfected by Fugene6 with 6 μg of pCR3.1-Flag-MIR2 or pCR3.1-HA-MIR2 and 6 μg of pCR3.1. In parallel, another dish was transfected with 6 μg of each of the tagged versions of MIR2. 48 hours posttransfection, the cells were scraped from the dishes and protein lysates were made by resuspending in RIPA buffer (PBS with 1% Igepal, 0.5% Deoxycholic acid sodium salt, and 0.1% SDS). The lysates were cleared of debris, split in half and incubated with either mouse anti-Flag M2 antibody or rabbit anti-HA antibody and then incubated with protein A/G+ agarose beads (Santa Cruz Biotech.). Immunoprecipitated proteins were separated on a 4-20% polyacrylamide gel, transferred to nitrocellulose and western

blotted with either mouse anti-Flag M2, mouse anti-HA or mouse anti-GFP, then incubated with goat-anti mouse Ig HRP and visualized with Luminol (Santa Cruz).

Flow cytometry analysis. Cells were washed in PBS 1% + BSA and incubated with specific mAb's (1 μg/10^6 cell) for 60 minutes at 4°C. Cell surface fluorescence was analyzed with a Becton Dickinson FACScalibur (Becton Dickinson, San Jose, California, USA).

Dominant Negative Assay 293T cells in 6 well dishes were transfected with 2 μg of vector alone, or 0.5 μg of pCR3.1-MIR2-EGFP and either 1.75 μg of pCR3.1 vector or pCR3.1-Flag-MIR2-ΔPHD. After 36 hours, the cells were removed from their dishes by using cell dissociation buffer (Gibco-BRL) according to the manufacture's instructions. The cells were washed with PBS, incubated in mouse anti-HLA (APC conjugated; Caltag laboratories) in PBS + 1% BSA at a dilution of 1:300 for one hour, washed twice with PBS. Cell surface fluorescence was analyzed with a Becton Dickinson FACScalibur (Becton Dickinson, San Jose, California, USA). In Figs 5 and 6, the FACS analysis was

gated on EGFP positive (i.e. transfected) cells and the level of MHC Class I on the surface of such cells was quantified.



RESULTS

Transmembrane orientation and oligomeric state of MIR2.

MIR2 contains no recognizable signal sequence in its primary amino acid sequence, but the protein is predicted to contain two transmembrane domains. To determine the transmembrane topology of the MIR2 protein, we employed a recently-described system involving selective permeabilization-based immunofluorescence [16, 17].

As shown in Figure 1B, a form of MIR2 was constructed in which a Flag epitote tag was placed at the amino terminus of the molecule and an HA tag was placed between the two transmembrane domains. This doubly-tagged protein is fully functional for B7-2 and ICAM-1 downregulation (data not shown). CV-1 cells were stably transduced with a retroviral vector encoding this version of MIR2. These cells were fixed and subjected to permeabilization with either (i) digitonin, which selectively permeabilizes the plasma membrane but leaves the ER membrane intact or (ii) Saponin, which under these conditions permeabilizes both the plasma membrane and the internal membranes of the cell [16, 17] . Following detergent treatment, cells were stained for the Flag tag (detected by FITC-conjugated second antibody) or the HA tag (stained with Texas Red–conjugated

second antibody). Digitonin permeabilization allows immunofluorescent tagging of only the Flag tag (Fig. 1C) and not the HA tag (Fig. 1D) suggesting that the amino terminus of MIR2 is in the cytosol while the intermembranous loop is in the lumen. This is confirmed by permeabilization with saponin (Figs.1E and 1F), which allows immunofluorescent staining of both the tags. This orientation disposes the Zn finger/PHD domain on the cytoplasmic face of the membrane (Fig. 1G). Since PHD domains are known to mediate protein-protein interactions [11, 12, 18], this would place it in a context where it could be available for interactions with cytosolic constituents. Similar studies with a C-terminal tagged version of MIR2 show that the C terminus is also in the cytoplasm (not shown).

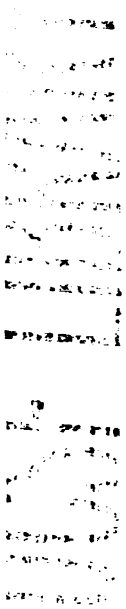
Western blotting of MIR2 in whole cell lysates often shows high molecular weight bands (Fig 2A), raising the possibility that MIR2 may form homo- or hetero-oligomeric structures. To test if MIR2 can homo-oligomerize, the protein was tagged with either a Flag epitope or an HA epitope at its amino terminus. The tagged versions of MIR2 were transfected alone (Figure 2A and Figure 2B, lanes 1 and 2) or together (Figure 2B, lane 3) into 293T cells and cell lysates were prepared. Figure 2A shows that, as expected, there is no serologic crossreaction between the HA and Flag tags. As shown

in lane 3 of Figure 2B, immunoprecipitation with anti-HA antibodies and Western blotting with anti-Flag antibodies shows that the differentially tagged versions of MIR2 can coimmunoprecipitate. This coprecipitation is dependent upon coexpression of the two proteins and indicates the formation of stable homooligomers. Similar findings have been made for MIR 1 (data not shown). A truncated form of MIR2 containing the two transmembrane regions and only a portion of the CR (Fig. 2C) is able to specifically coimmunoprecipitate with full length MIR2 (Fig. 2D) showing that oligomerization likely happens by interactions within the transmembrane domain.

The zinc finger and downstream conserved region (CR): cytosolic domains required for MIR2 function.

To map regions of MIR2 that are important for its function, we generated a series of MIR2 truncations and examined their ability to downregulate surface MHC-I and B7.2 (Figure 3A). As outlined in the legend to Fig 3, most of the truncations were expressed as MIR2-GFP fusion proteins. (Wild-type MIR2 is fully functional when fused to GFP in this fashion; Figure 3C and LC, unpublished data). Following transfection, the FACS analysis was gated on transfected (e.g. GFP-positive) cells, whose surface MHC-I and

B7.2 levels were then examined. All truncations were stably expressed as judged by immunofluorescence analysis and flow cytometry (not shown). As expected from previous results **[10]**, a deletion of the amino terminus to the first transmembrane region (thereby removing the PHD zinc finger) abrogates the ability of MIR2 to downregulate either MHC Class I or B7-2 from the cell surface (Fig. 3D). However, the PHD zinc finger by itself is not sufficient to induce the removal of target molecules from the cell surface (Figure 3E). Consistent with this, removal of the entire C-terminal cytosolic tail also destroys function, indicating that even a PHD Zn finger tethered to the membrane does not suffice to upregulate endocytosis (Figure 3F). This is despite the fact that it is stably expressed and accumulates in the ER as efficiently as WT MIR2 (not shown). However, addition of a small portion of the downstream cytosolic tail encompassing the CR sequences restores full function for B7-2 downregulation and partial function for MHC-I downregulation (Figure 3G). Thus, each cytosolic domain contains conserved sequences that are necessary, but not sufficient, for MIR function.



The transmembrane regions of MIR2 are involved in target selection.

To map regions of MIR responsible for determining the selection of its targets, we took advantage of the fact that MIR2 can downregulate B7.2 and ICAM-1 as well as MHC-I, while MIR1 can only downregulate MHC-I chains [9, 10]. To determine which regions of MIR2 account for its expanded target range, we constructed chimeras between these two highly related viral proteins (Figure 4A) and analyzed the ability of the resulting chimeras to downregulate surface MHC-I, B7.2 and ICAM-1 following transient transfection of BJAB cells (Figure 4B). (Unlike Figure 3, the FACS analysis of Figure 4B does not gate on the transfected cells; it displays the entire BJAB population, about 30-50% of which are transfected). A chimeric molecule that replaces the amino terminus of MIR2 with that of MIR1 does not affect the downregulation of B7.2 (Figure 4B, construct I). Similarly, replacement of both the N and C terminal cytosolic domains of MIR2 with their cognate regions of MIR1 does not affect the target selectivity of the protein – like WT MIR2, this chimera also downregulates both MHC-I and B7.2 (Figure 4B, construct III). Thus, this selectivity must reside in the TM domains and intermembranous loop of MIR2. As expected, the chimera encoded by construct II, which bears the TM and intermembranous regions of MIR1, has the target range of MIR1. Finally, replacement of the intermembranous loop of MIR2 with that of MIR1 has no

impact on MIR2 function (data not shown), suggesting that it is the TM domains of MIR2 that account for its target selectivity. In all cases, the ability of the chimeras to downregulate B7.2 and ICAM-1 were inseparable (data not shown), so only the B7.2 data are depicted here.

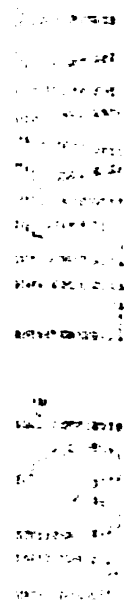
Truncated forms of MIR2 lacking the PHD or CR regions can act as dominant negatives.

The above experiments establish that there are several domains of MIR2 that are required for the MIR2-mediated endocytosis of molecules from the cell surface; some of these affect target selectivity, while others affect functions common to all targets. In other multifunctional proteins, deletion of a single effector domain often generates dominant negative mutants. Such mutants can have many bases – formation of nonfunctional hetero-oligomers, competition for binding to an essential partner, etc. Accordingly, we sought to establish whether deletion of one domain of MIR2 might engender a dominant negative mutant. For this we initially selected the MIR2 ΔPHD mutant (see Figure 3A), which lacks the N-terminal cytosolic domain (including the conserved PHD domain). Cells were cotransfected with a fixed quantity of WT MIR2 and either the empty vector

or a vector expressing equal quantities of the □PHD mutant. As expected, wild-type MIR2 expression reduces the surface level of MHC Class I when cotransfected with vector alone (Fig. 5A). When MIR2 was cotransfected with the MIR2 ΔPHD mutant, the surface level of MHC Class I was restored to a level comparable to the vector-transfected controls (Fig. 5B), indicating that MIR2 ΔPHD acts as a dominant negative mutant. As a specificity control, expression of MIR2 ΔPHD did not restore surface levels of MHC-I that had been reduced by cotransfection with a vector expressing the herpes simplex ICP47 protein (Fig.5C). ICP 47 acts by inhibiting the TAP transporter, preventing the loading of assembling MHC-I chains with antigenic peptides and thereby blocking egress of MHC-I chains from the ER [19, 20]. These results indicate that the negative effects of the ΔPHD mutant are selective for MHC-I downregulation mediated by MIR.

Similar findings were made with mutant MIR2ΔC1, which lacks the C-terminal cytosolic region including the CR element (see Fig 3A). As shown in Fig. 6A, WT MIR2 cotransfected with an empty expression vector produced the expected reduction in cell surface MHC-I chains. However, this reduction could be abrogated by coexpression of the MIR2ΔC1 mutant (Figure 6B). Thus, deletion of either the N- or C- terminal cytosolic

tail of MIR2 generates mutants that can block the ability of the WT protein to regulate the selective endocytosis of its MHC-I protein target.



DISCUSSION

These data establish that MIR2, a novel viral mediator of immune evasion, is an oligomeric, type III membrane protein with two transmembrane domains flanked by cytosolic regions at the N and C termini. Each cytosolic tail contains conserved sequences: the N-teminal region harbors a conserved Zn finger of the PHD subfamily, while the C-terminal tail contains a shorter conserved region (CR) of unknown function. Analysis of MIR1/MIR2 chimeras indicates that the transmembrane regions are the major determinants of target selectivity. We do not know whether one or both TM regions are required for this activity, nor do we yet understand the biochemical basis for target selection. The simplest model would be that the TM regions of MIR2 interact, directly or indirectly, with the TM domains of its target(s). Consistent with this idea, we and others [8] have recently found that MIR2 can downregulate human but not mouse MHC-I chains, and chimeras between human and mouse targets implicate the TM domain of the target in conferral of MIR2 regulation [21]. However, more indirect models are also possible.

This raises an interesting conundrum, since MIRs localize principally in the ER [5, 22] but their targets are endocytosed from the plasma membrane [5]. It is possible that

the interaction between effector and target takes place in the ER, leading to "marking" of the target chains for later endocytosis. However, in an earlier study [5] we showed that MHC-I chains made in the absence of MIR proteins could be downregulated following subsequent introduction of MIR expression vectors. If so, this would make "ER marking" an unlikely model. We think it more likely that a small fraction of MIR chains escape the ER and travel to the plasma membrane, where they can carry out their function. However, since flow cytometric and surface labeling experiments (LC and DG, unpublished) have been unable to reproducibly observe such chains above the background level, we estimate that putative surface MIR proteins would have to represent <5% of the total intracellular pool.

Our analysis of deletion mutants and chimeras supports the notion that the MIR2 protein is comprised of multiple domains, with the TM regions determining target selection and the cytosolic regions mediating other function(s) that are common to the endocytosis of all target proteins. Our experiments do not suffice to define the nature of these cytosolic function(s), but indicate that, whatever they may be, both cytosolic domains are required for their action. Deletion of cytosolic regions not only inactivates MIR2 function but, consistent with the aforementioned domain organization, creates

dominant negative mutants. Since only the TM and intermembranous regions are required for oligomer formation, such phenotypes might result from the creation of mixed, nonfunctional oligomers with WT MIR2. Alternatively (or additionally) the mutants might act by titrating away a putative essential cellular cofactor required for either interaction with the substrate or downstream interactions of a putative MIR-substrate complex with the endocytic machinery. We are currently screening for host factors that might interact with these regions, using a variety of genetic and biochemical screens. Given the remarkable selectivity of MIR-regulated endocytosis, delineation of the pathways that are engaged by MIR proteins can be expected to further inform our understanding of the cell biology of regulated endocytosis.

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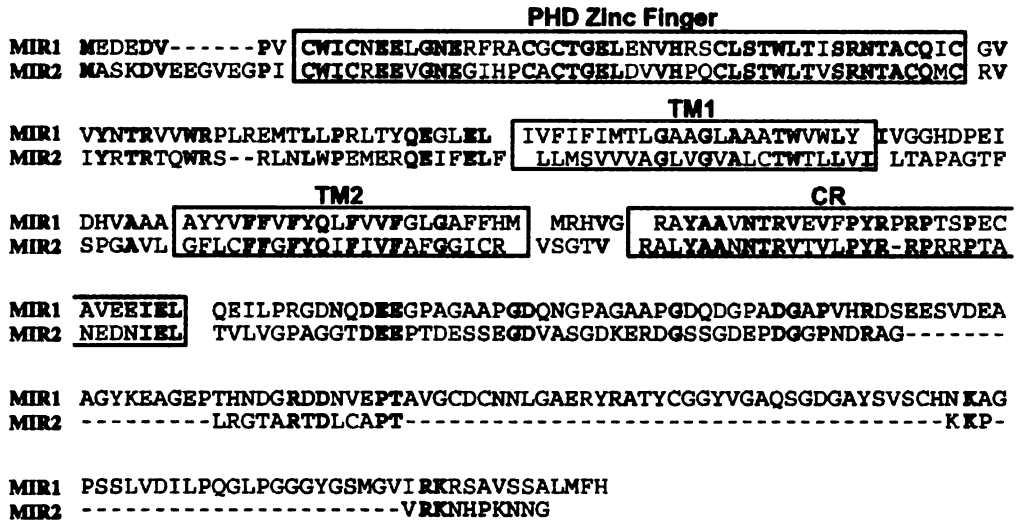
Fig. 1. Orientation of MIR2 in the ER membrane. (A) MIR1 and MIR2 amino acid seqeunces are aligned, with the PHD zinc finger, transmembrane domains 1 (TM1) and 2 (TM2), and the conserved region (CR) boxed. CV-1 cells were stably transduced with retrovirus vectors expressing Flag-MIR2-HA (shown in B). The plasma membrane was selectively permeabilized with 5 mg/ml digitonin (C and D), which allows access of the antibody only to the cytosolic compartment, or the cells were completely permeabilized with 0.25% Saponin (E and F). All samples were then coincubated with mouse anti-Flag and rabbit anti-HA, washed and coincubated with donkey anti-mouse IgG FITC conjugate and donkey anti-rabbit Texas Red conjugate. The proposed orientation of MIR2 within the ER membrane is shown in G.

