2012

Down-modulation of MICA on Malignant Glioma Cells by Herpes Simplex Virus

Carl Irwin Odom
University of Alabama at Birmingham

DOWN-MODULATION OF MICA ON MALIGNANT GLIOMA CELLS BY HERPES SIMPLEX VIRUS

by

Carl Irwin Odom

James M. Markert, CHAIR
Kevin A. Cassady
G. Yancey Gillespie
Peter R. Smith
Mark N. Prichard

A THESIS

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2012

SURFACE DOWN-MODULATION OF MICA IN MALIGNANT GLIOMA CELLS BY HSV

Carl Irwin Odom

BASIC MEDICAL SCIENCE

ABSTRACT

Glioblastoma Multiforme is a primary malignancy of the central nervous system and is fatal for patients despite surgical resection and radiotherapy. Oncolytic Herpes Simplex Virus (oHSV) vectors deleted of the $\gamma_1$34.5 neurovirulence gene are potential therapies for treating glioblastoma tumors. Although replication in permissive cells is considered the primary mechanism of oHSV mediated tumor clearance, there is evidence that the immune system is part of the tumor clearance mechanism during treatment with oncolytic viruses. Specifically, natural killer (NK) cells may be important for tumor clearance during oHSV therapy. NK cell activation relies partially on the stimulation of activating or inhibitory receptors and their respective ligands encountered on the surfaces of stressed cells. One of the most important and well studied receptor/ligand pairs is the natural killer cell activating receptor Natural Killer Group 2 member D (NKG2D) and the ligand MHC-class-I-polypeptide-related chain A (MICA). Members of the herpes family of viruses have evolved mechanisms of down-modulating MICA surface expression to prevent NK cell activation. This phenomenon is observed with HSV type-1 infection of epithelial tumor lines, yet the mechanism of action is currently unknown. In this work, we tested the hypothesis that HSV infection down-modulates MICA on glioma cells, a phenomenon which could negatively impact the NK cell mediated anti-tumor activity of oHSV. Specificity of MICA down-modulation was studied by analyzing another surface membrane protein, transferrin receptor. The contribution of Virion Host Shut-off (VHS)

protein to MICA mRNA degradation as a possible mechanism to surface protein down-modulation was studied by utilization of a VHS deleted virus. These studies demonstrate that MICA is down-modulated from the surface and interior of glioma cells during HSV infection and that down-modulation is specific to MICA compared to levels of transferrin receptor, which in contrast were increased. Comparison of mRNA and protein levels during wild type and VHS deleted recombinant infection suggests that VHS contributes to MICA mRNA degradation but not to protein down-modulation. Continued studies to identify the mechanism of MICA surface down-modulation and will allow for design of oHSV vectors able to optimize anti-tumor immune responses and prolong patient survival.

Keywords: Glioblastoma Multiforme, Herpes Simplex Virus, Natural Killer cell, MHC-class-I-polypeptide-related chain A (MICA), Virion Host Shut-off, Natural Killer Group 2 member D (NKG2D)

ACKNOWLEDGMENTS

This thesis was supported by NIH P01-CA 071933-13 (K. A. Cassady, J. M. Markert, R. J. Whitley), P20CA151129 (K. A. Cassady, J. M. Markert, G. Y. Gillespie), NIH training grant 5T32 NS048039 (D. C. Gaston), and the UAB Center for Clinical and Translational Science Grant Number TL1TR000167 from the National Center for Advancing Translational Sciences (NCATS) and National Center for Research Resources (NCRR) component of the National Institutes of Health (NIH).

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

This thesis fulfills part of the requirements to complete the Master of Science degree in basic medical science and the TL1 training program offered through the Center for Clinical and Translation Science. It is composed of a review article focused on the herpesvirdae mechanisms for evading natural killer (NK) cell recognition and an original research manuscript analyzing herpes simplex virus type-1 (HSV-1) down-modulation of MHC-class-I-polypeptide-related sequence A (MICA) on HeLa and malignant glioma cells. MICA is a stress induced ligand found to be upregulated on numerous tumor cell lines, including malignant gliomas (1, 2), that engages the NK cell activating Natural Killer Group 2 Member D (NKG2D) receptor.

The presence of immuno-modulating proteins has implications for the treatment of such tumors. *Glioblastoma multiforme* (GBM) is a primary tumor of the central nervous system that primarily affects adults and has a very poor prognosis despite surgical resection and radiotherapy (3). Oncolytic herpes simplex viruses (oHSV) are oncolytic vectors developed for treatment of GBM with proven clinical safety in phase I clinical trials (4, 5). These viruses are attenuated through deletions or mutations of the HSV-1 $\gamma_1$34.5 neurovirulance gene, allowing replication and lysis within tumor cells. In addition to direct lysis, studies utilizing oHSVs expressing immunomodulating cytokines demonstrate a role for the immune response in tumor clearance. An increase in immune infiltration and anti-tumor efficacy against murine glioma models has been reported in treatment with oHSV engineered to express immunostimulatory cytokine IL-12 or

chemokine CCL2 (6). In a phase Ib clinical trial utilizing G207, a clinical grade oHSV, gene expression studies of GBM resections from patients with a greater response to treatment as measured by prolonged survival demonstrated an increased expression of NK cell associated markers CD16, NKG2D, and DAP-10 along with mediators of cytotoxicity namely perforin, granzyme, and TRAIL (unpublished data). This is suggestive that in addition to direct lysis, the NK cell response may be important for the efficacy of tumor clearance during treatment with oHSV vectors leading to prolonged patient survival.

The review article presented, which is accepted for publication in the journal *Advances in Virology,* was written in order to gain better insight into the current literature regarding not only NKG2D and MICA, but also other NK cell regulatory receptors, their ligands, and modulation during herpes virus infection. The publication summarizes the activating and inhibitory receptors on the surfaces of NK cells and provides references for their structure and function. The corresponding ligands of these receptors are also summarized with references to their regulation on healthy and stressed cells and interaction with receptors. The publication also summarizes the immunoevasins that target NK cell regulatory receptors or ligands used by each member of the herpes virus family. This includes the current understanding of MICA modulation during HSV-1 infection and highlights the discrepancies between viruses in the knowledge regarding their innate immune evasion tactics.

Based on the current knowledge of MICA modulation by HSV-1 infection, experiments were performed which form the basis for the original research manuscript under preparation presented here. Very few studies have investigated the modulation of

MICA during HSV-1 infection and none have reported any modulation specifically on malignant glioma cells. It is hypothesized that HSV-1 down-modulates MICA from the surface of glioma cells in order to prevent NK cell activation, and because of the potential importance of the NK cell response in oHSV anti-tumor efficacy, this MICA down-modulation could adversely affect oHSV treatment. The studies performed test this hypothesis and analyze the behavior of MICA presentation and translation during HSV-1 infection compared to uninfected conditions and the behavior of another trans-membrane protein. Studies also attempt to elucidate the mechanism of HSV-1 mediated down-modulation. A better understanding of this mechanism can lead to engineered oHSV vectors able to optimize NK cell activation and anti-tumor activity as a result.