A


PHD Zinc Finger
TM1
TM2
CR
MIR1 MEDEDV------PV CWICNEELGNERFRACGCTGELENVHRSCLSTWLTISRNTACQIC GV
MIR2 MASKDVEEGVEGPI CWICREEVGNEGIHPCACTGELDVVHPQCLSTWLTVSRNTACQMC RV
MIR1 VYNTRVVWRPLREMTLLPRLTYQEGLEL IVFIFIMTLGAAGLAAATWVWLY IVGGHDPEI
MIR2 IYRTRTQWRS--RLNLWPEMERQEIFELF LLMSVVVAGLVGVALCTWTLLVI LTAPAGTF
MIR1 DHVAAA AYYVFFVFYQLFVVFGLGAFFHM MRHVG RAYAAVNTRVEVFPYRPRPTSPEC
MIR2 SPGAVL GFLCFFGFYQIFIVFAFGGICR VSGTV RALYAANNTRVTVLPYR-RPRRPTA
MIR1 AVEEIEL QEILPRGDNQDEEGPAGAAPGDQNGPAGAAPGDQDGPADGAPVHRDSEESVDEA
MIR2 NEDNIEL TVLVGPAGGTDEEPTDESSEGDVASGDKERDGSSGDEPDGGPNDRAG-------
MIR1 AGYKEAGEPTHNDGRDDNVEPTAVGCDCNNLGAERYRATYCGGYVGAQSGDGAYSVSCHNKAG
MIR2 ---------LRGTARTDLCAPT-----------------------------------KKP-
MIR1 PSSLVDILPQGLPGGGYGSMGVIRKRSAVSSALMFH
MIR2 ----------------------VRKNHPKNNG

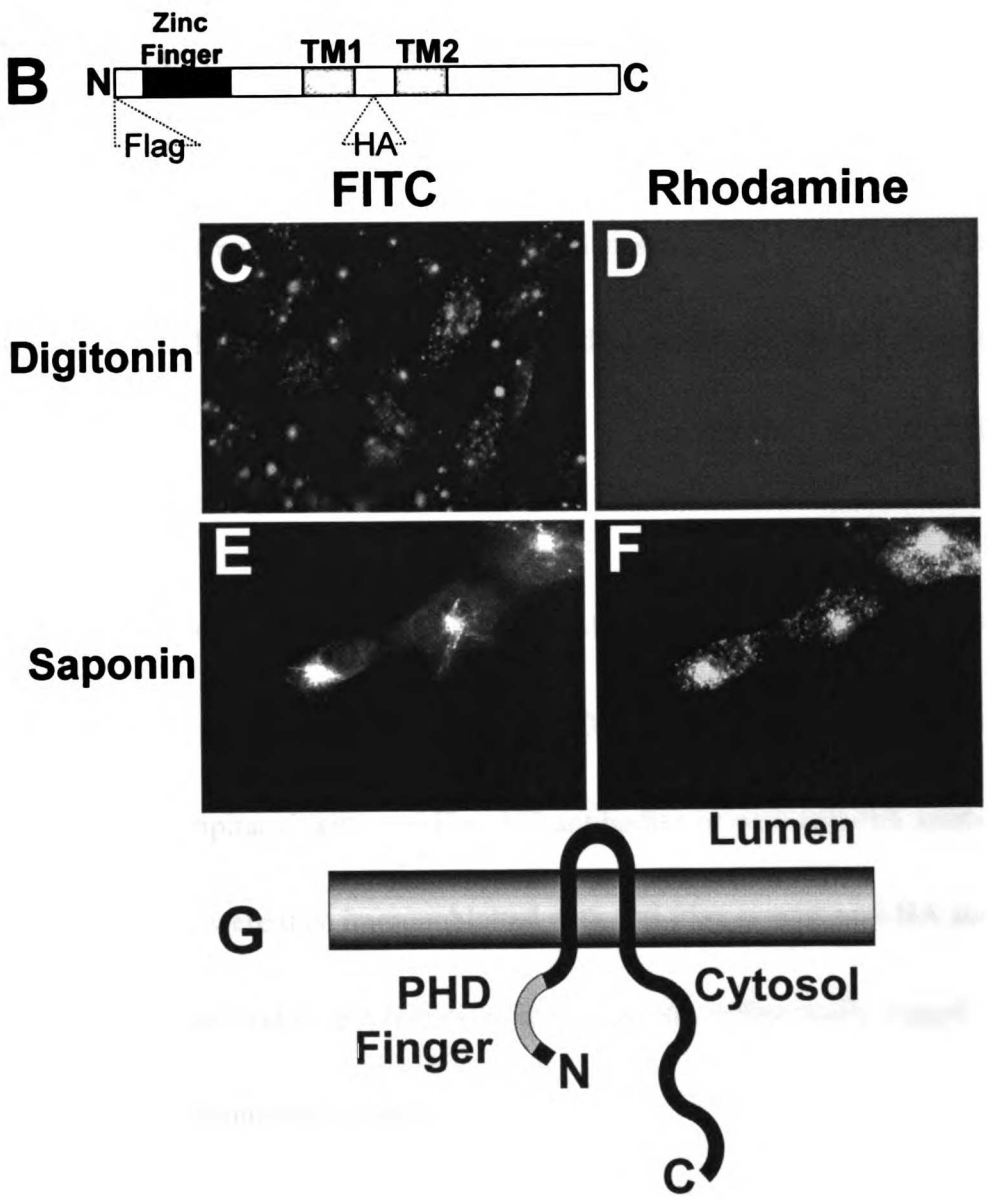
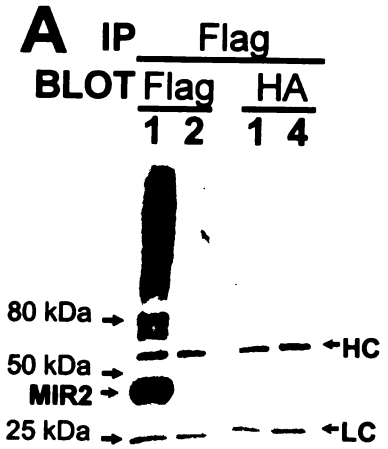

B
Zinc
Finger
TM1
TM2
N
C
Flag
HA
FITC
Rhodamine
C
D
Digitonin
E
F
Saponin
G
Lumen
PHD
Finger
N
Cytosol
C

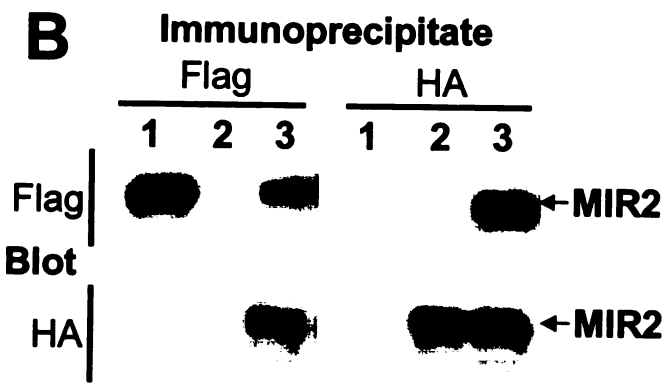
Fig. 2. MIR2 Forms Oligomers. (A) 293T cells were transfected with expression vectors encoding Flag-tagged MIR2 (lanes 1 and 3) or HA-tagged MIR2 (lanes 2, 4, and 5). After lysate preparation, extracts were precipitated with either anti-Flag (lanes 1-4) or anti-HA (lane 5). Immune precipitates were separated by SDS_PAGE, transferred to membranes and immunoblotted with anti-Flag (lanes 1 and 3) or anti-HA (lanes 1, 4 and 5) murine mAbs. Reactive bands were identified by reaction with HRP- conjugated goat anti-mouse Ig antibodies. HC and LC denote murine antibody heavy chain and light chain respectively.

(B) 293T cells were transfected with pCR3.1-Flag-MIR2 (lane 1), pCR3.1-HA-MIR2 (lane 2), or both pCR3.1-Flag-MIR2 and pCR3.1-HA-MIR2 (lane 3). Protein extracts were immunoprecipitated with anti-Flag M2 antibodies or with anti-HA antibodies. The separated proteins were then immunoblotted with anti-Flag or with anti-HA and then with HRP conjugated secondary antibodies to show that the differentially tagged versions of MIR2 could coimmunoprecipitate.

(C) A truncated form of MIR2 (MIR2ΔNΔC-EGFP) with only the transmembrane domains (1and 2) and amino acids into the CR region through the conserved PYR residues (see Fig.1) was fused to EGFP and ligated into the pCR3.1 expression vector.

(D) 293T cells were transfected with pCR3.1 and pCR3.1- MIR2ΔNΔC-EGFP (lane 1), or both pCR3.1-Flag-MIR2 and pCR3.1- MIR2ΔNΔC-EGFP (lane 2). Protein extracts were immunoprecipitated with anti-GFP antibodies (left panel) or with anti-Flag antibodies (right panel) and visualized by incubation with a secondary mouse anti-. The separated proteins were then immunoblotted with anti-GFP and then HRP conjugated goat anti-mouse Ig antibodies to show that the MIR2ΔNΔC-EGFP construct could coimmunoprecipitate with Flag-MIR2.


A
IP Flag
BLOT Flag HA
1 2 1 4
80 kDa
50 kDa
MIR2
25 kDa
HC
LC


B
Immunoprecipitate
Flag HA
1 2 3 1 2 3
Flag
Blot
HA
MIR2
MIR2

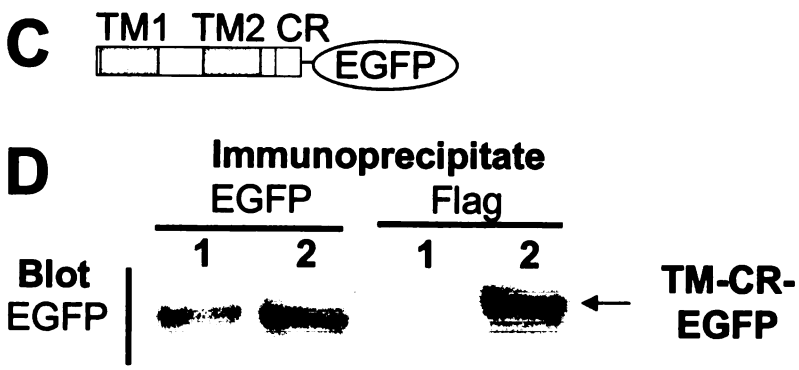

C
TM1 TM2 CR
EGFP
D
Immunoprecipitate
EGFP
Flag
1
2
1
2
Blot
EGFP
TM-CR-
EGFP

Fig. 3. Regions of MIR2 required for function. (A) Schematic depiction of MIR2 deletion mutants. These constructs were fused to EGFP (or tagged with FLAG in the case of MIR2ΔTMΔC)and ligated into the pCR3.1 eukaryotic expression vector for the studies of Fig. 3B.

(B-G)BJAB cells were transiently transfected with the indicated constructs from Fig. 3*A*. After 36 hours the cells were stained with mouse anti-HLA Class I conjugated to APC and mouse anti-B7-2 conjugated to phycoerythrin and analyzed by flow cytometry, gating on those cells that were transfected (either EGFP positive or FLAG positive cells), using a Becton Dickinson FACSCalibur.

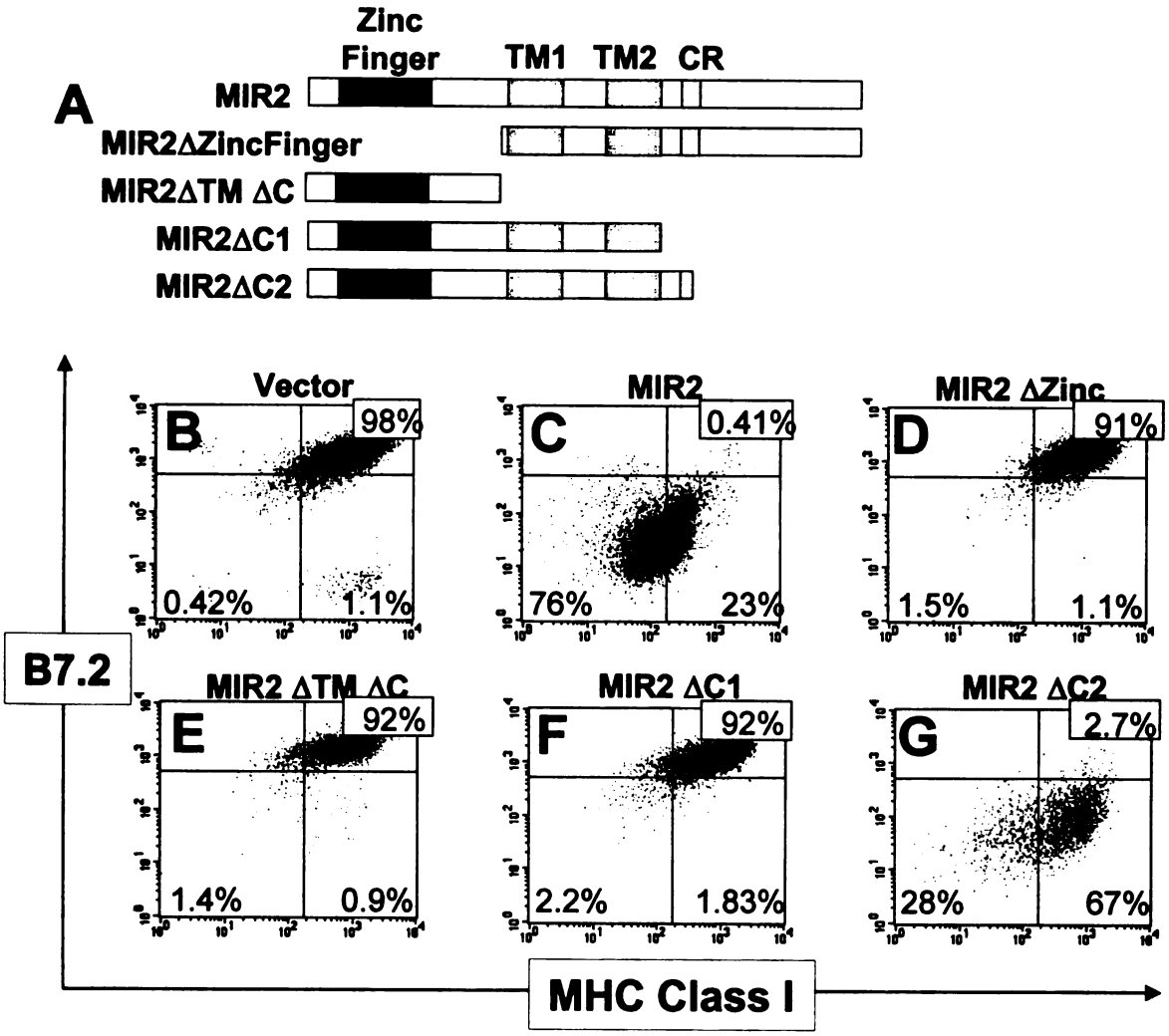

A
Zinc Finger
TM1
TM2
CR
MIR2
MIR2ΔZincFinger
MIR2ΔTM ΔC
MIR2ΔC1
MIR2ΔC2
B7.2
MHC Class I
Vector
B
98%
0.42%
1.1%
MIR2
C
0.41%
76%
23%
MIR2 ΔZinc
D
91%
1.5%
1.1%
MIR2 ΔTM ΔC
E
92%
1.4%
0.9%
MIR2 ΔC1
F
92%
2.2%
1.83%
MIR2 ΔC2
G
2.7%
28%
67%

Fig. 4. Transmembrane regions of MIR2 mediated B7-2 downregulation.

(A) Schematic depiction of MIR1/MIR2 chimeras. These constructs were ligated into pCR3.1.

(B) BJAB cells were transiently transfected with the indicated constructs from *A*. After 36 hours the cells were stained with mouse anti-HLA Class I conjugated to FITC and mouse anti-B7-2 conjugated to phycoerythrin and the total viable cell population analyzed by flow cytometry using a Becton Dickinson FACSCalibur.

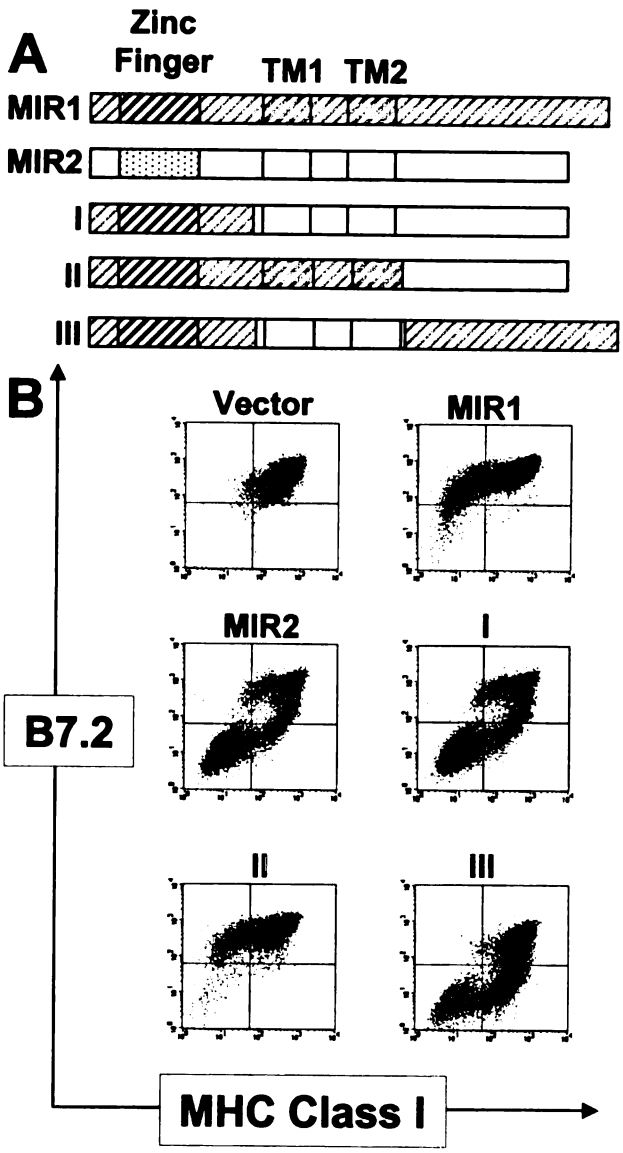

A
Zinc
Finger
TM1
TM2
MIR1
MIR2
I
II
III
B
Vector
MIR1
MIR2
I
II
III
B7.2
MHC Class I

Fig. 5. An N-terminal truncated form of MIR2 can act as a dominant negative to block MIR2 downregulation of HLA Class I from the cell surface.

293T cells were transfected with either pCR3.1, pBMN-MIR2-GFP and pCR3.1 (A) or pBMN-MIR2-EGFP and pCR3.1-MIR2 ΔPHD (B). After 36 hours, cells were stained with mouse anti-HLA Class I conjugated to APC and analyzed by flow cytometry using a Becton Dickinson FACSCalibur. GFP positive cells were gated and the levels of HLA Class I on the cell surface was measured. (C) MIR2ΔPHD does not affect MHC-I downregulation by HSV ICP 47.

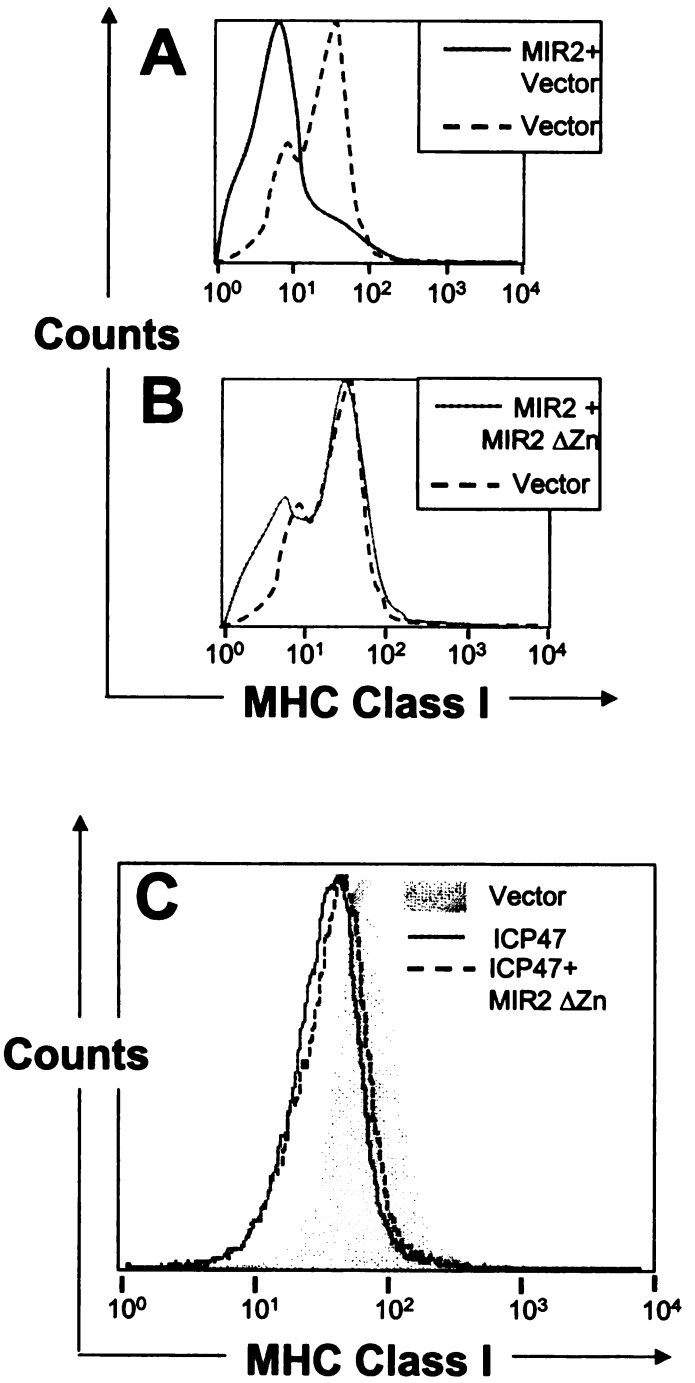
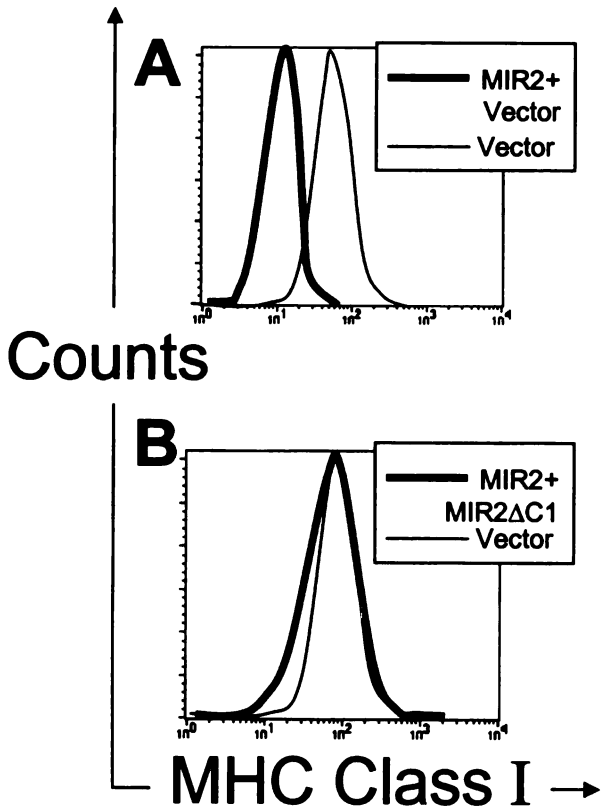

A
MIR2+
Vector
Vector
10^0 10^1 10^2 10^3 10^4
Counts
B
MIR2 +
MIR2 ΔZn
Vector
10^0 10^1 10^2 10^3 10^4
MHC Class I
C
Vector
ICP47
ICP47+
MIR2 ΔZn
Counts
10^0 10^1 10^2 10^3 10^4
MHC Class I

Fig. 6 A C-terminal truncation of MIR2 has a dominant negative phenotype

293T cells were transfected with either pCR3.1 (4X), pCR3.1-MIR2 (Flag tagged) (1X) and (A) or pCR3.1-MIR2 (Flag tagged) (1X) and pCR3.1-MIR2 ΔC1 (4X) (B). After 36 hours, cells were stained with mouse anti-HLA Class I conjugated to APC and analyzed by flow cytometry using a Becton Dickinson FACSCalibur. Flag positive cells were gated and the levels of HLA Class I on the cell surface was measured.


A
MIR2+
Vector
Vector
Counts
B
MIR2+
MIR2ΔC1
Vector
MHC Class I →

CHAPTER 3

The Kaposi's Sarcoma Associated Herpesvirus Modulators of Immune Recognition (MIR) Proteins Influence Innate Immunity by Downregulating CD1d

Summary

Little is known about the role of CD1d-restricted T cells in anti-viral immune responses. Here we show that the lytic replication cycle of the Kaposi's sarcoma-associated herpevirus (KSHV) promotes downregulation of cell surface CD1d. This is attributable to expression of the two MIR (modulator of immune recognition) proteins of the virus, each of which promotes the loss of surface CD1d expression following transfection into uninfected cells. Inhibition of CD1d surface display is due to ubiquitination of the chains on a unique lysine residue in their cyoplasmic tail, which triggers endocytosis. Unlike MIR-mediated MHC-I downregulation, however, internalized CD1d does not appear to undergo accelerated lysosomal degradation. MIR2-induced down-regulation of CD1d resulted in reduced activation of CD1d-restricted T cells *in vitro*. KSHV modulation of CD1d expression represents a strategy for viral evasion of innate host immune responses that implicates CD1d-restricted T cells in control of this viral infection.

Introduction

Recognition of a viral infection by the innate immune system is critical to both the efficient control of the earliest steps in the viral infection and production of cytokine signals for activation of the adaptive component of an immune response. In most infections, (bacterial as well as viral) an innate response is normally activated in the hours immediately following infection and is cued by the production of generic, infection-induced signals (e.g. dsRNA) and pathogen-induced host cell signaling (e.g. via toll-like receptors) [1]. Virus-infected cells release type I interferons that can render surrounding cells less susceptible to viral infection and replication. Many cells of the innate immune system participate in the early response, most notably NK cells, which can recognize and kill infected cells and release abundant quantities of antiviral cytokines [2]. Additionally, T cells that are restricted by CD1 molecules – a family of antigen presenting molecules distantly related to class I and class I molecules of the MHC – probably participate in early host responses (Gumperz and Brenner 2001).

Unlike MHC-encoded antigen presenting molecules that present peptides, CD1 molecules have been found to present lipids and glycolipids as antigens to T cells (Porcelli and Modlin 1999). Certain CD1 isoforms (i.e. CD1a, b, and c) have been shown to present pathogen-specific glycolipids [3] [4-6]. In contrast, it remains unclear whether CD1d molecules present foreign antigens, but this isoform has been shown to present self-glycolipids to T cells [7, 8]. CD1d chains are nonpolymorphic and are expressed only on a selected number of cell types, including B cells, dendritic cells, hepatoctyes and enterocytes [9]. CD1d-restricted T cells appear evolutionarily conserved, and comprise a population of T cells (known as "NKT" cells) that express an invariantly

rearranged TCRα chain, as well as other T cells that utilize diversely rearranged TCRs [9] [10]. The physiological ligands of CD1d-restricted T cells are unknown, but cells of the semi-invariant TCR subset ("iNKT" cells) are strongly activated by a synthetic glycolipid called α-galactosylceramide (α-GalCer), which was originally derived from a marine sponge. This lipid has often been used as a surrogate antigen in experimental studies of iNKT cells, and results in potent release of IFN-γ and IL-4 [11]. CD1d-restricted T cells that use diversely rearranged TCRs do not appear to respond to α-GalCer, and have been less well studied than their iNKT cell counterparts.

Recently, several lines of work have begun to suggest possible roles for CD1-restricted T cells in the response to viral infection. For example, nonclassical (i.e. α-GalCer-nonreactive) NKT cells are involved in the development of acute hepatitis in a mouse model of Hepatitis B viral (HBV) infection [12]. In fact, expression of HBV genes in mouse liver is associated with upregulation of CD1d expression in hepatocytes (J Baron and DG, unpublished data), suggesting that CD1 may be one target of an as-yet unknown pathway of pathogen-induced signaling. In addition, models of Respiratory Syncytial Virus (RSV), Herpes Simplex Virus (HSV) and coxsackievirus B3 infection in CD1d-deficient mice have also suggested possible involvement of CD1d in antiviral responses [13]; [14]; [15]. However, viral ligands for CD1d have yet to be discovered, and it has not been obvious how lipid recognition might be tied to viral infection. As a result, the role of CD1-restricted cells in antiviral defense has remained poorly understood.

One way to infer a biological role for a protein in antiviral immunity is to search for viral effects that subvert the function or impair expression of that protein.

Herpesviruses have developed several methods to modulate the immune response directed against them, including proteins that block the TAP peptide transporter, and a variety of proteins that impair MHC-I assembly, transport or stability (Reviewed in [16]; [17]. The large number of redundant inhibitors of MHC-I suggests an important role for CTLs in anti-herpesviral defense. Similarly, many herpesviruses encode proteins that impair type I IFN induction or action [18]; [19]; [20]; [21], and recently CMV has been found to encode proteins that block NK cell-activating receptors [22].

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is a B-lymphotropic herpesvirus that is the causative agent of at least two lymphoproliferative diseases (primary effusion lymphoma and multicentric Castleman's disease) as well as Kaposi's Sarcoma, a tumor of endothelial origin (Reviewed in [23] [24] [25]. Like other herpesvirus family members, it encodes proteins that block MHC-I display on the cell surface[26]; [27]; [28] [29]. These are known as MIR proteins (for Modulators of Immune Recognition); MIR1 and MIR2 are expressed during lytic replication and are encoded by the open reading frames K3 and K5, respectively. They function as membrane bound ubiquitin ligases, leading to ubiquitination of their target proteins on lysine residues in their cytosolic tails [30]. The result of this ubiquitination is to enhance the endocytosis of the target chain and its delivery (via the multivesicular body) to the lysosome, where it is proteolytically destroyed. Both MIR1 and MIR2 reduce the cell surface levels of MHC Class I; MIR2 also selectively targets the costimulatory molecule B7.2 and the Intracellular Adhesion Molecule-1 (ICAM-1) both of which are involved in the enhancement of helper T cell signaling [31]; [32]. From these activities it can be inferred that both CTL and CD4 T cell action are likely to play important roles in host

defense against KSHV. Here we show that lytic KSHV infection also diminishes the surface display of CD1d, and that both MIR1 and MIR2 can carry out this downregulation. The downregulation is sufficient to impair NKT cell activation. The existence of a pathway for viral evasion of CD1d-restricted T cell responses suggests a potentially important role for these T cells in the control of herpesviral infection.

Results

KSHV Lytic Replication Decreases CD1d Levels

To investigate how KSHV infection affects CD1d expression, we analyzed CD1d cell surface expression on BCBL-1 lymphoma cells, a cell line that is latently infected by KSHV, and normally expresses only a small subset of viral genes. Flow cytometric analysis of these cells showed that in their uninduced state they express high levels of CD1d on their surface (Figure 1A). By treatment with phorbol esters, BCBL cells can be induced to enter the lytic replication cycle, whereupon a temporally regulated cascade of transcription occurs, ultimately resulting in expression of the vast majority of viral genes and the production of infectious viral progeny. However, induction is generally inefficient, with only 5-20% of the cells displaying markers of lytic replication. Therefore, in this study, entry into the lytic cycle was monitored by the expression of the virus-encoded cell surface glycoprotein K8.1 [33]. As shown in Figure 1B, by 4 days post lytic induction, flow cytometric analysis showed that the levels of CD1d were approximately ten times lower in K8.1 positive cells than in the K8.1 negative cells that escaped induction (Figure 1B, top panel). This loss of surface CD1 was selective: K8.1 positive cells retained normal surface levels of the control transferrin receptor protein. (Figure 1B, lower panel). Thus, lytically infected cells showed reduced CD1d cell

surface expression, and the loss of CD1d was not due to nonspecific toxicity resulting from the progression of lytic KSHV infection.

MIR1 and MIR2 induce CD1d Downregulation

Because of the known ability of MIR1 and 2 to modulate immunologically relevant host cell surface proteins (e.g. MHC I and B7.2), we investigated the effects of these viral proteins on CD1d expression. For these experiments, we employed fusion proteins in which MIR1 and MIR2 were fused to EGFP (Enhanced Green Fluorescent Proteins). We have earlier shown that these chimeras are stably expressed and fully functional for downregulation of MHC-I chains upon either transient or stable transfection into cells [34]. Accordingly, each chimera was transfected into BJAB cells, which express low-moderate levels of CD1d on their surface. By gating on EGFP-positive cells we could identify cells expressing MIR, and the levels of CD1 in these cells was quantitated by flow cytometry. Fig 2A (lower panel) confirms that both MIR-EGFP fusions were able to down-regulate MHC class I expression in these cells. As shown in Figure 2A (upper panel), both MIR1 and MIR2 transfected cells showed a marked decrease in the levels of CD1d on the cell surface, down to a level comparable with nonstained BJAB cells. Notably, there was no significant change in the level of CD1c expression (data not shown).

The modest resting level of CD1d on BJAB cells limits the dynamic range of the CD1d reduction assay and may lead to underestimation of the magnitude of the inhibition. Therefore, HepG2 hepatoblastoma cells, which have high levels of endogenous CD1d on their surface (Figure 2B) were selected for further study. HepG2

cells were transduced with a retrovirus encoding a functional, Flag-tagged MIR2 and a neomycin resistance marker and neo-resistant HepG2 cells were selected as a bulk population; as a control, a similar population was selected following transduction with a lacZ/neo retrovirus. Each cell population was then analyzed by flow cytometry. As shown in Figure 2B, left panel, the levels of CD1d were reduced up to 100-fold in the MIR2 stable cells as compared to control HepG2 cells that stably express lacZ. This is an even greater reduction than is observed on MHC-I chains in the same populations of cells (Fig 2B, right panel). Thus, the effect of MIR2 on CD1d appears very robust. Attempts to generate stable transductants of either HepG2 or BJAB with MIR1 were unsuccessful, as the resulting transductants rapidly lost their initially high level of expression of MIR1. Nonetheless, transient expression of MIR1-GFP indicates that it possesses substantial ability to downregulate CD1d (Fig. 2A). Thus, CD1d regulation, like MHC-I but unlike B7.2 and ICAM-1 downregulation, has been conserved in both MIR family members. As a control, the levels of HLA-DR as shown to be not affected by either MIR1 or MIR2. MHC Class II is an important trafficking partner of CD1d and it is important to note that the levels do not decrease. Also, Fas, a non involved protein is not affected by MIR expression. Of note is the levels of CD1c which are not perturbed by expression of MIR1 but are downregulated by MIR2.