HUMAN HERPESVIRIDAE METHODS OF NATURAL KILLER CELL EVASION

by

CARL I. ODOM, DAVID C. GASTON, JAMES M. MARKERT, KEVIN A. CASSADY

Accepted to *Advances in Virology*

Human Herpesviridae Methods of Natural Killer Cell Evasion

Carl I. Odom[1], David C. Gaston[1], James M. Markert, M.D.[1,2,4], and Kevin A. Cassady, M.D.[1,3,4]

[1]University of Alabama School of Medicine, University of Alabama at Birmingham, 1600 6th Ave S., CHB 118C, Birmingham, AL 35233-1701
ciodom@uab.edu, dcgaston@uab.edu
[2]Division of Neurosurgery, Department of Surgery, University of Alabama at Birmingham, 1530 3rd Ave S., FOT 1060, Birmingham, AL 35294-3410
markert@uab.edu
[3]Division of Infectious Disease, Department of Pediatrics, University of Alabama at Birmingham, 1600 7th Ave. South, CHB 118, Birmingham, AL 35233-1701
kcasssady@peds.uab.edu
[4]Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham 1530 3rd Ave. South, Birmingham, AL 35294-3410

kcassady@peds.uab.edu, corresponding author

Abstract

Human herpesviruses cause diseases of considerable morbidity and mortality, ranging from encephalitis to hematologic malignancies. As evidence emerges about the role of innate immunity and natural killer (NK) cells in the control of herpesvirus infection, evidence of viral methods of innate immune evasion grows as well. These methods include interference with the ligands on infected cell surfaces that bind NK cell activating or inhibitory receptors. This review summarizes the most extensively studied NK cell receptor/ligand pairs and then describes the methods of NK cell evasion used by all eight herpesviruses through these receptors and ligands. Although great strides have been made in elucidating their mechanisms, there is still a disparity between viruses in the amount of knowledge regarding innate immune evasion. Further research of herpesvirus innate immune evasion can provide insight for circumventing viral mechanisms in future therapies.

I. Introduction (Herpesviridae and Disease)

The human herpes family of viruses includes human cytomegalovirus (HCMV), Kaposi's sarcoma herpesvirus (KSHV), Herpes Simplex Virus types 1 and 2 (HSV-1, 2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesvirus 6 and 7 (HHV6, 7). These viruses share similar characteristics: all contain linear double stranded DNA, are enveloped, and undergo latent and lytic lifecycles. However, there are important differences between these viruses in terms of infection niche and immune evasion strategies for persistent infection.

Herpesviridae evasion of adaptive immune responses has been previously described [1-4]. This article will focus on Herpesvirus innate immune evasion,

specifically viral evasion of the natural killer (NK) cells response. Reviews on broad interactions between viruses and NK cells can be found in references [5-8]. The role of NK cells in controlling herpes viral infections become apparent in consideration that multiple herpes infections have been documented in patients lacking NK cells [9] and evidence of NK activation during viral infection [10-13].

II. NK cells and activation

NK cells are important innate immune cells involved in the regulation of viral infection [14, 15]. They are a lymphocyte subset of the innate immune system that kill without prior exposure and sensitization to antigens via release of granzymes, perforin, TRAIL, and FAS ligand [16]. NK cells are regulated through surface receptor interactions with ligands expressed on stressed cells, such as virally infected or malignantly transformed cells. NK cells possess both activating and inhibitory cell surface receptors - it is the balance of ligand interactions with these receptors that determine NK cell activation. The structures, functions, and signaling mechanisms of these receptors and their ligands are comprehensively reviewed in references [16-21]. In addition to receptor-mediated regulation, cytokines induced during viral infection (IL-15, IL-12, IL-8, IFN-α, and IFN-β), can indirectly activate NK cells as well [6]. A summary of the receptors present on NK cells and associated ligands most relevant to immune evasion by human herpesviruses is provided below.

A. Activating Receptors and Ligands

Natural Killer Group 2 Member D (NKG2D) receptor: NKG2D is a receptor found prominently on NK cells that provides activation signals through the co-receptor DAP-10 upon ligand binding. The ligands that bind NKG2D include: 1) the MHC-I like

molecules MHC-class-I-polypeptide-related sequence A (MICA) and B (MICB), 2) UL16 Binding Proteins (ULBP1-4 and 6) and 3) Retinoic Acid Early Transcript 1G (RAET1G). This interaction with multiple activating ligands is unique to NKG2D and does not occur with the other NK cell activating receptors [22, 23]. Investigators have proposed that this development of multiple activating ligands is a co-evolutionary responses to viral or tumor pressure [22]. The structures of MICA and MICB are similar to MHC-I with alpha domains, however they do not engage β2-microglobulin [24-26]. Surface expression of these ligands is normally absent or low on healthy cells and increases upon events of cellular stress such as viral infection, DNA damage, oxidative stress, and oncogenic stress [22, 27-30]. MICA is noted to have a large polymorphic distribution, with over 73 alleles identified [31]. A subset group of MICA alleles contains a frameshift mutation resulting in a premature stop codon and subsequent truncation of the cytoplasmic C-terminus. Interestingly, the MICA allele *008 encodes a truncated protein and is the most frequently distributed MICA allele in various populations across the world [32-39]. ULBP1-4 and RAET1 have alpha1 and alpha2 domains similar to MICA/B, however unlike MICA/B they do not contain alpha3 domains and their mRNA is expressed at low levels even in normal cells without corresponding surface expression [23, 40].

Natural Cytotoxicity Receptors (NCRs): The NCRs contain immunoglobulin (Ig)-like domains and include NKp30, NKp44, NKp46, and NKp80 [41, 42]. A role for NCRs has been implicated in the prognosis of leukemia [43, 44] and the recognition/killing of various solid tumors [45, 46]. Only NKp30 has a confirmed ligand, the tumor ligand B7-H6 [47, 48]. Additional ligands for the NCRs are unknown, although

possible ligands have been identified and include nuclear factor BAT3 [49] and a number of viral hemaglutinin proteins and heperan sulfate structures [50, 51].

DNAX accessory molecule-1 (DNAM-1): DNAM-1 is a member of the Ig super family that recognizes CD112 (nectin-2) and the polio virus receptor [17]. Similar to other activating receptors, there is expression of DNAM-1 ligands on various tumors resulting in DNAM-1-mediated killing alone or in concert with other receptors [52-56]. Aberrations in DNAM-1 expression or DNAM-1 expressing NK cells have also been linked to a variety of autoimmune diseases [57-59].

B. Inhibitory Receptors and Ligands

The primary inhibitory receptors include the Killer Ig-like Inhibitory Receptors (KIRs) and CD94-NKG2A lectin-like inhibitory receptor. The KIRs and CD94-NKG2A bind to MHC-I molecules and diminish NK cell activation. There has been no evidence to date for their binding to MHC-II molecules. The receptor-ligand interactions for both KIRs and CD94-NKG2A are MHC-I isotype specific [60-62]. In accordance with the "Missing Self" hypothesis first proposed by Karre et al., the lack of MHC-I on target cells removes the inhibitory signals from NK cells, thus leading to unopposed activation [63, 64].

Killer Ig-like Inhibitory Receptors (KIRs): The KIRs are members of the Ig super-family that recognize MHC-I molecules of the HLA-C isotype on surrounding cells [17, 19]. The absence of HLA-C on tumor and virus infected cells can result in loss of NK cell inhibition [21, 65, 66].

Leukocyte Ig-like receptor (LIR)-1: Like the KIRs, LIR-1 contains Ig domains and binds MHC-I, but with a lower affinity than other inhibitory receptors [17, 19]. LIR-1 expression is more variable on NK cells than other immune cells [17].

CD94-NKG2A lectin-like inhibitory receptor: This receptor is a C-lectin like heterodimer that recognizes MHC-I molecules of the HLA-E isotype. Ligation of HLA-E by CD94-NKG2A leads to inhibition of NK cells, yet HLA-E ligation can activate NK cells if CD94 is complexed to NKG2C or -E [17, 19]. Similar to KIRs, the CD94-NKG2A complex results in loss of NK cell inhibition in the absence of HLA-E. However, the uninhibited activity is not as strong as that mediated by KIRs [65].

III. Herpesviridae methods of NK cell evasion

Human herpesviruses have evolved multiple mechanisms to dampen NK cell cytotoxicity, interacting with many of the factors influencing the balance of NK cell activation and inhibition. A summary of these mechanisms is provided in **Table 1**. A number of methods employed by human herpesviruses hinder the expression of NK cell ligands on infected cells. This method of immune evasion has been studied in different members of the herpesvirus family, defining marked similarities and stark differences between family members. Multiple mechanisms offset the indirect NK cell activation prompted by lack of MHC-I surface expression. As many human herpesviruses diminish MHC-I presentation of viral antigens to avoid detection by cytotoxic T lymphocytes, these mechanisms may offset the loss of NK cell inhibition from "missing self" [64, 67].

A. CMV: The HCMV product UL18 is an MHC-I homologue that binds the inhibitory NK cell receptor LIR, possibly as a means of increasing the inhibitory signal [68, 69]. However, inhibition via this ortholog is controversial [70-72]. CMV also

encodes UL40, which stabilizes and promotes surface expression of the HLA-E isotype. This diminishes NK cell activation by increased ligation of the CD94-NKG2A receptor [73-75]. US11 targets HLA-A; US2 and US3 target HLA-A and HLA-G while sparing HLA-E; and US6 targets HLA-A,G, and E for degradation to diminish cytotoxic T cell detection [64, 67, 76].

In addition to inducing an inhibitory response, HCMV also suppresses activating ligands that bind NKG2D. HCMV UL16 binds MICB, ULBP1, and ULBP2 to sequester these proteins in the ER of infected cells but is unable to bind to RAET1G [23, 77-80]. The crystal structure of UL16-MICB complex has been characterized in reference [81]. The HCMV protein UL142 blocks surface expression of some MICA alleles by interacting with the cytosolic carboxyl-terminal region of the transmembrane protein and retaining it in the golgi network, limiting surface expression of the activating ligand. HCMV UL142 cannot downmodulate truncated forms of MICA lacking the intracellular carboxy terminus. It is interesting that of the >70 MICA allelic forms the MICA*008 truncated form is present in a majority of the population and may provide a selective advantage [82, 83]. There is also evidence that HCMV encodes the microRNA mIR-UL112 that decreases MICB production to escape NKG2D detection [84, 85].

In summary, HCMV has multiple means of NK cell ligand manipulation. UL18 is a mock MHC-I molecule that takes advantage of NK cell inhibitory receptors while UL40 prolongs the inhibitory signals of actual host MHC-I molecules. UL16 retains NKG2D ligands (except MICA and ULBP3) to prevent activation while UL142 down-modulates MICA in an allele dependent manner. All of the methods of immune evasion used by CMV are more comprehensively reviewed in references [2, 86].

B. KSHV: KSHV encodes proteins that target MHC-I to prevent viral antigen presentation to T-lymphocytes as does HCMV, however, the molecular mechanisms differ. KSHV K3 and K5 are E3 ubiquitin ligases that transfer ubiquitin to the cytoplasmic tails of proteins [82, 87]. The K5 protein targets HLA-A and HLA-B for endocytosis from cell surfaces while the K3 protein targets HLA-A,B,C, and E [87-89]. No interactions with HLA-G are known.

Like UL142 of CMV, the KSHV protein K5 also blocks surface expression of MICA and MICB, but is unable to down-regulate the MICA*008 allele due to the absence of a cytoplasmic tail and lysine ubiquitin sites [90]. Ubiquitinated MICA proteins are endocytosed from the infected cell surface and retained in cytoplasmic vesicles without increased degradation [90]. K5-mediated down-modulation protects infected cells from NK cell cytotoxicity [90]. K5 also down-modulates the activating ligands B7-2, AICL, and ICAM-1 by a similar mechanism [90, 91]. In contrast to acute lytic infection, chronic infection with KSHV results in higher levels of MHC-I, MICA/B, PVR, and CD112 expression [92]. Akin to HCMV, KSHV encodes the microRNA miRK-12-7 inhibiting MICB expression [93]. Additionally, KSHV has been reported to infect NK cells, leading to down modulation of the activating NCRs and NKG2D receptors on NK cell surfaces [92].

To summarize, KSHV is similar to CMV in that both viruses encode proteins down-modulating MHC-I and MICA/B. The mechanism by which KSHV proteins function diverges from HCMV proteins in that KSHV K3 and K5 ubiquinate and promote endocytosis of targeted proteins, whereas HCMV UL142 and UL16 prevent protein maturation and surface expression. Similar to HCMV UL142, KSHV K5 down-

modulation of MICA is allele dependent. The mechanistic basis is the absence of ubiquitin sites in the truncated cytoplasmic tail. To date there is no evidence of KSHV mechanisms affecting the expression of the ULBP or RAET ligands. KSHV specific immune evasion is reviewed in more detail in references [82, 94].