MIR2 downregulation of CD1d can lower CD1d restricted T cell activation

The effect of MIR-mediated downregulation of CD1d on the activation of CD1d restricted T cells was investigated in an *in vitro* co-culture system. In this system, BJAB cells that stably expressed either lacZ or MIR2 were incubated with CD1d-restricted

NKT cell clones in the presence of α-GalCer, and interferon-gamma release was measured by ELISA as described (Brigl et al. 2003). As shown in Figure 3A, CD1d-restricted T cell clones incubated with MIR2-expressing BJABs showed reduced IFNγ production compared to clones incubated with the lacZ-expressing BJAB cells. This effect appeared statistically significant and was consistent among three different CD1d-restricted T cells clones. Next, IFN-γ release induced by the MIR2 and lacZ transfected BJAB cells was monitored in the presence and absence of α-GalCer. As expected, IFNγ production by the T cell clones was greatly increased in the presence of α-GalCer compared to that in its absence (Fig 3B). Notably, whereas MIR2 expression led to a marked reduction in IFNγ secreted in response to α-GalCer, it had no detectable effect on the modest amount of IFNγ produced by the T cells in the absence of α-GalCer (Figure 3B). [We also examined the effects of anti-CD1d antibody on activation. As shown in Fig 3C, anti-CD1d reduced activation by control BJAB/LacZ cells to an extent similar to that produced by MIR2 expression. Addition of anti-CD1d to BJAB/MIR2 cells in the assay further reduced the level of activation seen in this context, indicating the presence of a low level of residual CD1d on the surface of these cells. Thus, MIR2 expression sharply reduces but does not entirely eliminate residual CD1d-dependent signaling.

MIR2 induced ubiquitination and internalization of CD1d and/or CD1d- associated proteins

In earlier studies of MIR-mediated downregulation of MHC-I and B7.2, we [30] and others [35] showed that downregulation of the target proteins was dependent upon the ubiquitination of cytosolic lysine residues in the target. Ubiquitination triggers

internalization of these chains and directs the nascent endosome into the multivesicular body (MVB) pathway [36], where its contents are sorted to lysosomes and degraded by lysosomal proteases [30]. Although the steps involved in this sorting pathwayare not fully understood, it is known that ubiquitin also plays prominent roles in these processes. For example, many components of the MVB machinery are either ubiquitinated or harbor Ub-binding motifs (Reviewed in [37]).

Given the importance of ubiquitination in MIR activity we asked if CD1d or chains associated with it undergo ubiquitination in a MIR-dependent fashion. HepG2 cells stably transduced with either lacZ or MIR2 were lysed in detergent; CD1d was immunoprecipitated, separated by SDS-PAGE, transferred to solid supports and immunoblotted with anti-ubiquitin conjugated to HRP. As shown in Figure 4, immunoprecipitation with CD1d induces a strong and heterogeneous ubiquitination signal only in MIR2-expressing cells and not in the lacZ-expressing control cells. As human CD1d has been shown to form stable complexes with other proteins (i.e. MHC class II) [38], the ubiquitinated bands in Figure 4 may represent polyubiquitinated CD1 chains, ubiquitination of multiple associated proteins, or both.

MIR2 induced downregulation and ubiquitination of CD1d is dependent on intracytoplasmic lysines

The CD1d intracytoplasmic tail contains a single lysine, which may be a target of the MIR ubiquitin ligases (Figure 5A). In previous studies, the removal of lysines from the cytoplasmic tail of potential MIR downregulation targets abolished the ubiquitination of the target proteins and their subsequent downregulation [30]. Interestingly, murine CD1d

has no cytosolic lysines and is not downregulated by MIR2, even when expressed in human CD1d-positive cells (not shown). Therefore, to investigate the role of the sole lysine in the cytoplasmic tail of human CD1d, it was mutated to an arginine (Figure 5A). As previous work has shown that the MIR proteins use the transmembrane regions as a site for target specificity; the TM regions of human CD1d were included in our chimeras [34] [30]. To allow specific detection of the transfected CD1d molecules in human BJAB cells, chimeric CD1d proteins were created that contain the extracellular domains of mouse CD1d, fused to the transmembrane region and cytoplasmic tail of human CD1d (Figure 5B). These chimeras can be specifically detected with an anti-mouse CD1d mAb, which does not cross-react with human CD1d Mouse As shown in Figure 5C, transient cotransfection of an eGFP expression vector and these chimeric CD1d constructs in BJAB cells resulted in equivalent expression levels of the chimeric CD1d containing the wild type cytoplasmic tail and that with the mutated tail (Figure 5C).. When these constructs are cotransfected with an expression vector for the functional MIR2-eGFP fusion protein, however, a markedly different result is obtained. The wild-type chimera is strongly downregulated by MIR2, and this effect is abolished by the K→R mutation (Fig 5C).

To confirm that the role of the lysine was to serve as a ubiquitination substrate, BJAB cells that were stably expressed either lacZ or MIR2 were stably transduced with either the wildtype chimeric CD1d or the K→R mutant chimera. From each of the 4 resulting lines the chimeric CD1d chains were immunoprecipitated with mouse anti-CD1d, and after SDS-PAGE and electroblotting to membranes, the blots were probed with anti-ubiquitin antibody. As expected, no ubiquitinated proteins were detected in the

CD1d precipitate in the absence of MIR2 (Figure 5D). More importantly, no ubiquitinated bands were observed in the MIR2-expressing cells that were transfected with the arginine mutant chimera (Fig 5D). This result suggests that at least one of the bands in the precipitate of the WT chimera in MIR2 cells is the ubiquitinated CD1d itself and that the recruitment of all other ubiquitinated species into this complex is dependent upon ubiquitination of the CD1d chimera.

MIR2 promotes endocytosis of cell-surface CD1d but does not strongly enhance its degradation

The disappearance of cell-surface CD1d could be due to shedding, proteolysis or internalization (endocytosis). To further investigate the mechanism of CD1d loss, we took advantage of the fact that endocytosis can be selectively blocked by expression of dominant negative alleles of the GTPase dynamin, whose activity is required for endosome formation [39]. BJAB cells were transiently transfected with the wild-type mouse-human CD1d chimera and either a vector control or MIR2. In addition, each culture received a vector expressing either a WT or dominant-negative mutant dynamin (these constructs also expressed a GFP gene). Using flow cytometry, we then gated upon GFP–expressing (i.e. transfected) cells and analyzed them for cell surface CD1d. As expected, MIR2 expression in the presence of WT dynamin led to strong downregulation of CD1d (Fig 6A, top panel). However, when the dynamin dominant-negative mutant K44E was expressed, this downregulation was completely abrogated (Fig 6A, center panel). This effect was dependent upon MIR2 expression, as K44E expression had little

impact on surface CD1d in the absence of MIR 2 (Fig 6A, bottom panel). Thus, the disappearance of cell surface CD1d appears to be due to internalization via endocytosis.

MIR-mediated downregulation of MHC Class I induces a rapid degradation of the MHC Class I chains in a lysosome-dependent manner [26]. To examine the half-life of CD1d chains in the presence of the MIR proteins, we carried out a pulse chase experiment to track the metabolism of newly-sythesized CD1d polypeptides. HepG2 cells expressing either lacZ or MIR2 were labeled for 20 minutes with S^{35}-methionine and chased in unlabeled medium for 1, 2 or 12 hours thereafter; then, CD1d chains were immunoprecipitated and examined by SDS-PAGE. (HepG2 cells were used for this experiment because of their more abundant expression of endogenous CD1d). Comparison of the profiles of CD1d in MIR2 and lacZ expressing cells revealed no clear change in the half-life of the protein in the presence of MIR2 (Fig 6B, left panel, rows 1 and 3). Moreover, the addition of chloroquine (C), an inhibitor of lysosomal acidification and proteolysis, during the chase failed to lead to substantial accumulation of labeled CD1d chains (Fig 6B, left, rows 3 and 4), though it did partially rescue the appearance of MHC-I chains under these conditions (Fig 6B, right panel). Thus, in contrast to MHC class I, in the presence of MIR2, CD1d is ubiquitinated and removed from the cell surface, but does not appear to undergo enhanced degradation. Correspondingly, flow cytometric analysis of steady-state intracellular CD1d levels revealed no appreciable change in the presence or absence of MIR2 (Figure 6C).

Discussion

Our finding that cell surface CD1d can be downregulated in lytic KSHV infection and by expression of KSHV MIR proteins represents the first report of modulation of the CD1d system by a virus and suggests a role for this system in the control of KSHV infection. We propose that CD1d downregulation may assist KSHV during the early phases of a new infection by impairing the local activation of CD1d-restricted T cells, thereby (i) reducing the direct release of antiviral cytokines (e.g. interferons) into the microenvironment in which viral replication has begun, and (ii) reducing the signals that promote a subsequent adaptive immune response to the virus. This would be analogous to roles we have earlier proposed for MIR2 based on its abrogation of CD4 T cell activation through downregulation of B7.2 [31]. The ability of the MIRs to downregulate both innate and adaptive components of the immune recognition apparatus is testimony to the remarkable ability of viruses to craft highly versatile inhibitors of host immune responses.

KSHV is not the only virus to modulate the innate immune system. Recent studies in cytolomegalovirus (CMV) have focused on the selective modulation of NK cell activating receptors by viral proteins [22] [40]. This is thought to not only impair NK-mediated lysis of infected cells but also to delay NK cell activation of the adaptive response and subsequent polarization of the T-helper response [41]. CD1d restricted T cells or NKT cells may represent a parallel system for the rapid detection of viral infection and the enhancement of adaptive immunity. Blocking activation of CD1d restricted T cells may also modulate the activation of NK cells [42]. If so, KSHV may

achieve indirectly what CMV achieves by direct blockade of NK activating receptors on infected cells

Many questions remain to be answered concerning the role of the CD1d system in the control of a viral infection. Chief among these have been the determination of the ligand presented by CD1d during a viral infection, and how CD1d-restricted T cells become specifically activated. While it is not clear whether the physiological ligands of CD1d-restricted T cells include foreign compounds, many CD1d-restricted T cell clones seem to be autoreactive against lipids of host origin (Chiu et al. J Exp Med 1999, Gumperz et al, 2000). It has recently been shown that this reactivity to self antigens can be exploited in a novel activation strategy that may allow for the rapid triggering of CD1d-restricted T cells in a wide variety of pathogenic infections (Brigl et al. 2003). Under normal circumstances CD1d-restricted T cell recognition of self antigens results in little cytokine secretion; however, in the presence of the cytokines IL-12 and IL-2, which are produced by APCs in response to microbial products, the effect of self antigen recognition is amplified, leading to potent IFNγ production by CD1d restricted T cells. This mechanism appeared responsible for the early activation of CD1d-restricted T cells observed in vivo in a bacterial infection (Brigl et al., 2003), and may be sufficiently generic that it also allows for the activation of CD1d-restricted T cells in viral infections. Alternatively, in viral infections may trigger the production of new classes of self lipids that are part of a stress activated system; these could then be introduced into the CD1d antigen processing pathway. For example, as a result of viral infection normal lipids could become differently glycosylated or different types of lipids could gain access to CD1d-antigen loading sites, leading to the presentation of novel lipids (i.e. "altered self")

on infected B cells. Uninfected APCs might also acquire and present these lipids following endocytosis of cell membranes from lysed host cells or phagocytosis of infected or apoptotic cells. . A third hypothesis is that viruses like KSHV might encode highly hydrophobic proteins, peptides from which might also be presented in the hydrophobic binding pocket of CD1d. Although only fragmentary and controversial evidence links the CD1d system to peptide presentation (Castano et al 1995), direct presentation of viral peptides remains a formal possibility.

Interestingly, whereas we observed a marked effect of MIR2 expression on activation of CD1d-restricted T cells in response to the exogenously added antigen α-GalCer, the modest IFNγ secretion that was obtained in the absence of α-GalCer – presumably the response of the CD1d-restricted T cells to self antigens presented by the APCs –was notsignificantly reduced. Further investigation will be required to determine the cause and significanceof this difference., However we suspect it could be due to a differential effect of the MIR proteins on CD1d loading of endogenously vs. exogenously derived antigens. CD1d molecules traffick extensively through the endocytic system (Sugita M et al. Immunity 2002, Elewaut D et al., J Exp Med 2003, Cernadas M et al., JI 2003) and are capable of presenting both exogenously derived (Prigozy et al Science 2001) and endogenously derived antigens (Chiu et al. J Exp Med 1999, Zhou et al., Science Dec 18 2003). However, it is not clear in precisely which compartments the relevant physiological ligands of CD1d-restricted T cells are loaded, and it is possible that exogenous and endogenous cellular lipids may be loaded at distinct sites. If so, MIR proteins may direct CD1d polypeptides to compartments in which loading of exogenous lipids is inefficient, while still allowing the loading of endogenous ones..

Our results also shed light on how the MIR proteins influence the trafficking of molecules involved in immune recognition. In all cases, the target proteins are ubiquitinated and endocytosed.,. In the case of MHC-I chains, enhanced endocytosis results in chains being delivered via the MVB to lysosomes for degradation. Since the MVB sorting steps also prominently involve ubiquitinated proteins, it has been suggested that MIRs may have additional ubiquitination targets downstream of the endocytic step. However, in the case of CD1d, we have found little evidence for enhanced lysosomal delivery of the protein, despite its efficient endocytosis under the influence of the MIR proteins. This decoupling of the ubiquitin mediated endocytosis and degradation by the MIR proteins favors the notion that the primary MIR-regulated step is endocytosis. Perhaps the difference between CD1d and MHC-I reflects the different pathways normally taken by these proteins. Murine CD1d has a long half-life and is known to be actively recycled from internal vesicles to the plasma membrane; indeed, at steady state much of the cellular complement of the protein resides in internal vesicles ([43]). This suggests that lysosomal degradation plays at best a modest role in the turnover of CD1d polypeptides. Thus, in the case of both CD1 and MHC, the simplest explanation for MIR action is that it directly targets the endocytic step. Where the protein is naturally sorted to lysosomes (e.g. MHC) this results in enhanced turnover; where not (e.g. CD1) the result is enhanced internalization. By carefully analyzing the complement of ubiquitinated host proteins that complex with MHC-I and CD1d in the presence of MIR2 we hope to be able to dissect the biochemical details of the downregulatory mechanism, and in so doing to shed further light on the trafficking of these important mediators of immune recognition.

Experimental Procedures

Cell Culture

BCBL-1, an established KSHV infected human B-cell line, that is free of EBV infection was grown in RPMI with glutamine, sodium bicarbonate and β-mercaptoethanol, HepG2, a hepatocellular carcinoma cell line was grown in Minimal Essential Medium. Phoenix cells, a 293T based retroviral packaging cell line was grown in Dulbecco's modified Eagle's medium (DMEM), and the BJAB B cell lymphoma cell line was grown in RPMI medium 1640. All media were additionally supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin. BJAB cells were transfected by electroporation (250 V, 950 microfarads) of 20 μg of plasmid DNA into 10^7 cells in 0.5 ml of serum-free medium. The cells were then transferred to complete RPMI, which was preincubated to 37 °C. The transfection efficiency of BJAB cells was routinely 30-50% under these conditions [34].

Stable BJAB and HepG2 cells expressing MIR proteins were created by retroviral transduction. Upon transfection with the retroviral vectors (pBMN or pBMP, containing either neomycin resistance or puromycin resistance), the Phoenix packaging cell line produces replication-defective viral particles that were used to stably transduce recipient HepG2 or BJAB cells. Transient transfections of Phoenix cells were performed using Fugene6 (Roche Molecular Biochemicals) according to the manufacturer's suggested protocol. Phoenix cells were transfected, media was changed 24 hours post-transfection and the virus-containing supernatant was harvested 48 h after the transfection, filtered through a 0.45-μm syringe filter, and diluted with Polybrene (4 μg/ml final dilution). HepG2 or BJAB cells (in six-well dishes) were infected by spin infection (800 X *g* for

2 h at 22 °C) using 2 ml of viral supernatant per well of a six well dish. Selection of transduced HepG2 or BJAB was started 36 h after infection by adding 1.5 mg/ml or 0.8 mg/ml of G418 for BJAB or HepG2 respectively or 1μg/ml of puromycin for BJAB cells. All cell lines were selected and used as pooled populations.

Derivation of CD1d-restricted T cell clones

CD1d-restricted T cell clones were derived by staining human PBMC with α-GalCer loaded human CD1d tetramers as described [44]. Positively stained T cells were single cell sorted by flow cytometry and cultured in the presence of irradiated allogeneic human PBMC in RPMI culture medium containing 10% heat inactivated fetal bovine serum, 1% human AB serum, penicillin and streptomycin, L-glutamine, PHA, and 2nM IL-2. Cloned T cell lines were expanded in culture using the same medium but lacking PHA, and were re-stimulated periodically by exposure to irradiated allogeneic PBMC in the presence of PHA.

Coculture and Interferon Release Assays

Cocultures were designed with 10^4 target cells and recently stimulated CD1d restricted T cells in target to effector ratios of 1:1 or 1:2. These cells were cocultured in 96 well dishes for 18 hours at 37 degrees after which the supernatants were removed and the levels of interferon gamma were analyzed. DMSO dissolved α-Galactoceramide (α-GalCer) was kept at 4 degrees at a concentration of 100 μg/ml. It was sonicated at 37 degrees in a water bath sonicator before used at a concentration of 50ng/ml. Quantitation of IFN-γ production was determined by averaging triplicate wells and using linear

regression from a standard curve and determined by a standard ELISA using a BD Biosciences OptEIA™ Set.

Construction of Chimeric CD1d Expression Vectors

Mouse CD1d (mCD1d) was PCR amplified from ATTC clone Mouse 948632 VK35G05.R' using the forward primer 5'-GTACTGGATCCGCCGCCACCATGCGGTACCTACCATGGCTGTT – 3' and the reverse primer 5'-TGCATGCGGCCGCTCACCGGATGTCTTGATAAGCGCTTCTCCTTCTCCAGATA TAGTAGACTACAGCACCC – 3' using PfuUltra (Stratagene) per the manufacturer's instructions. The fragment was cloned into the BamHI and NotI sites of pCR3.1 (Clontech). Human CD1d was PCR amplified from the Mammalian Gene Collection MGC:34622; IMAGE:5229534 clone using forward primer 5' – ATCATGGATCCGCCGCCACCATGGGGGTGCCTGCTGTTTCTGCTG – 3' and for the reverse primer, either 5' – TATATGCGGCCGCTCACAGGACGCCCTGATAGGAAGTT – 3' for the wildtype cytoplasmic tail or 5' – TATATGCGGCCGCCACAGGACGCCCTGATAGGAAGTTTGCCTGCGAAACCGG GAGGTAAAGCCCACAATGAGG – 3' for a lysine to arginine mutation. All products were recovered and cloned into the BamHI and NotI sites of pCR3.1 (Clontech). Chimeric CD1d was generated by creating fragments of the extracellular domain of mouse CD1d with the mCD1d forward primer and the reverse primer 5'-

CAGGACTGCCAAGGCAATCAAGCCCATGGGTGCTTGCCTGGCATCCC - 3' while the transmembrane and cytoplasmic tail of human CD1d was generated by the reverse primers for human CD1d (either wild-type of mutant) and the forward primer 5' – GGGATGCCAGGCAAGCACCCATGGGCTTGATTGCCTTGGCAGTCCTG – 3' with PfuUltra according to the manufacturer's directions. The fragments were gel purified, mixed in single PCR reaction with the forward primer for full length mouse CD1d and the reverse primer for the full length human CD1d (either wildtype or mutant) using Vent DNA Polymerase (New England Biolabs) according the manufacturer's directions. The chimeric fragments were recovered, and cloned into the BamHI and NotI sites in pCR3.1 for expression studies or subcloned into pMX-PIE (a gift from M. Lodoen and L. L. Lanier) for creation of retroviruses for stable transduction.

Flow Cytometry

BCBL-1 cells were fixed in 4% paraformaldehyde and washed 3 times with PBS. The cells were then blocked with a wash of PBS + 1% BSA. BCBL-1 cells were then incubated with rabbit anti-K8.1 in 100µl of PBS + 1% BSA for 45 minutes on ice, washed with PBS + 1% BSA, incubated with mouse anti-rabbit conjugated to FITC (Sigma) in 100µl, and washed with PBS + 1% BSA. The cells were then further incubated with a mixture of phycoerythrin conjugated mouse anti-transferrin receptor (Caltag) and Zenon-one - APC (Molecular Probes) labeled mouse anti-CD1d (Calbiochem) in 100µl of PBS + 1% BSA. After this last incubation, the cells were washed extensively with PBS and resuspended in 200µl of PBS for analysis by a FACScaliber (Becton Dickinson).

For BJAB and HepG2 experiments, cells were washed once with PBS + 1% BSA, and incubated without fixation with the appropriate antibody on ice. CD1d was analyzed by using CD1d antibody (Calbiochem) that was Zenon-one – APC (Molecular Probes) labeled. Extracellular mouse CD1d was analyzed by rat developed anti-mouse CD1d antibodies (BD Biosciences, Pharmingen) (1:100 dilution) with a goat derived anti-rat secondary antibody couple with APC (Caltag) (1:100 dilution). In addition, mouse anti-Human Class I (W6/32) pre-coupled to PE (Caltag) was used at a dilution of 1:100 in PBS + 1% BSA in these experiments. After incubation and extensive washings in PBS, cells were analyzed by flow cytometry with a FACScaliber.

Coimmunoprecipitation and Western Blotting

A confluent 10-cm dish of stable HepG2 cells or 5 X 10^6 BJAB cells were washed 3 times with ice cold PBS, and 1ml of ice cold PBS + 1% Igepal (Sigma, NP-40 type detergent) was added to the dish or tube. The cells were suspended in the lysis buffer and passed twice through a 21-gauge syringe and the lysate was cleared by spinning in a microcentrifuge for 10 minutes at maximum speed at 4 degrees. The lysates were incubated with 2μg of mouse anti-CD1d antibody ([45], a gnerous gift of Steven Porcelli) which recognizes the β-2 complexed form of CD1d or rat-anti mouse CD1d (BD Biosciences, Pharmingen). After one hour of mixing at 4 degrees, 20μl of protein A/G + agarose beads (for the Mouse antibody) or protein G beads (for the rat antibody) (Santa Cruz Biotech) were then added to mix with the lysates overnight at 4 degrees. The immunoprecipitation beads were then washed 4 times with cold PBS and then

resuspended in SDS loading buffer. The immunoprecipitates were then separated by SDS-PAGE (4-20% Tris-HCl gradient gel, Bio-Rad) and the proteins were transferred to nitrocellulose (Immobolin-P). After blocking overnight in TBS+Tween20+5% milk, the membrane was incubated with mouse-anti-ubiquitin (N-19) antibody (Santa Cruz Biotech) that is preconjugated to HRP for one hour. After extensive washings (3 times for 15 minutes and 3 times for 5 minutes) with TBS + Tween20 + 5% milk, the antibodies were revealed with Luminol (Santa Cruz Biotech).

Dynamin Dominant Negative Experiments

BJAB cells were transfected with 24μg of DNA in a 3:2:1 ratio consisting of pCR3.1 expressing the wildtype chimeric CD1d (Figure 5D), either empty of MIR expressing pCR3.1, and pIRES2-EGFP with either wildtype or a dominant negative allele (K44E) of dynamin [39]. The wildtype and K44E dynamin constructs were a kind gift of R. Vallee. After 40 hours, the cells were stained for the surface levels of chimeric CD1d was measured by flow cytometry. The GFP expressing cells were gated upon and the levels of chimeric CD1d were analyzed.

Pulse Chase Analysis

HepG2 (6 cm dish per time point) stable for the expression of MIR2 (or lacZ as a control) were used. Cell were preincubated with cysteine/methionine-free DMEM (Sigma) (for the HepG2) supplemented with 10% (vol/vol) FCS and penicillin-streptomycin for 1 h at 37°C. The cells were labeled for 20 min with 400 μCi of Trans S^{35}-Label (ICN) in a final volume of 2 ml. After washes, cells were chased for the indicated time in 50 ml of

complete MEM medium. At the end of chase periods, cells were lysed in 500 µl of PBS containing 1% Nonidet P-40 and protease inhibitor mixture (Roche) on ice for 30 min. Nuclei and insoluble debris were removed by centrifugation. The remaining extract was incubated with 2 µg of anti-CD1d or anti-MHC Class I (W6/32) mAb for 1 h at 4°C. The mixture was supplemented with BSA to a final concentration of 1% and incubated overnight with 40 µl of protein A/G beads (Santa Cruz Biotechnology) at 4°C. Protein A/G beads were washed 4 times with ice cold PBS, and the bound proteins were analyzed by electrophoresis in SDS-PAGE (4-20% Tris-HCl gradient gel, Bio-Rad). Gels were dried, and analyzed by using a PhosphorImager Storm 860 (Molecular Dynamics). Chloroquine was added from a 100X stock in PBS to a final concentration of 10µm at the beginning of the chase period directly to the chase medium.

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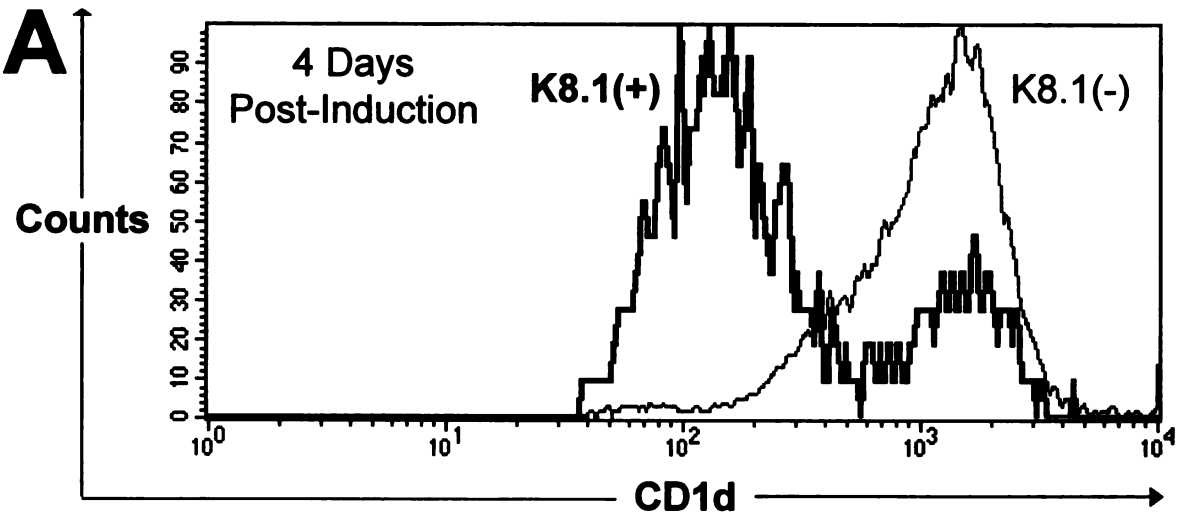
Figure Legends

Figure 1 ***KSHV Lytic Replication Decreases CD1d Levels***

A. The levels of CD1d on uninduced BCBL-1 cells.

B. BCBL-1 cells were induced to enter the lytic replication cycle, and were allowed to proceed to 4 days post-induction where the cells in the late portion of the lytic cycle could be monitored by K8.1 expression. The cells were stained with a rabbit primary antibody against K8.1 and a anti-rabbit secondary, and also a Zenon-One-APC labeled antibody against CD1d and a PE conjugated antibody against Transferrin receptor. The levels of CD1d for both the K8.1 positive and K8.1 negative populations. To assure that induction of lytic replication does not cause a general downregulation the normal level of transferrin receptor was determined by analyzing a bulk population and creating a gate in which 80% of the cells fell into. As shown, the K8.1 positive and K8.1 negative populations both show similar levels of Tranferrin receptor thus even in a nongated population, most cells had normal levels of cell surface receptors.


A
4 Days
Post-Induction
K8.1(+)
K8.1(-)
Counts
CD1d

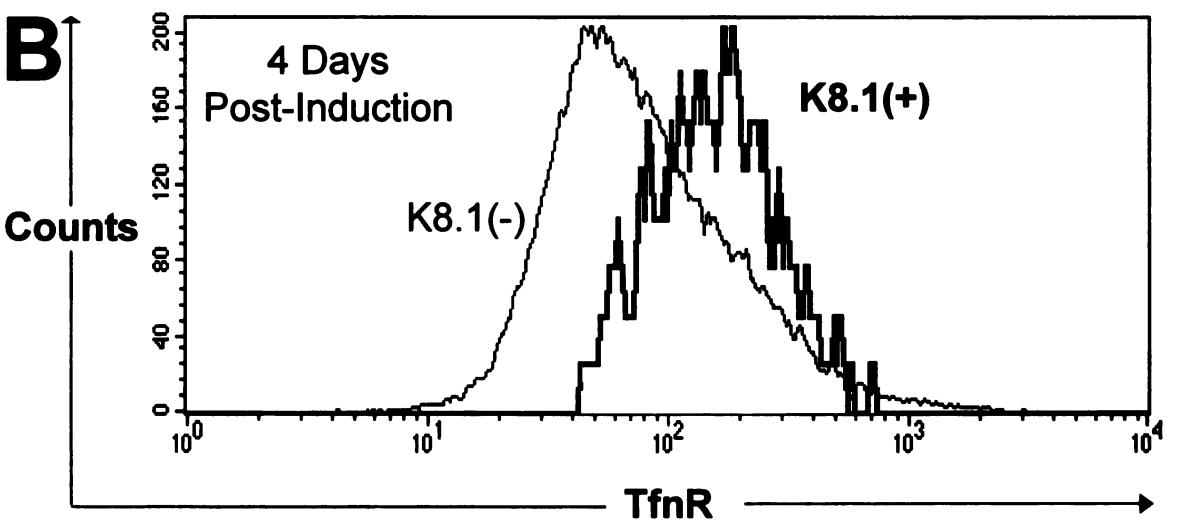

B
4 Days
Post-Induction
K8.1(+)
K8.1(-)
Counts
TfnR

Figure 2 ***MIR1 and MIR2 Induce CD1d Downregulation***

A. BJAB cells were transiently transfected by electroporation with expression vectors for eGFP, MIR1-eGFP and MIR2-eGFP. 36 hours post transfection, the cells were stained with Zenon-One labeled mouse monoclonal antibodies against either CD1d or HLA Class I. For reference, the nonstained population is also displayed. In addition, the levels of HLA-DR, CD1c and Fas are included as controls.

B. HepG2 cells, which express a high level of endogenous level of CD1d, were stabely transduced with retroviral vectors encoding either lacZ or MIR2 that is Flag tagged. A mixed population of stable cells were selected, stained with Zenon-One labeled monoclonal antibodies and analyzed by flow cytometry. As a reference, the histogram for the nonstained control is also displayed.

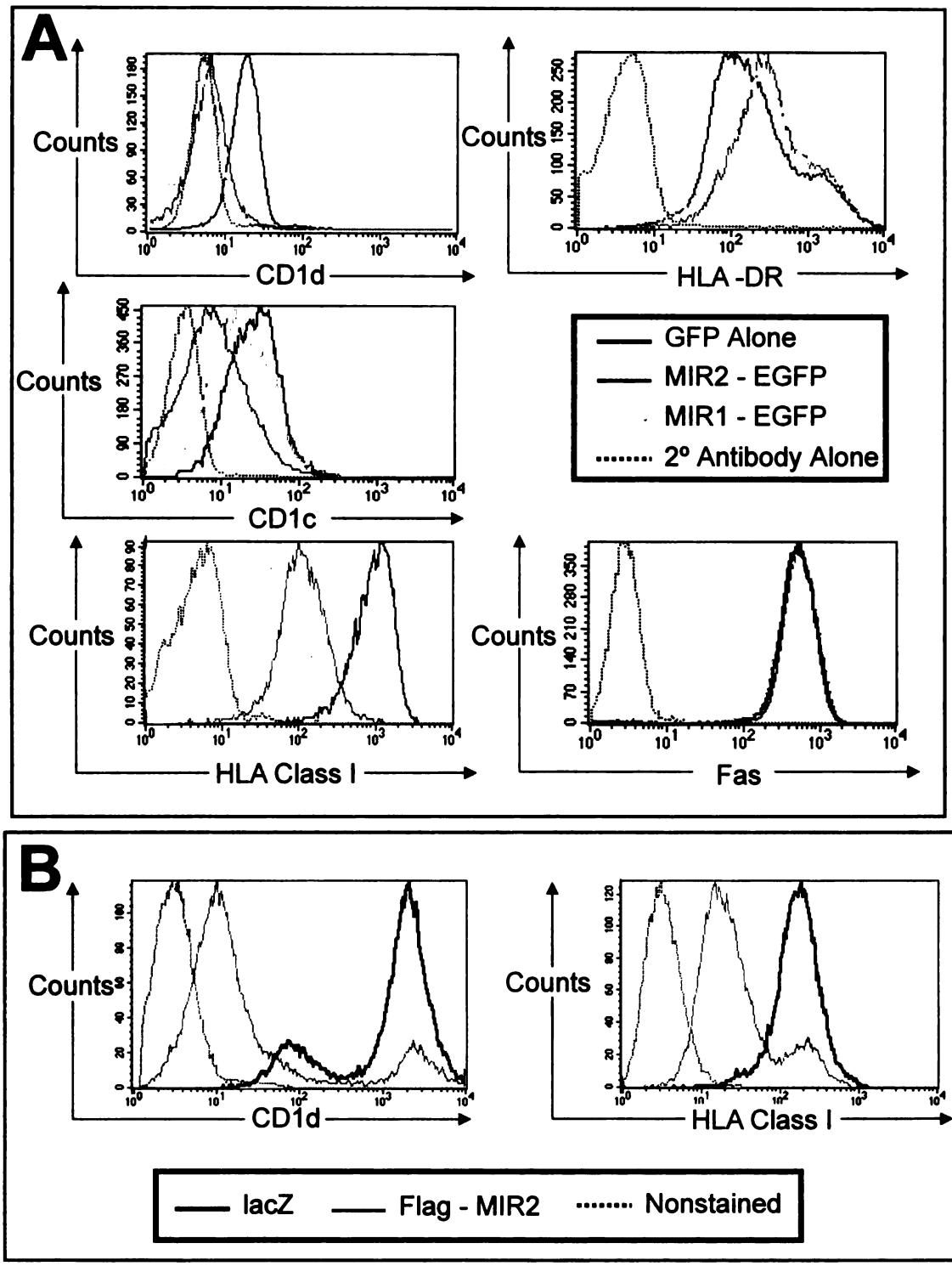

A
Counts
CD1d
Counts
HLA -DR
Counts
CD1c
GFP Alone
MIR2 - EGFP
MIR1 - EGFP
2° Antibody Alone
Counts
HLA Class I
Counts
Fas
B
Counts
CD1d
Counts
HLA Class I
lacZ
Flag - MIR2
Nonstained

Figure 3 ***MIR2 downregulation of CD1d lowers CD1d restricted T cell activation***

A. BJAB cells stabely transduced with either lacZ (gray bars) as a control or MIR2 (white bars) were cocultured with CD1d restricted T cells. This coculture was done in the presence of α-GalCer to provide an activating ligand for the T cells. Each coculture was done in triplicate. After 18 hours of coculture, the supernatant was assayed for IFN-γ release by ELISA and the results were graphed along with the standard deviation bars.