C. HSV-1 and 2: The exact mechanisms by which HSV-1 modulates NK cell inhibitory and activating ligands are less studied than those for HCMV and KSHV. The HSV-1 and 2 US12 gene product (infected cell protein 47, ICP47) down-modulates MHC-I surface expression by suppressing MHC-I transport from the ER [64, 95]. ICP47 binds to the transporter associated with antigen presentation (TAP) and in doing so inhibits MHC-I antigen loading and expression of antigenic peptides generated by proteasomal degradation that then translocate from the cytosol to the ER lumen [96-99]. Cells engineered to express ICP47 failed to express antigenic peptides [98]. Interestingly, HSV-1 induces expression of certain HLA-G isoforms while decreasing the surface expression of others [100].

The consequences of MHC-I down-modulation on NK cell recognition of HSV-1 and HSV-2 infected cells are controversial. Studies using antibody blocking of KIRs and MHC-I in conjunction with exogenous ICP47 expression suggest that the protective properties of MHC-I via KIR inhibitory signaling are rendered ineffective upon infection with HSV-1 [64]. However, some studies utilizing ICP47 deleted recombinant HSV-1 and anti-KIR antibodies suggest that NK cell inhibitory effects of MHC-I molecules are not significant enough alone to diminish cytotoxicity and that the viral product ICP47 is not necessary in inducing susceptibility to NK cell killing [101]. There are yet other findings that suggest a qualitative change in MHC-I molecules, such as the binding site

shape presented to NK cells during HSV infection rather than the quantity of molecules presented, may contribute to NK recognition and killing [102].

There are few studies examining the influence of HSV-1 and HSV-2 infection upon the NK cell activating ligands. Experiments utilizing HSV-1 recombinants deleted of all IE genes except for ICP0 were able to induce NK cell induced lysis of human fibroblasts. Fibroblasts expressing ICP0 yielded similar results [101]. Infection with these recombinants also demonstrated an up-regulation of unknown ligands binding to NCRs with cyotoxicity dependent upon their presence, yet this only occurred at low multiplicities of infection (MOIs). ICP0 independent mechanisms were reported at higher MOIs [101]. In contrast, studies of HSV effects on NKG2D ligands demonstrated decreased surface expression of MICA in HeLa and U373 cells infected with HSV-1 or HSV-2 with no difference in total protein levels [95]. Interestingly, this HSV mediated down-modulation of MICA occurs with both the full-length and the truncated protein encoded by the MICA*008 allele. This diverges significantly from the inability of HCMV and KSHV to down-modulate truncated MICA variants. Down-modulation of MICA was reported with ICP0 deleted recombinant HSV but not with PAA blocking of late gene expression, suggestive that this phenomenon is dependent on late-gene expression [95].

These studies together suggest HSV-1 and HSV-2 employ both early and late gene modulation of NK activating ligands, each with potentially different consequences for virus infected cells. ICP0 might be sufficient to trigger NK cell cytotoxicity at low MOIs through upregulation of NCR ligands, which would be deleterious for virus survival. Yet at higher MOIs mechanisms other than ICP0 contribute to infected cell

susceptibility. MICA down-modulation was shown to be independent of ICP0 expression and may be caused by late gene products. As posited by Schepis et al, HSV-1 may cause infected cells to be particularly susceptible to NK cell mediated killing early in infection due to ICP0 up-regulation of NCR ligands while attempting immuno-evasion later in infection by NKG2D ligand and MHC-I down-regulation [95]. Although HSV-1 and HSV-2 microRNAs have been documented, none have been found to interfere with NK cell pathways.

D. EBV: As with the previously described human herpesviruses, EBV has methods of interfering with MHC-I to prevent presentation of viral antigens to cytotoxic T-cells. During active B cell infection, EBV expresses a viral homolog of interleukin-10 (vIL-10) [103] and BNLF2a, a lytic-phase viral protein [104, 105]; both have been reported to down-regulate the expression of TAP and in turn decreases surface MHC-I. BILF1 is a protein also expressed during lytic EBV infection that mediates both increased endocytosis/degradation and decreased exocytosis/presentation of MHC-I [106]. Down-modulation of all isotypes of MHC-I during EBV lytic infection, and subsequent decrease in inhibitory binding to KIRs and CD94-NKG2A, results in increased sensitivity to NK cell mediated killing [107]. Instead of down-regulation of activating ligands to off-set this decrease in inhibition, the same studies found an increase in ULBP1 and CD112 expression that contributed to NK cell activation [107]. The only reported mechanism to possibly offset this indirect NK cell activation is a microRNA (miR-BART2-5p) inhibiting MICB expression [93]. EBV may possibly interfere with NKG2D activation through down-modulation of the NKG2D receptor itself via indoleamine-2, 3-dioxygenase metabolites, although the functional consequences have yet to be reported

[108]. Similar to KSHV, EBV can infect NK cells, causing aberrantly high expression of the inhibitory CD94-NKG2A receptor but diminished expression of the KIRs [109].

E. HHV-6 & HHV-7: The involvement of NK cells in the control of HHV-6 and HHV-7 infection has been documented through studies of IL-2 and IL-15 enhancement of cytotoxicity [110-112]. However, the only documented HHV-7 protein involved in NK cell ligand modulation is U21, a transmembrane protein capable of down-modulating both MHC-I and MICA/B. U21 binds and re-directs MHC-I trafficking to lysosomal compartments most similarly to HCMV MHC-I interfering proteins [113-115]. The effect on NK cell killing through this method has not been established. U21 also binds to ULBP1 for redirection to lysosomes and decreases surface MICA and MICB by an undefined mechanism that results in decreased NK cell cytotoxicity [116]. The A and B variants of HHV-6 express proteins analogous to U21 that also bind MHC-I for lysosome redirection [117], but the effect on NK cells or identification of mechanisms affecting activating ligands has not been established.

F. VZV: Although NK cells have long been implicated in the control of VZV infection [118-120], specific interactions with infected cells through NK cell receptors have not been extensively studied. VZV down-regulates MHC-I on infected cell surfaces via the viral protein kinase ORF66, leading to retention of MHC-I molecules in the golgi [121, 122]. However, any functional consequences of ORF66 on NK cell recognition and killing have not been demonstrated. Likewise, no methods of VZV interference with NK cell activating ligands have been reported to date.