B. As in A, coculture experiments were done with CD1d restricted T cells and the BJAB cells, this time either in the presence or absence of α -GalCer. Cocultures were done with α-GalCer lacZ (gray bars) as a control or MIR2 (white bars) or without α-GalCer and lacZ (black bars) as a control or MIR2 (dark gray bars). After 18 hours, the supernatants were recovered and the IFN- γ in the supernatant was assayed by ELISA.

C. As in A, coculture experiments were done with CD1d restricted T cells and the BJAB cells, this either in the presence mouse anti-HLA Class I as a control (lacZ (gray bars) as a control or MIR2 (white bars)) or mouse anti-CD1d antibodies (lacZ (black bars) as a control or MIR2 (dark gray bars)). After 18 hours, the supernatants were recovered and the IFN- γ in the supernatant was assayed by ELISA.

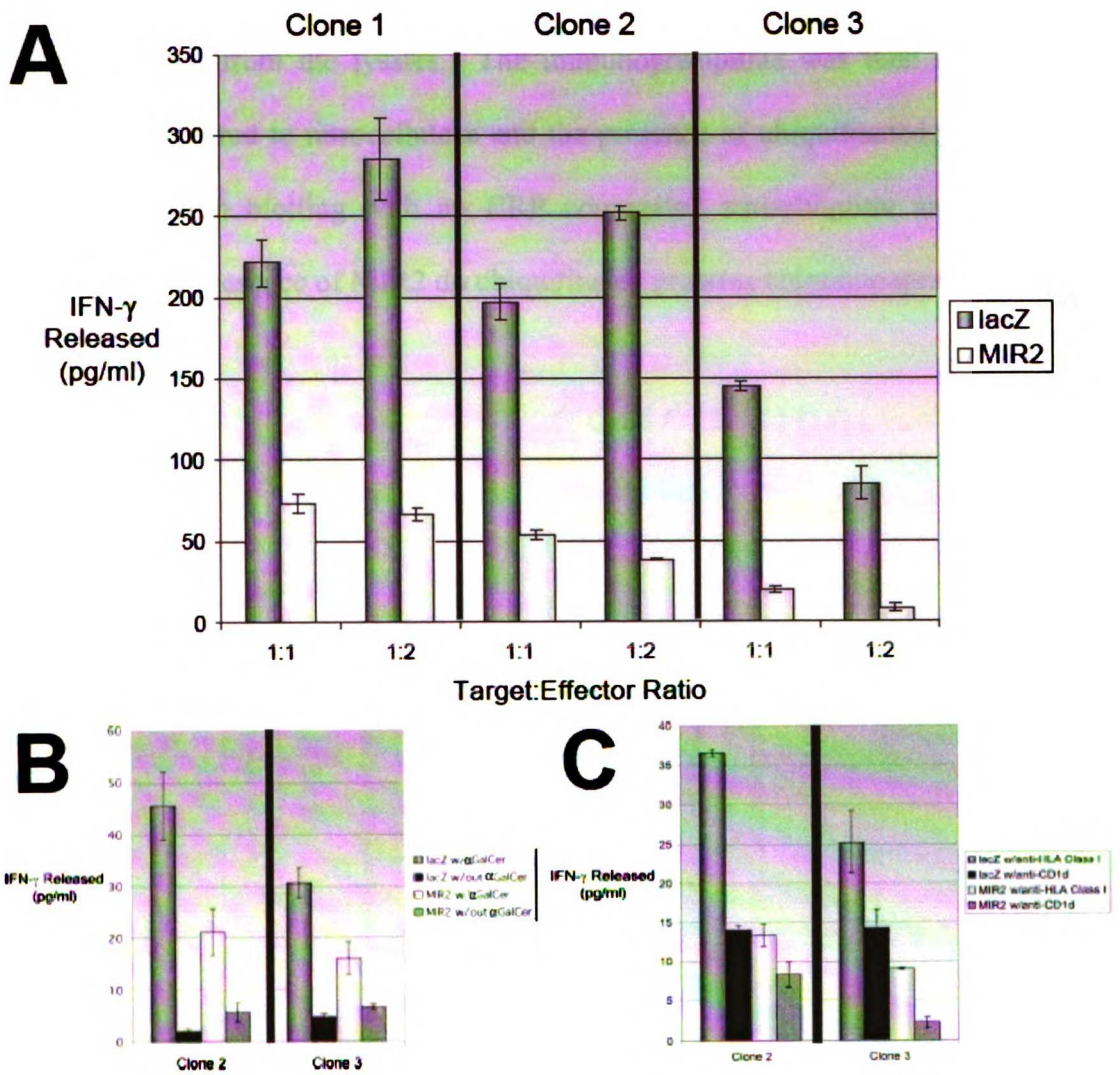

A
Clone 1
Clone 2
Clone 3
350
300
250
200
150
100
50
0
IFN-γ Released (pg/ml)
lacZ
MIR2
1:1
1:2
1:1
1:2
1:1
1:2
Target:Effector Ratio
B
IFN-γ Released (pg/ml)
Clone 2
Clone 3
C
IFN-γ Released (pg/ml)
Clone 2
Clone 3

Figure 4 ***MIR2 expression leads to the coimmunoprecipitation of ubiquitinated proteins with CD1d***

HepG2 cells stabely transduced with either lacZ or MIR2 were lysed and CD1d was immunoprecipitated from the lysates. The immunoprecipitate was then separated by SDS-PAGE, transferred to nitrocellulose and the presence of ubiquitinated proteins was revealed by Western blotting with an HRP conjugated anti-ubiquitin antibody. As shown, only in the presence of MIR2 do ubiquitinated proteins coimmunoprecipitate with CD1d.

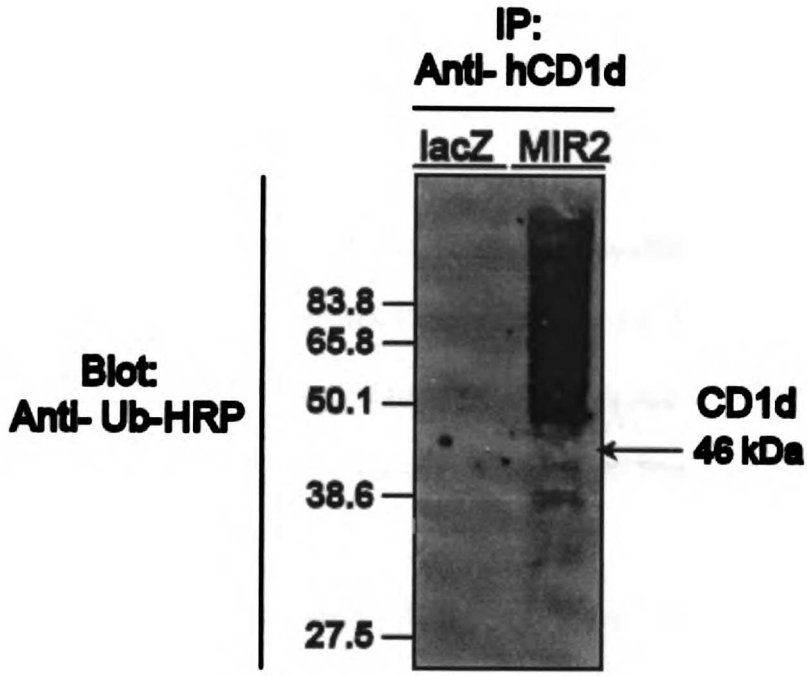

IP:
Anti- hCD1d
lacZ
MIR2
Blot:
Anti- Ub-HRP
83.8
65.8
50.1
38.6
27.5
CD1d
46 kDa

Figure 5 ***CD1d Cytoplasmic Lysines are required for MIR2 mediated downregulation and ubiquitination of CD1d***

A. Alignment of the transmembrane and cytoplasmic tails of CD1d of human and mouse origin show that a single lysine exists in human CD1d but not CD1d of mouse origin.

B. Several chimeric proteins were created to confirm the requirement of cytoplasmic lysines for the ability of the MIR proteins to downregulate CD1d. This schematic shows the theoretical domain organization of chimeric CD1d molecules that were constructed and the mutations that were introduced.

C. BJAB cells were cotransfected with either of the two chimeric CD1d (wildtype or lysine to arginine mutant) with an expression vector for eGFP (left panel) or MIR2 fused to eGFP (right panel). The levels of chimeric CD1d was determined by staining with anti-mouse CD1d antibodies and the cells were analyzed by flow cytometry, gating upon eGFP positive cells.

D. BJAB cells were stabely transduced with either lacZ (as a control) or MIR2 and then stabely transduced with either a wildtype chimeric CD1d or a lysine to arginine mutantion. The cells were then lysed, chimeric CD1d was immunoprecipitated and the immunoprecipitate was blotted for the presence of ubiquitin.

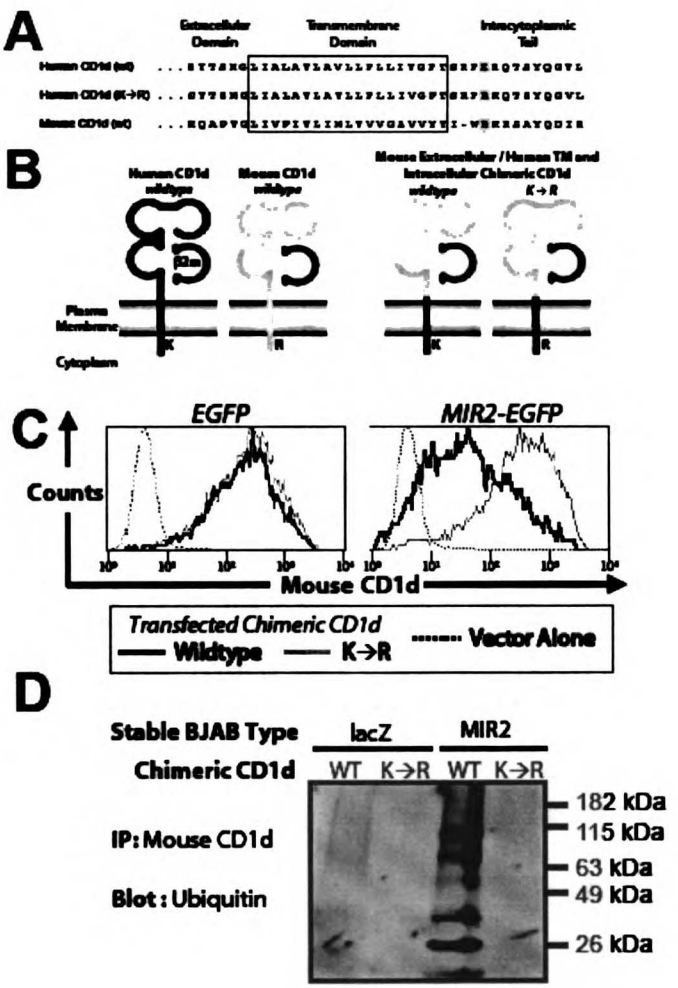

A
Extracellular Domain
Transmembrane Domain
Intracytoplasmic Tail
B
Human CD1d wildtype
Mouse CD1d wildtype
Mouse Extracellular / Human TM and Intracellular Chimeric CD1d
wildtype
K→R
Plasma Membrane
Cytoplasm
K
R
C
EGFP
MIR2-EGFP
Counts
Mouse CD1d
Transfected Chimeric CD1d
Wildtype
K→R
Vector Alone
D
Stable BJAB Type
lacZ
MIR2
Chimeric CD1d
WT
K→R
WT
K→R
IP: Mouse CD1d
Blot : Ubiquitin
182 kDa
115 kDa
63 kDa
49 kDa
26 kDa

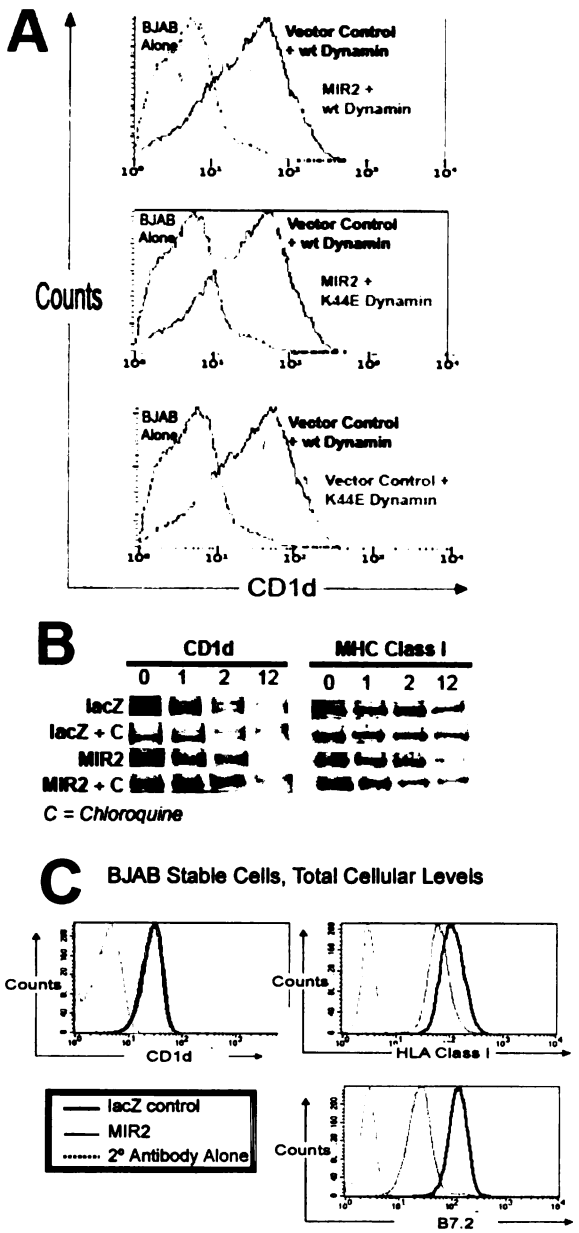
Fig. 6 ***MIR2 downregultaion of CD1d is due to endocytosis but not enhanced degradation***

A. BJAB cells were transiently transfected with expression vectors for the wildtype chimeric CD1d molecule (see Figure 5B), either empty of MIR expressing, and either wild-type of dominant negative dynamin. All panels show the levels of chimeric CD1d in the presence of wild-type Dynamin (black solid line) as well as the levels of antibody cross reactivity in the cells transfected with empty vector instead of one expressing a chimeric CD1d (broken black line). In the top panel, the gray line represents chimeric CD1d levels when contransfected with MIR2 and wild-type dynamin while the center panel's gray line represents cotransfection with MIR2 and dominant negative dynamin and thus restoration of cell surface CD1d. The bottom panel's gray line represents cotransfection with empty vector instead of MIR2 as well as dominant negative dynamin.

B. HepG2 cells were pulse for 30 minutes with S^{35} trans-label and chased for the indicated amounts of time. HepG2 cells stabley expressing lacZ or MIR2 were chased in normal media or media with chloroquine (denoted as "+C") added to block lysosomal degradation. Lysates were immunopreciptated for CD1d (left panel) or MHC Class I (right panel) as a control. As shown, the presence of the MIR2 gene does not change the half-life of the CD1d chains as compared to the MIR2 mediated degradation of MHC Class I.

C. BJAB cells that were stable for expression of MIR2 or lacZ as a control were fixed and permeabilized for determination of total cellular levels of protein by flow cytometry. Cells were stained for either B7.2, MHC Class I or CD1d. As shown, the presence of the

MIR2 gene does not change the total levels of CD1d even though the surface levels of CD1d are decreased. This is contrasted with the levels of B7.2 and MHC Class I which both decrease in the presence of MIR2.