IV. Conclusion

Human herpesvirurses possess multiple mechanisms for evading both innate and adaptive immune responses. A summary of NK cell receptors, their ligands, and viral mechanisms interfering with each is provided in **Table 1**. A primary point of interest is the diversity of NK cell evasion mechanisms employed by human herpesviruses. Other than mechanisms shared by the highly similar HSV-1 and HSV-2, the human herpesviruses have evolved different mechanisms for subverting the immune response. It is also notable that these immunoevasion mechanisms do not group with herpesvirus subfamilies. For example, HCMV (a betaherpesvirus) and KSHV (a gammaherpesvirus) both encode microRNA's targeting MICB, yet down-modulate MICA via different mechanisms. The NK cell evasion mechanisms are unique to each human herpesvirus likely reflect selection pressures encountered in the various infection niches occupied by the viruses. The lack of well-defined mechanisms of NK cell immunoevasion by given herpesviruses (i.e. HSV and VZV) is puzzling. There is well-documented persistence of HSV in patients with NK cell defects and the importance of NK cell involvement in the control of disease. Continuing research will likely reveal as yet unknown mechanisms of immunoevasion by the alpha herpes viruses.

Human herpesviruses cause substantial morbidity and mortality. HSV-1 is regarded as one of the most common causes of viral encephalitis, an infection carrying significant risk of mortality [123, 124]. EBV, HCMV, and KSHV infections have the potential to not only cause severe manifestations during acute infection, but also the development of hematologic or solid malignancies [125, 126]. A variety of herpesviruses also cause cutaneous and ocular infections with potential for life-long morbidity [127-129]. Thorough knowledge of specific viral immune evasion mechanisms may provide

avenues for developing more effective therapies against disease related to human herpesviruses. Understanding NK cell evasion may improve oncolytic herpesvirus therapies for cancer [130-132]. Insight into viral abilities to evade the immune system may also yield better markers for clinical prognosis and monitoring of active and latent infection [109, 133]. Continued research into these mechanisms of NK cell evasion will not only deepen basic understandings of human herpesviruses, but may also serve to ultimately alleviate disease burden and guide strategies for clearance of persistent infection in immunocompromised patients

Acknowledgments

The authors would like to thank Dr. Yancey Gillespie for his input and facilitation of this review. This publication was supported by NIH P01-CA 071933-13 (K. A. Cassady and J. M. Markert), P20CA151129 (K. A. Cassady and J. M. Markert), NIH training grant 5T32 NS048039 (D. C. Gaston), and the National Center for Advancing Translational Sciences 5TL1 RR025775-04 (C. I. Odom).

References

1. Chan, T., et al., *Innate and adaptive immunity against herpes simplex virus type 2 in the genital mucosa.* J Reprod Immunol, 2011. **88**(2): p. 210-8.
2. Jackson, S.E., G.M. Mason, and M.R. Wills, *Human cytomegalovirus immunity and immune evasion.* Virus Res, 2010. **157**(2): p. 151-60.
3. Coscoy, L., *Immune evasion by Kaposi's sarcoma-associated herpesvirus.* Nat Rev Immunol, 2007. **7**(5): p. 391-401.
4. Wiertz, E.J., et al., *Herpesvirus interference with major histocompatibility complex class II-restricted T-cell activation.* J Virol, 2007. **81**(9): p. 4389-96.
5. Jonjic, S., et al., *Immune evasion of natural killer cells by viruses.* Curr Opin Immunol, 2008. **20**(1): p. 30-8.
6. Lanier, L.L., *Evolutionary struggles between NK cells and viruses.* Nat Rev Immunol, 2008. **8**(4): p. 259-68.
7. Groth, A., et al., *Mechanisms of tumor and viral immune escape from natural killer cell-mediated surveillance.* J Innate Immun, 2011. **3**(4): p. 344-54.
8. Lisnic, V.J., A. Krmpotic, and S. Jonjic, *Modulation of natural killer cell activity by viruses.* Curr Opin Microbiol, 2010. **13**(4): p. 530-9.
9. Biron, C.A., K.S. Byron, and J.L. Sullivan, *Severe herpesvirus infections in an adolescent without natural killer cells.* N Engl J Med, 1989. **320**(26): p. 1731-5.

10. Fitzgerald, P.A., M. Mendelsohn, and C. Lopez, *Human natural killer cells limit replication of herpes simplex virus type 1 in vitro.* J Immunol, 1985. **134**(4): p. 2666-72.
11. Paya, C.V., R.A. Schoon, and P.J. Leibson, *Alternative mechanisms of natural killer cell activation during herpes simplex virus infection.* J Immunol, 1990. **144**(11): p. 4370-5.
12. Fitzgerald-Bocarsly, P., et al., *Immediate-early gene expression is sufficient for induction of natural killer cell-mediated lysis of herpes simplex virus type 1-infected fibroblasts.* J Virol, 1991. **65**(6): p. 3151-60.
13. Lopez, C., et al., *Studies of the cell lineage of the effector cells that spontaneously lyse HSV-1 infected fibroblasts (NK(HSV-1)).* J Immunol, 1982. **129**(2): p. 824-8.
14. Marras, F., F. Bozzano, and A. De Maria, *Involvement of activating NK cell receptors and their modulation in pathogen immunity.* J Biomed Biotechnol, 2011. **2011**: p. 152430.
15. Lee, S.H. and C.A. Biron, *Here today--not gone tomorrow: roles for activating receptors in sustaining NK cells during viral infections.* Eur J Immunol, 2010. **40**(4): p. 923-32.
16. Farag, S.S. and M.A. Caligiuri, *Human natural killer cell development and biology.* Blood Rev, 2006. **20**(3): p. 123-37.
17. Lanier, L.L., *NK cell recognition.* Annu Rev Immunol, 2005. **23**: p. 225-74.
18. Bryceson, Y.T., et al., *Molecular mechanisms of natural killer cell activation.* J Innate Immun, 2011. **3**(3): p. 216-26.
19. Joyce, M.G. and P.D. Sun, *The structural basis of ligand recognition by natural killer cell receptors.* J Biomed Biotechnol, 2011. **2011**: p. 203628.
20. Zafirova, B., et al., *Regulation of immune cell function and differentiation by the NKG2D receptor.* Cell Mol Life Sci, 2011. **68**(21): p. 3519-29.
21. Purdy, A.K. and K.S. Campbell, *Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR).* Cancer Biol Ther, 2009. **8**(23): p. 2211-20.
22. Eagle, R.A. and J. Trowsdale, *Promiscuity and the single receptor: NKG2D.* Nat Rev Immunol, 2007. **7**(9): p. 737-44.
23. Raulet, D.H., *Roles of the NKG2D immunoreceptor and its ligands.* Nat Rev Immunol, 2003. **3**(10): p. 781-90.
24. Li, P., et al., *Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand.* Immunity, 1999. **10**(5): p. 577-84.
25. Li, P., et al., *Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA.* Nat Immunol, 2001. **2**(5): p. 443-51.
26. Holmes, M.A., et al., *Structural studies of allelic diversity of the MHC class I homolog MIC-B, a stress-inducible ligand for the activating immunoreceptor NKG2D.* J Immunol, 2002. **169**(3): p. 1395-400.
27. Moretta, L., et al., *Surface NK receptors and their ligands on tumor cells.* Semin Immunol, 2006. **18**(3): p. 151-8.
28. Stern-Ginossar, N. and O. Mandelboim, *An integrated view of the regulation of NKG2D ligands.* Immunology, 2009. **128**(1): p. 1-6.
29. Gasser, S., et al., *The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor.* Nature, 2005. **436**(7054): p. 1186-90.

30. Yamamoto, K., et al., *Oxidative stress increases MICA and MICB gene expression in the human colon carcinoma cell line (CaCo-2).* Biochim Biophys Acta, 2001. **1526**(1): p. 10-2.
31. Frigoul, *MICA: standardized IMGT allele nomenclature, polymorphisms and diseases.* recent res. devel. human genet., 2005. **3**: p. 95-145.
32. Petersdorf, E.W., et al., *Population study of allelic diversity in the human MHC class I-related MIC-A gene.* Immunogenetics, 1999. **49**(7-8): p. 605-12.
33. Zhu, F., et al., *Distribution of MICA diversity in the Chinese Han population by polymerase chain reaction sequence-based typing for exons 2-6.* Tissue Antigens, 2009. **73**(4): p. 358-63.
34. Marin, M.L., et al., *MICA polymorphism in a sample of the Sao Paulo population, Brazil.* Eur J Immunogenet, 2004. **31**(2): p. 63-71.
35. Gao, X., et al., *Diversity of MICA and linkage disequilibrium with HLA-B in two North American populations.* Hum Immunol, 2006. **67**(3): p. 152-8.
36. Lucas, D., et al., *Allelic diversity of MICA gene and MICA/HLA-B haplotypic variation in a population of the Murcia region in southeastern Spain.* Hum Immunol, 2008. **69**(10): p. 655-60.
37. Tian, W., et al., *MICA, HLA-B haplotypic variation in five population groups of sub-Saharan African ancestry.* Genes Immun, 2003. **4**(7): p. 500-5.
38. Cambra, A., et al., *MICA-HLA-B haplotype diversity and linkage disequilibrium in a population of Jewish descent from Majorca (the Balearic Islands).* Hum Immunol, 2009. **70**(7): p. 513-7.
39. Durmanova, V., et al., *Characterization of MICA gene polymorphism of HLA complex in the Slovak population.* Ann Hum Biol, 2011. **38**(5): p. 570-6.
40. Cosman, D., et al., *ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor.* Immunity, 2001. **14**(2): p. 123-33.
41. Bottino, C., et al., *The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering.* Hum Immunol, 2000. **61**(1): p. 1-6.
42. Vitale, M., et al., *Identification of NKp80, a novel triggering molecule expressed by human NK cells.* Eur J Immunol, 2001. **31**(1): p. 233-42.
43. Fauriat, C., et al., *Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction.* Blood, 2007. **109**(1): p. 323-30.
44. Costello, R.T., et al., *Expression of natural killer cell activating receptors in patients with chronic lymphocytic leukaemia.* Immunology, 2012. **135**(2): p. 151-7.
45. Glasner, A., et al., *Recognition and Prevention of Tumor Metastasis by the NK Receptor NKp46/NCR1.* J Immunol, 2012(188): p. 000-000.
46. Morgado, S., et al., *NK cell recognition and killing of melanoma cells is controlled by multiple activating receptor-ligand interactions.* J Innate Immun, 2011. **3**(4): p. 365-73.
47. Li, Y., Q. Wang, and R.A. Mariuzza, *Structure of the human activating natural cytotoxicity receptor NKp30 bound to its tumor cell ligand B7-H6.* J Exp Med, 2011. **208**(4): p. 703-14.
48. Kaifu, T., et al., *B7-H6/NKp30 interaction: a mechanism of alerting NK cells against tumors.* Cell Mol Life Sci, 2011. **68**(21): p. 3531-9.

49. Pogge von Strandmann, E., et al., *Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells.* Immunity, 2007. **27**(6): p. 965-74.
50. Hecht, M.L., et al., *Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences.* J Proteome Res, 2009. **8**(2): p. 712-20.
51. Jarahian, M., et al., *Activation of natural killer cells by newcastle disease virus hemagglutinin-neuraminidase.* J Virol, 2009. **83**(16): p. 8108-21.
52. Carlsten, M., et al., *DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells.* Cancer Res, 2007. **67**(3): p. 1317-25.
53. Castriconi, R., et al., *Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction.* Cancer Res, 2004. **64**(24): p. 9180-4.
54. Chan, C.J., et al., *DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases.* J Immunol, 2010. **184**(2): p. 902-11.
55. Lakshmikanth, T., et al., *NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo.* J Clin Invest, 2009. **119**(5): p. 1251-63.
56. Zhang, Z., et al., *Identification and functional analysis of ligands for natural killer cell activating receptors in colon carcinoma.* Tohoku J Exp Med, 2012. **226**(1): p. 59-68.
57. Huang, Z., et al., *Involvement of CD226+ NK cells in immunopathogenesis of systemic lupus erythematosus.* J Immunol, 2011. **186**(6): p. 3421-31.
58. Maiti, A.K., et al., *Non-synonymous variant (Gly307Ser) in CD226 is associated with susceptibility to multiple autoimmune diseases.* Rheumatology (Oxford), 2010. **49**(7): p. 1239-44.
59. Tjon, J.M., et al., *DNAM-1 mediates epithelial cell-specific cytotoxicity of aberrant intraepithelial lymphocyte lines from refractory celiac disease type II patients.* J Immunol, 2011. **186**(11): p. 6304-12.
60. Wagtmann, N., et al., *Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer.* Immunity, 1995. **3**(6): p. 801-9.
61. Braud, V.M., et al., *HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C.* Nature, 1998. **391**(6669): p. 795-9.
62. Borrego, F., et al., *Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis.* J Exp Med, 1998. **187**(5): p. 813-8.
63. Karre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. **319**(6055): p. 675-8.
64. Huard, B. and K. Fruh, *A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells.* Eur J Immunol, 2000. **30**(2): p. 509-15.
65. Yu, J., et al., *Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands.* J Immunol, 2007. **179**(9): p. 5977-89.