A
BJAB Alone
Vector Control + wt Dynamin
MIR2 + wt Dynamin
BJAB Alone
Vector Control + wt Dynamin
MIR2 + K44E Dynamin
BJAB Alone
Vector Control + wt Dynamin
Vector Control + K44E Dynamin
Counts
CD1d
B
CD1d
MHC Class I
0 1 2 12
0 1 2 12
lacZ
lacZ + C
MIR2
MIR2 + C
C = Chloroquine
C
BJAB Stable Cells, Total Cellular Levels
Counts
CD1d
Counts
HLA Class I
lacZ control
MIR2
2° Antibody Alone
Counts
B7.2

CHAPTER 4

Modulation of the CD1 System by Kaposi's Sarcoma-associated Herpesvirus

ABSTRACT

The CD1 system is know seen as a pathogen recognition system of the immune system that is potentiated by foreign antigens and enhances the efficacy of cytokine signals from the innate immune system. Previous finding by our group showed that KSHV was able to directly modulate the CD1d system by downregulating CD1d from the cell surface by expression of either MIR1 or MIR2. These studies spearheaded the search for proteins of KSHV that are recognized by the CD1d system either directly or indirectly. Here we show that several KSHV proteins including the spectrum of the glycoproteins os KSHV are able to induce an upregulation of cell surface CD1d. In addition, we further went on to study the CD1c system which seems to be specifically targeted by the MIR2 protein. We see that many of the glycoproteins can induce the transient and stable increase in cell surface CD1c. Finally, we see that in addition to MIR2, stable expression of K8.1A but not the shorter splice variant, K8.1B, is able to induce the downregulation of CD1c. These findings have many implication for the understanding of how KSHV is recognized by the innate T cells of the CD1 system and how KSHV is able to subvert these mechanisms.

INTRODUCTION

The CD1 system is a highly conserved pathogen recognition system of the immune system [1]. The CD1 proteins themselves are a family of cell surface proteins that are currently believed to survey the internal compartments of the cell and recognize either cellular stress or pathogens directly [2] [3]. The proteins in the CD1 family are structurally analogous to MHC Class I with the typical structure of the alpha chain as well as beta-2-microglobulin interactions, but are distinct in that in place of a hydrophilic charged peptide binding groove of MHC Class I, these proteins contain a deep hydrophobic binding groove that is believed to bind lipids [4]. For some CD1 family members, including CD1b[5], these lipids are believed to be of pathogen origin, while others such as CD1d are increasingly seen as presenters of lipids derived from host origin [6] [7]. CD1 a,b,c are mainly expressed by professional antigen presenting cells such as B cells and dendritic cells, while CD1d is expressed in other tissues such as monocytes, hepatocytes and enterocytes [1].

As a structural homologue of MHC Class I, CD1 acts as a ligand for the T cell receptor of T cells that are restricted for recognition of CD1 proteins[8]. These T cells are often seen as innate in nature, primarily because the CD1 family itself is invariant and does not display the level of polymorphism that the MHC Class I system does[9]. As such these cells are often classified as an arm of the innate immune system. The functional significance of these CD1 restricted T cells is only now being understood [10]. CD1c restricted T cell lines were originally derived by screening cells cytotoxicity to cells that express Mycobacterial, protease-resistant antigens [11].

Our initial studies into CD1c began by looking at their ability to be downregulated by MIR1 and MIR2, the KSHV modulators of immune recognition. MIR1 and MIR2 are able to downregulate CD1d and as a specificity control, we went on to see their ability to modulate CD1c (Chapter 3). While MIR1 does not change the levels of CD1c, MIR2 is able to downregulate surface CD1c levels. This specific downregulation lead us to examine if other, non-MIR proteins might upregulate CD1d or CD1c.

Here we see that expression of a set of KSHV genes from the limited and specific set that we screened leads to a modest upregulation of CD1d on the cell surface. As an initial survey into viral modulation of these systems, we began to look at selected proteins that might modulate the CD1 levels within the cell. In addition, the CD1c levels increases after transient transfection with the most of the glycoproteins of KSHV. K8.1A though is able to reduce cell surface levels of CD1c, pointing out a potential new mechanism of immune evasion.

MATERIALS AND METHODS

Cell Culture

BCBL-1, an established KSHV infected human B-cell line, that is free of EBV infection was grown in RPMI with glutamine, sodium bicarbonate and β-mercaptoethanol. Phoenix cells, a 293T based retroviral packaging cell line was grown in Dulbecco's modified Eagle's medium (DMEM), and the BJAB B cell lymphoma cell line was grown in RPMI medium 1640. All media were additionally supplemented with 10% (v/v) fetal calf serum and penicillin/streptomycin. BJAB cells were transfected by electroporation (250 V, 950 microfarads) of 20 μg of plasmid DNA into 10^7 cells in 0.5 ml of serum-free medium. The cells were then transferred to complete RPMI, which was preincubated to 37 °C. The transfection efficiency of BJAB cells was routinely 30-50% under these conditions [12].

Stable expressing proteins were created by retroviral transduction. Upon transfection with the retroviral vectors (pBMN or pBMP, containing either neomycin resistance or puromycin resistance), the Phoenix packaging cell line produces replication-defective viral particles that were used to stably transduce recipient BJAB cells. Transient transfections of Phoenix cells were performed using Fugene6 (Roche Molecular Biochemicals) according to the manufacturer's suggested protocol. Phoenix cells were transfected, media was changed 24 hours post-transfection and the virus-containing supernatant was harvested 48 h after the transfection, filtered through a 0.45-μm syringe filter, and diluted with Polybrene (4 μg/ml final dilution). HepG2 or BJAB cells (in six-well dishes) were infected by spin infection (800 X *g* for 2 h at 22 °C) using 2 ml of viral supernatant per well of a six well dish. Selection of transduced BJAB was

started 36 h after infection by adding 1.5 mg/ml or 0.8 mg/ml of G418 for BJAB respectively or 1μg/ml of puromycin for BJAB cells. All cell lines were selected and used as pooled populations.

KSHV Expression Constructs

All KSHV expression constructs were derived by PCR from the appropriate BAC containing the KSHV genomic locus. For the spliced genes, such as K8.1A, RT PCR was performed to capture the cDNA structure. All of the glycoprotein were derived by l. Coscoy and A. Grundhoff and the other KSHV genes were derived by B. Glaunsinger.

Flow Cytometry

BCBL-1 cells were fixed in 4% paraformaldehyde and washed 3 times with PBS. The cells were then blocked with a wash of PBS + 1% BSA. BCBL-1 cells were then incubated Zenon-one - APC (Molecular Probes) labeled mouse anti-CD1d (Calbiochem) in 100μl of PBS + 1% BSA. After this last incubation, the cells were washed extensively with PBS and resuspended in 200μl of PBS for analysis by a FACScaliber (Becton Dickinson).

For BJAB experiments, cells were washed once with PBS + 1% BSA, and incubated without fixation with the appropriate antibody on ice. CD1d was analyzed by using CD1d antibody (Calbiochem) that was Zenon-one – APC (Molecular Probes) labeled or a labeled CD1c antibody (Santa Cruz Biotech). In addition, mouse anti-Human Class I (W6/32) pre-coupled to PE (Caltag) was used at a dilution of 1:100 in PBS + 1% BSA in

these experiments. After incubation and extensive washings in PBS, cells were analyzed by flow cytometry with a FACScaliber.

RESULTS and DISCUSSION

KSHV Proteins Influence CD1d Surface Levels

Previous work from our lab showed that the cell culture system that we use to culture KSHV showed very high levels of CD1d on its cell surface (Figure 1A left panel). This BCBL-1 cell line (Body Cavity Based Lymphoma) can be seen to have levels of CD1d that are at least three logs higher than those present in the BJAB cell line (Figure 1A, right panel). To see if expression of KSHV proteins leads to upregulation of cell surface CD1d, a bank of expression vectors was screened for their ability to upregulate CD1d surface levels.

Our initial attempts at understanding CD1d upregulation on the latently infected BCBL-1 cell line, pushed us to examine the latent proteins as well as proteins that may influence membrane homestasis within the cell (e.g. multi-transmembrane proteins as well as glycoproteins). Disruption of the membrane systems within the cell either by ER stress or protein folding burdens may induce an unfolded protein response that could then lead to enhanced lipid metabolism and possible enhanced loading of CD1 molecules. As shown in Figure 1B, expression of most of the latent and unique signaling molecules of KSHV (K1, viral FLIP, kaposinA, viral Cyclin, K15, and LANA) produce no change in transfected cells. The viral G-protein coupled receptor that is highly expressed in lytic infection shows a modest increase in CD1d levels on the cell surface.

For expression of the KSHV glycoproteins, a mix of the glycoprotein expression constructs was used to facilitate proper maturation of the glycoproteins within the transfected cells. Notably, it is these proteins that are likely to be first seen by an infected cell as well as the proteins that are most likely to affect membrane biogenesis upon the high level production of glycoproteins seen at the late stages of herpesviral replication. Upon transfection of the glycoprotein mixture into the BJAB cells, a group of cells that express higher levels of CD1d are seen. Upon transfection with the ORF68 gene, a glycoprotein of unknown function, an even higher increase in CD1d is seen. Thus the glycoproteins and at least one of the multitransmembrane proteins (vGPCR) seem to modestly upregulate CD1d levels of the cell surface.

Long term expression of KSHV glycoproteins induces upregulation of CD1c

To see if the selected cells modulated other arms of the CD1 system, we looked at the change in CD1c levels on the BJABs that were stabely transduced by the KSHV glycoproteins. As shown in Figure 2, upon one week of transduction and stable selection for retroviral integrants, the levels of CD1c on the surface of cells, is upregulated in almost all the glycoprotein stable cells.

Transient Expression of the KSHV Glycoproteins induces Upregulation of CD1c

To see how transient expression of the glycorproteins influence the levels of CD1c on the cell surface, we transfected BJAB cells with expression constructs for selected glycoproteins. As shown in Figure 3, most glycoproteins at 15 hours post transfection

induced low level modulation of the CD1c levels on the surface. But upon 30 hours post transfection (Figure 3B), the levels of surface CD1c seem to be twice as high as control transfected cells. Because these experiments are carried out as cotransfection experiments, the dark grey bars represent the GFP positive cells in each reaction which the light grey bars represent the GFP negative cells.

Expression of the K8.1A Gene Induces CD1c Downregulation

Based on the fact that K8.1 stable lines did not have increased CD1c (Figure 3), BJAB cells were transiently transfected with expression constructs expressing K8.1 as a genomic construct, or the cDNA versions of K8.1A or the smaller spliced version K8.1B. As shown in Figure 4A, the GFP along transfection does not change the basal levels of CD1c on the BJAB cell surface. In contrast, the cells transfected with the genomic K8.1 construct or the cDNA expressing construct of K8.1A induce a downregulation of the CD1c levels on the cell surface. A striking finding is that the K8.1B construct which is mostly the exact K8.1A protein with a spliced to a different carboxy terminus is not able to induce this change.

These finding show that though some glycoprotein constructs are not reactive to the CD1c system or induce minimal upregulation, K8.1A but not K8.1B, is able to induce a downregulation of the surface levels of CD1c.

Thus it seems that KSHV is able to selectively downregulate both the CD1d and CD1c system. Though there is precedent for the importance of the CD1d system in the control of viral infection, there has of yet not been a clear correlation between the CD1c system and viral infection. This may represent an additional innate control mechanism of viral infection in which KSHV has evolved specific proteins to downregulate the CD1c levels. However, it cannot be ruled out that K8.1A is a signaling molecule that disrupts normal cellular networks and thus has a downstream consequence of downregulating CD1c

levels. Importantly, the CD1d levels do not seem to be significantly affected by the K8.1A expression; the effects of this glycoprotein appear to be selective for CD1c.

These studies of viral modulators of the CD1c and CD1d system do not yet allow construction of a model for how CD1 affects replication but lays the groundwork for future studies into how the innate immune system is able to modulate a viral infection. As of yet, the exact nature of what the CD1 systems monitor during a viral infection is contentious. Whether, a virally encoded ligand or perhaps the cellular stress of a viral infection itself is able to be monitored by the CD1 systems is still under investigation[13] [14]. However, in all of the CD1 systems, it is clear that increased levels of CD1 on the cell surface is often sufficient to allow for CD1 restricted T cells to react to the CD1 expressing cell [15] [16] [17] [18]. Thus potentiated CD1 restricted T cells may only need increased CD1 on target cells and not necessarily a particular antigen loaded onto the CD1. In addition, some studies have linked CD1c to restriction by gamma-delta T cells, thus implicating These studies set the groundwork to continue investigating how the innate T cell system restricted by CD1 is able to monitor and control the earliest steps in a viral infection.

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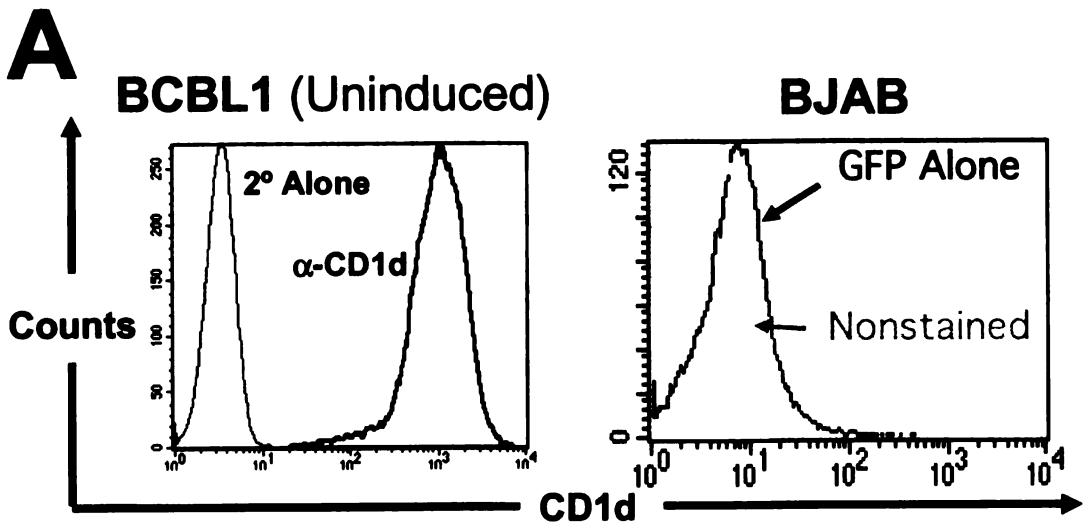
Figure 1 ***Expression of KSHV Genes Leads to Increases in Surface CD1d Levels***

A. BCBL-1 cells were stained for surface CD1d levels to show the high level on KSHV latently infected cells. BJAB cells were transfected with GFP alone to show the levels on transfected BJAB cells.

B. A set of KSHV genes was transfected into BJAB cells in a cotransfection experiment with GFP. 36 hours post transfection, the cells were stained for cell surface CD1d levels and the GFP positive cells were analyzed. The glycoproteins represent gB, gH, gL, gM, K8.1, ORF68 and vOX-2. The numbers represent the geomertric mean of the fluorescence intensity of that particular transfectant over the GFP alone level.


A
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BJAB
2° Alone
α-CD1d
GFP Alone
Nonstained
Counts
CD1d

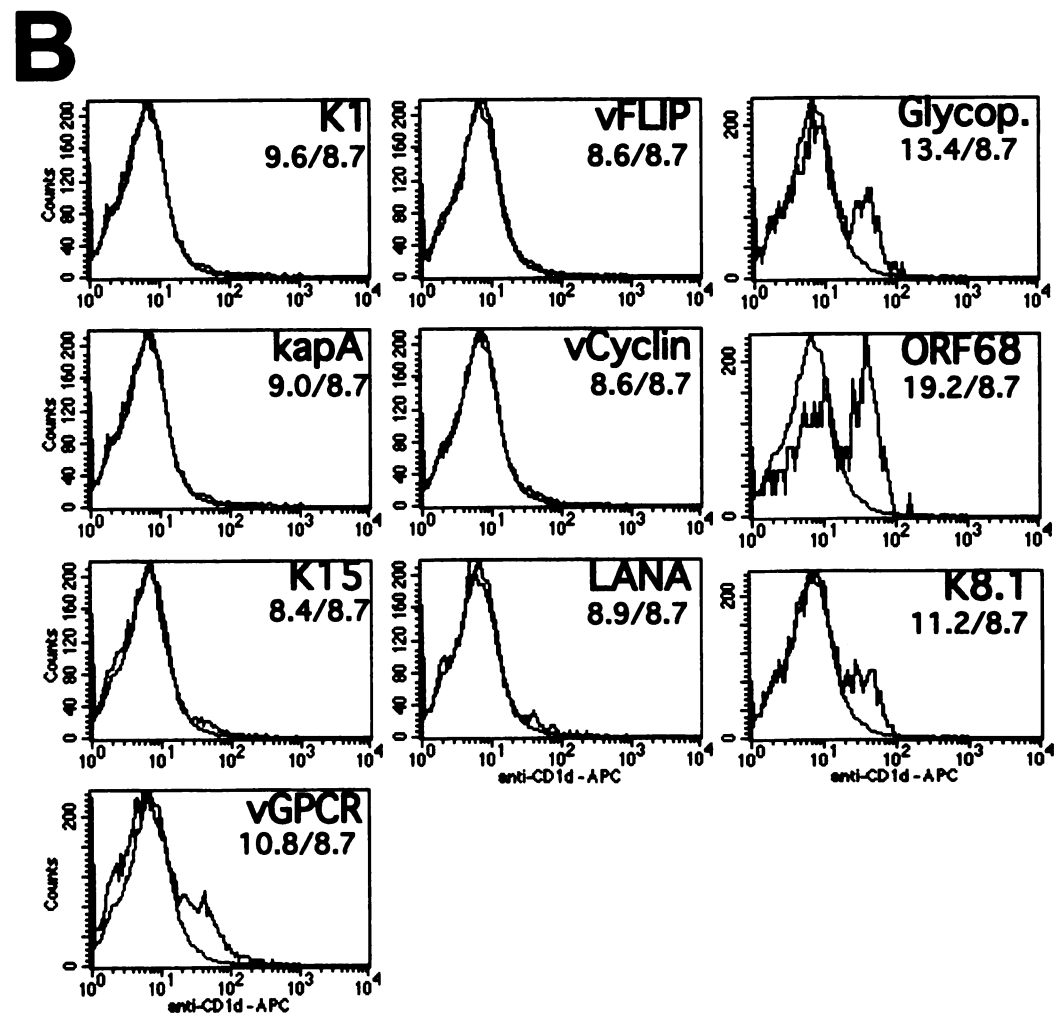

B
K1
9.6/8.7
vFLIP
8.6/8.7
Glycop.
13.4/8.7
kapA
9.0/8.7
vCyclin
8.6/8.7
ORF68
19.2/8.7
K15
8.4/8.7
LANA
8.9/8.7
K8.1
11.2/8.7
vGPCR
10.8/8.7
Counts
anti-CD1d-APC

FIGURE 2 ***Long term expression of the KSHV Glycoproteins Induces an Increase in CD1c Surface Levels***

Staining of the stable BJAB cells to reveal the surface levels of CD1c show that in fact, over a week of exposure to the KSHV glycoproteins, there is an increase in the cell surface levels of CD1c. All except K8.1A show an increase ranging from 1.2 times the normal level to 1.6 times the normal level of surface CD1c.