66. van Bergen, J., et al., *Cutting edge: killer Ig-like receptors mediate "missing self" recognition in vivo.* J Immunol, 2009. **182**(5): p. 2569-72.
67. Lin, A., H. Xu, and W. Yan, *Modulation of HLA expression in human cytomegalovirus immune evasion.* Cell Mol Immunol, 2007. **4**(2): p. 91-8.
68. Reyburn, H.T., et al., *The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells.* Nature, 1997. **386**(6624): p. 514-7.
69. Yang, Z. and P.J. Bjorkman, *Structure of UL18, a peptide-binding viral MHC mimic, bound to a host inhibitory receptor.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10095-100.
70. Leong, C.C., et al., *Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog.* J Exp Med, 1998. **187**(10): p. 1681-7.
71. Cosman, D., et al., *A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules.* Immunity, 1997. **7**(2): p. 273-82.
72. Wills, M.R., et al., *Human cytomegalovirus encodes an MHC class I-like molecule (UL142) that functions to inhibit NK cell lysis.* J Immunol, 2005. **175**(11): p. 7457-65.
73. Tomasec, P., et al., *Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40.* Science, 2000. **287**(5455): p. 1031.
74. Ulbrecht, M., et al., *Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis.* J Immunol, 2000. **164**(10): p. 5019-22.
75. Wang, E.C., et al., *UL40-mediated NK evasion during productive infection with human cytomegalovirus.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7570-5.
76. Llano, M., et al., *Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets.* Eur J Immunol, 2003. **33**(10): p. 2744-54.
77. Welte, S.A., et al., *Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein.* Eur J Immunol, 2003. **33**(1): p. 194-203.
78. Wu, J., et al., *Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein.* J Immunol, 2003. **170**(8): p. 4196-200.
79. Dunn, C., et al., *Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity.* J Exp Med, 2003. **197**(11): p. 1427-39.
80. Wittenbrink, M., J. Spreu, and A. Steinle, *Differential NKG2D binding to highly related human NKG2D ligands ULBP2 and RAET1G is determined by a single amino acid in the alpha2 domain.* Eur J Immunol, 2009. **39**(6): p. 1642-51.
81. Muller, S., et al., *Structure of the HCMV UL16-MICB complex elucidates select binding of a viral immunoevasin to diverse NKG2D ligands.* PLoS Pathog, 2010. **6**(1): p. e1000723.
82. Thomas, M., M. Wills, and P.J. Lehner, *Natural killer cell evasion by an E3 ubiquitin ligase from Kaposi's sarcoma-associated herpesvirus.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 459-63.

83. Chalupny, N.J., et al., *Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142.* Biochem Biophys Res Commun, 2006. **346**(1): p. 175-81.
84. Colonna, M., S. Jonjic, and C. Watzl, *Natural killer cells: fighting viruses and much more.* Nat Immunol, 2011. **12**(2): p. 107-10.
85. Stern-Ginossar, N., et al., *Host immune system gene targeting by a viral miRNA.* Science, 2007. **317**(5836): p. 376-81.
86. Mocarski, E.S., Jr., *Immune escape and exploitation strategies of cytomegaloviruses: impact on and imitation of the major histocompatibility system.* Cell Microbiol, 2004. **6**(8): p. 707-17.
87. Ishido, S., et al., *Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins.* J Virol, 2000. **74**(11): p. 5300-9.
88. Haque, M., et al., *Major histocompatibility complex class I molecules are down-regulated at the cell surface by the K5 protein encoded by Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8.* J Gen Virol, 2001. **82**(Pt 5): p. 1175-80.
89. Bartee, E., et al., *Downregulation of Major Histocompatibility Complex Class I by Human Ubiquitin Ligases Related to Viral Immune Evasion Proteins.* J Virol, 2004. **78**(3): p. 1109-1120.
90. Thomas, M., et al., *Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1656-61.
91. Ishido, S., et al., *Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein.* Immunity, 2000. **13**(3): p. 365-74.
92. Dupuy, S., et al., *Human Herpesvirus 8 (HHV8) sequentially shapes the NK cell repertoire during the course of asymptomatic infection and Kaposi sarcoma.* PLoS Pathog, 2012. **8**(1): p. e1002486.
93. Nachmani, D., et al., *Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells.* Cell Host Microbe, 2009. **5**(4): p. 376-85.
94. Means, R.E., et al., *Immune evasion strategies of Kaposi's sarcoma-associated herpesvirus.* Curr Top Microbiol Immunol, 2002. **269**: p. 187-201.
95. Schepis, D., et al., *Herpes simplex virus infection downmodulates NKG2D ligand expression.* Scand J Immunol, 2009. **69**(5): p. 429-36.
96. Hill, A.B., et al., *HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2.* J Immunol, 1994. **152**(6): p. 2736-41.
97. Hill, A., et al., *Herpes simplex virus turns off the TAP to evade host immunity.* Nature, 1995. **375**(6530): p. 411-5.
98. York, I.A., et al., *A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes.* Cell, 1994. **77**(4): p. 525-35.
99. Tomazin, R., et al., *Herpes simplex virus type 2 ICP47 inhibits human TAP but not mouse TAP.* J Virol, 1998. **72**(3): p. 2560-3.
100. Lafon, M., et al., *Modulation of HLA-G expression in human neural cells after neurotropic viral infections.* J Virol, 2005. **79**(24): p. 15226-37.

101. Chisholm, S.E., et al., *Expression of ICP0 is sufficient to trigger natural killer cell recognition of herpes simplex virus-infected cells by natural cytotoxicity receptors.* J Infect Dis, 2007. **195**(8): p. 1160-8.
102. Kaufman, D.S., R.A. Schoon, and P.J. Leibson, *Role for major histocompatibility complex class I in regulating natural killer cell-mediated killing of virus-infected cells.* Proc Natl Acad Sci U S A, 1992. **89**(17): p. 8337-41.
103. Sin, S.H. and D.P. Dittmer, *Cytokine homologs of human gammaherpesviruses.* J Interferon Cytokine Res, 2012. **32**(2): p. 53-9.
104. Croft, N.P., et al., *Stage-specific inhibition of MHC class I presentation by the Epstein-Barr virus BNLF2a protein during virus lytic cycle.* PLoS Pathog, 2009. **5**(6): p. e1000490.
105. Horst, D., et al., *EBV protein BNLF2a exploits host tail-anchored protein integration machinery to inhibit TAP.* J Immunol, 2011. **186**(6): p. 3594-605.
106. Zuo, J., et al., *The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways.* J Virol, 2011. **85**(4): p. 1604-14.
107. Pappworth, I.Y., E.C. Wang, and M. Rowe, *The switch from latent to productive infection in epstein-barr virus-infected B cells is associated with sensitization to NK cell killing.* J Virol, 2007. **81**(2): p. 474-82.
108. Song, H., et al., *IDO metabolite produced by EBV-transformed B cells inhibits surface expression of NKG2D in NK cells via the c-Jun N-terminal kinase (JNK) pathway.* Immunol Lett, 2011. **136**(2): p. 187-93.
109. Sawada, A., et al., *NK-cell repertoire is feasible for diagnosing Epstein-Barr virus-infected NK-cell lymphoproliferative disease and evaluating the treatment effect.* Am J Hematol, 2006. **81**(8): p. 576-81.
110. Kida, K., R. Isozumi, and M. Ito, *Killing of human Herpes virus 6-infected cells by lymphocytes cultured with interleukin-2 or -12.* Pediatr Int, 2000. **42**(6): p. 631-6.
111. Flamand, L., I. Stefanescu, and J. Menezes, *Human herpesvirus-6 enhances natural killer cell cytotoxicity via IL-15.* J Clin Invest, 1996. **97**(6): p. 1373-81.
112. Atedzoe, B.N., A. Ahmad, and J. Menezes, *Enhancement of natural killer cell cytotoxicity by the human herpesvirus-7 via IL-15 induction.* J Immunol, 1997. **159**(10): p. 4966-72.
113. Glosson, N.L., et al., *Insight into the mechanism of human herpesvirus 7 U21-mediated diversion of class I MHC molecules to lysosomes.* J Biol Chem, 2010. **285**(47): p. 37016-29.
114. May, N.A., N.L. Glosson, and A.W. Hudson, *Human herpesvirus 7 u21 downregulates classical and nonclassical class I major histocompatibility complex molecules from the cell surface.* J Virol, 2010. **84**(8): p. 3738-51.
115. Hudson, A.W., et al., *The ER-lumenal domain of the HHV-7 immunoevasin U21 directs class I MHC molecules to lysosomes.* Traffic, 2003. **4**(12): p. 824-37.
116. Schneider, C.L. and A.W. Hudson, *The human herpesvirus-7 (HHV-7) U21 immunoevasin subverts NK-mediated cytoxicity through modulation of MICA and MICB.* PLoS Pathog, 2011. **7**(11): p. e1002362.