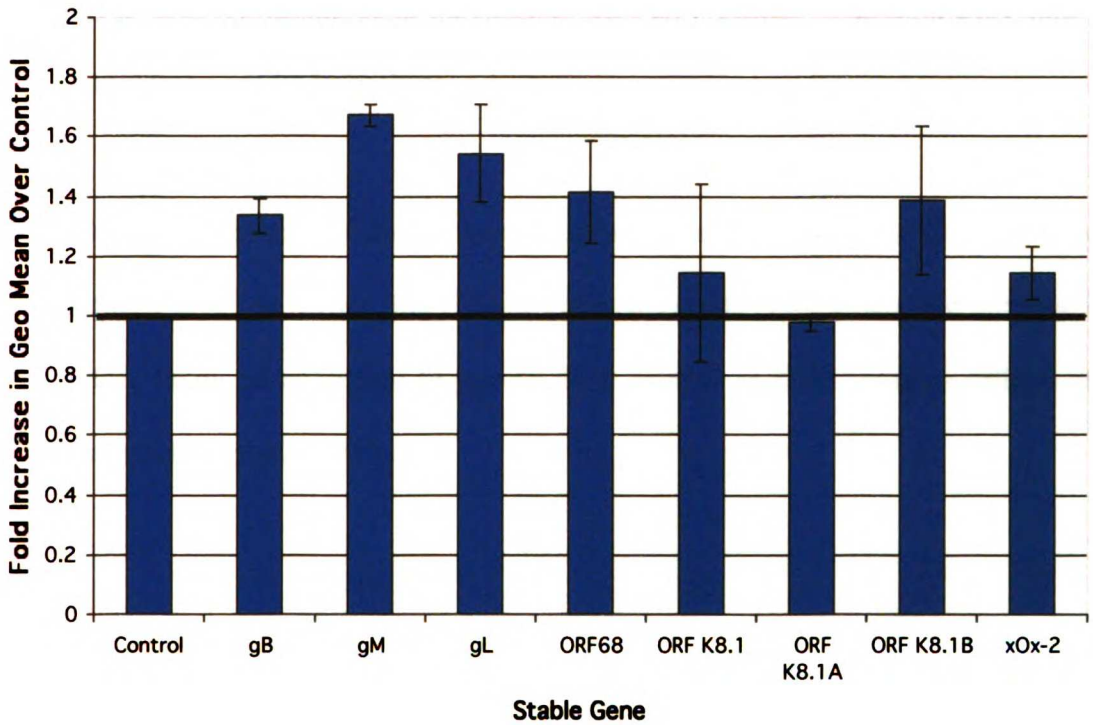
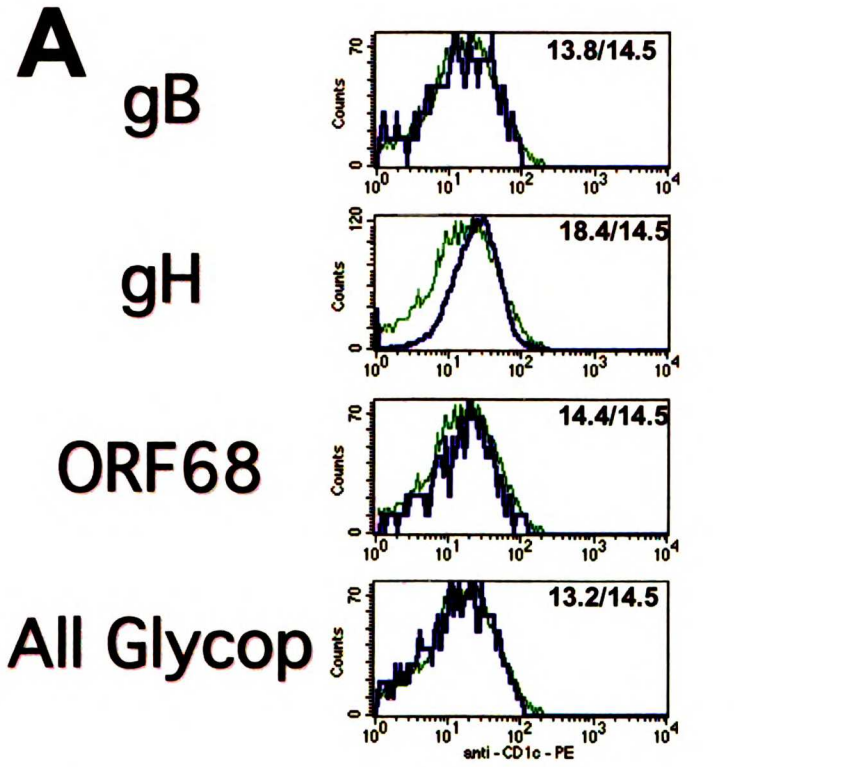

Fold Increase in Geo Mean Over Control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
gB
gM
gL
ORF68
ORF K8.1
ORF K8.1A
ORF K8.1B
xOx-2
Stable Gene

FIGURE 3 ***Expression of the KSHV Glycoproteins Increases CD1c Surface Levels***

A. A set of KSHV glycoprotein genes was transfected into BJAB cells in a cotransfection experiment with GFP. 18 hours post transfection, the cells were stained for cell surface CD1c levels and the GFP positive cells were analyzed. As shown, most the glycoprotein induce a minimal increase in CD1c levels. The blue line represents the cells transfected with the repective glycoprotein and the green line represents cells transfected with GFP along. The light grey line is nonspecific antibody background.

B. The cells were carried out for an additional 18 hours for a total of 36 hours post transfection. As shown, the GFP positive (red) cells show a two fold increase in CD1c levels as compared to control GFP as well as GFP negative cells in the transfection pool (light blue).


A
gB
gH
ORF68
All Glycop
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18.4/14.5
14.4/14.5
13.2/14.5
Counts
anti - CD1c - PE

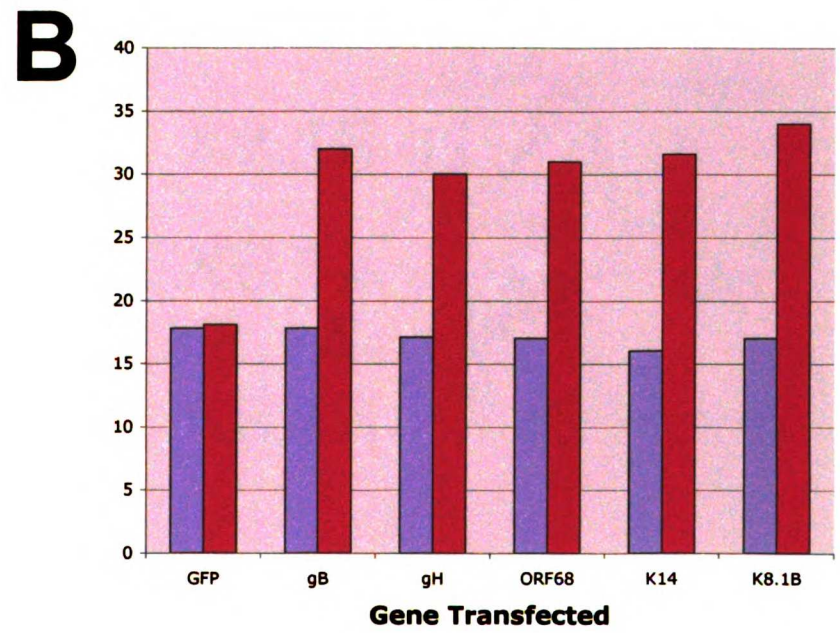
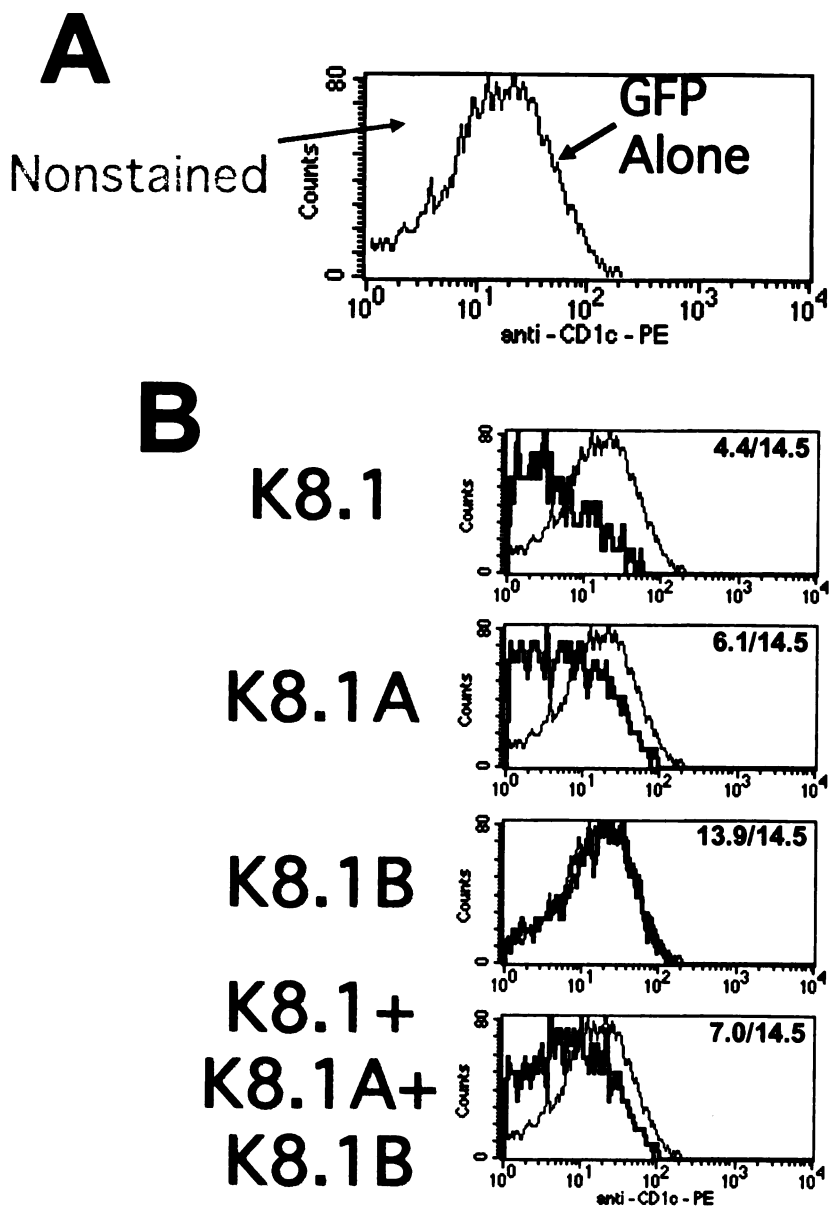

B
40
35
30
25
20
15
10
5
0
GFP
gB
gH
ORF68
K14
K8.1B
Gene Transfected

FIGURE 4 ***The K8.1A Glycoprotein of KSHV Reduces Surface Levels of CD1c***

A. BJAB cells were cotransfected with GFP. 36 hours post transfection, the cells were stained for cell surface CD1c levels and the GFP positive cells were analyzed. Here the GFP alone sample is shown to show the basal level of CD1c on the cell surface.

B. Transfection with the K8.1 genes either as a K8.1 genomic construct (top panel) or K8.1A or K8.1B cDNAs show that expression of the genomic construct, which should express all K8.1 forms or the K8.1A cDNA vector shows a marked decrease in cell surface CD1c. This effect is not seen when K8.1B is transfected alone. Values in top right of plot represent the geometric mean of the fluorescence for that particular sample, over the value for the GFP alone sample in A. The blue line represents the population transfected with the glycoprotein.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Nonstained
GFP
Alone
Counts
anti-CD1c-PE
B
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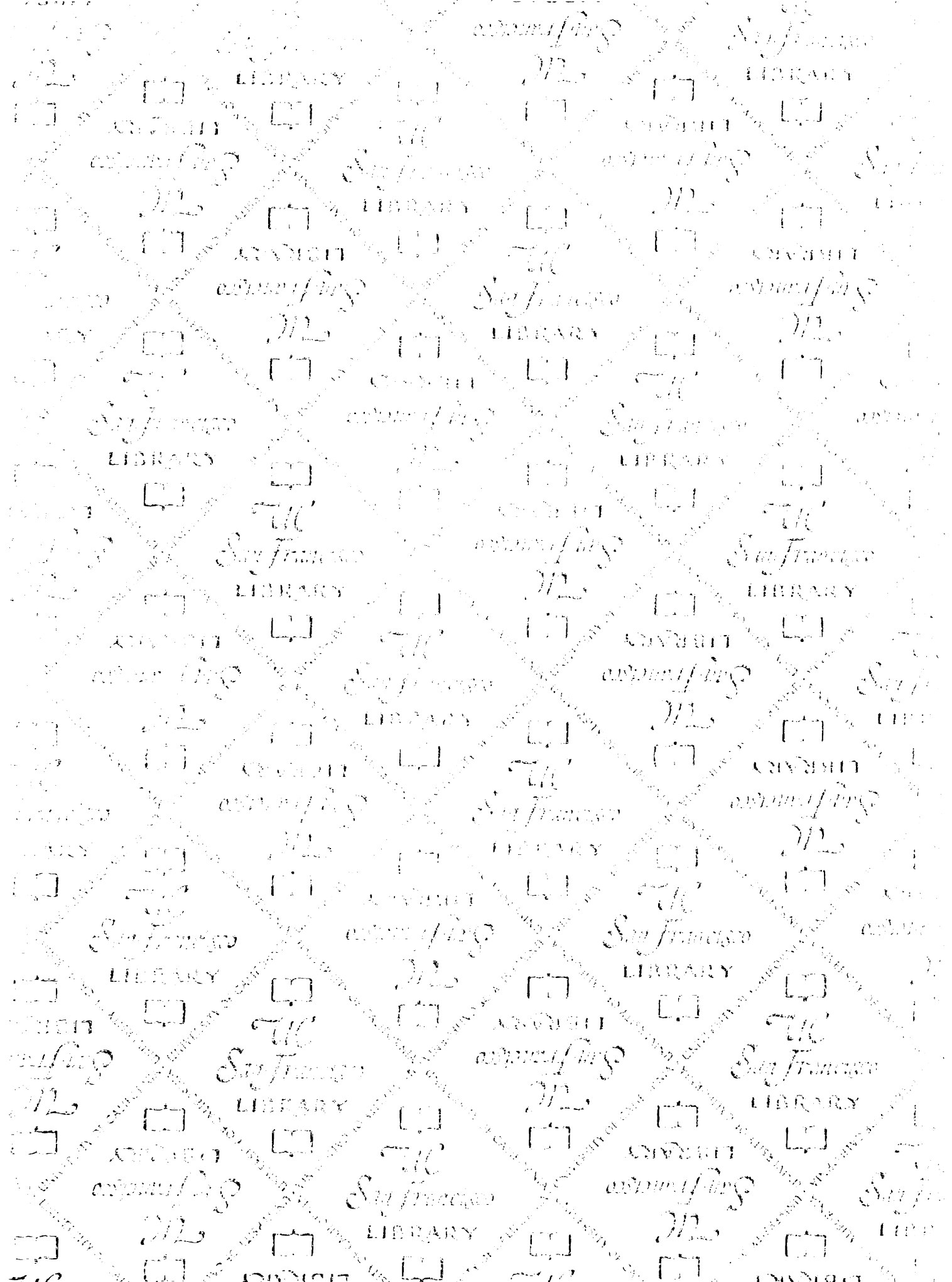
4.4/14.5
K8.1A
6.1/14.5
K8.1B
13.9/14.5
K8.1+
K8.1A+
K8.1B
7.0/14.5
anti-CD1c-PE


The San Francisco
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