117. Glosson, N.L. and A.W. Hudson, *Human herpesvirus-6A and -6B encode viral immunoevasins that downregulate class I MHC molecules.* Virology, 2007. **365**(1): p. 125-35.
118. Tilden, A.B., et al., *Demonstration of NK cell-mediated lysis of varicella-zoster virus (VZV)-infected cells: characterization of the effector cells.* J Immunol, 1986. **136**(11): p. 4243-8.
119. Ito, M., et al., *Interleukin 2 enhances natural killing of varicella-zoster virus-infected targets.* Clin Exp Immunol, 1986. **65**(1): p. 182-9.
120. Hayward, A.R., M. Herberger, and M. Lazslo, *Cellular interactions in the lysis of varicella-zoster virus infected human fibroblasts.* Clin Exp Immunol, 1986. **63**(1): p. 141-6.
121. Abendroth, A., et al., *Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells.* J Virol, 2001. **75**(10): p. 4878-88.
122. Eisfeld, A.J., et al., *Downregulation of class I major histocompatibility complex surface expression by varicella-zoster virus involves open reading frame 66 protein kinase-dependent and -independent mechanisms.* J Virol, 2007. **81**(17): p. 9034-49.
123. Rozenberg, F., C. Deback, and H. Agut, *Herpes simplex encephalitis : from virus to therapy.* Infect Disord Drug Targets, 2011. **11**(3): p. 235-50.
124. Stahl, J.P., et al., *Epidemiology of viral encephalitis in 2011.* Med Mal Infect, 2011. **41**(9): p. 453-64.
125. Gantt, S. and C. Casper, *Human herpesvirus 8-associated neoplasms: the roles of viral replication and antiviral treatment.* Curr Opin Infect Dis, 2011. **24**(4): p. 295-301.
126. Taylor, G.S. and D.J. Blackbourn, *Infectious agents in human cancers: lessons in immunity and immunomodulation from gammaherpesviruses EBV and KSHV.* Cancer Lett, 2011. **305**(2): p. 263-78.
127. Chisholm, C. and L. Lopez, *Cutaneous infections caused by Herpesviridae: a review.* Arch Pathol Lab Med, 2011. **135**(10): p. 1357-62.
128. Al-Dujaili, L.J., et al., *Ocular herpes simplex virus: how are latency, reactivation, recurrent disease and therapy interrelated?* Future Microbiol, 2011. **6**(8): p. 877-907.
129. Berardi, A., et al., *Neonatal herpes simplex virus.* J Matern Fetal Neonatal Med, 2011. **24 Suppl 1**: p. 88-90.
130. Fulci, G., et al., *Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(34): p. 12873-8.
131. Fulci, G., et al., *Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses.* Cancer Res, 2007. **67**(19): p. 9398-406.
132. Wakimoto, H., et al., *Altered expression of antiviral cytokine mRNAs associated with cyclophosphamide's enhancement of viral oncolysis.* Gene Ther, 2004. **11**(2): p. 214-23.
133. Haedicke, W., et al., *Expression of CD94/NKG2A and killer immunoglobulin-like receptors in NK cells and a subset of extranodal cytotoxic T-cell lymphomas.* Blood, 2000. **95**(11): p. 3628-30.

134. Ashiru, O., et al., *NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142.* J Virol, 2009. **83**(23): p. 12345-54.

Table 1. Summary of Known Interactions between NK cell Receptors, Ligands, and Herpesvirus Immunoevasins

Major Receptors	Ligand	Virus	Immunoevasin	Mechanism	References
Activating					
NKG2D	MICA	HCMV	UL142	Internal Retention	[83, 134]
		KSHV	K5	Ubiquitination/Sequestration	[82, 90]
		HSV	?	?	[95, 101]
		HHV-7	U21	?	[116]
	MICB	HCMV	UL16	Internal Retention	[23, 77-79]
		HCMV	miR-UL112	Translational Downregulation	[85]
		KSHV	K5	Ubiquitination/Sequestration	[82, 90]
		KSHV	miRK12-7	Translational Downregulation	[93]
		HSV	?	?	[95, 101]
		EBV	miR-BART2-5p	Translational Downregulation	[93]
		HHV-7	U21	?	[116]
	ULBP1-4	HCMV	UL16	Internal Retention	[23, 77-79]
		HSV	?	?	[95]
		HHV-7	U21	Lysosomal Degradation	[116]
NCRs	AICL	KSHV	K5		[90]
DNAM-1	PVR	?	?		
	CD112	?	?		
Inhibitory					
LIR-1, KIRs, CD94/NKG2A	MHC-I	HCMV	UL18	MHC-I Homologue	[68, 69]
		HCMV	UL40	Signal Prolongation	[73-75]
		HCMV	US2, US3, US6, US11	Retention/Degradation	[64, 67]
		KSHV	K5, K3	Endocytosis	[87-89]
		HSV	ICP47	TAP Interference	[96-98]
		EBV	vIL-10	IL-10 Homolog	[103]
		EBV	BNLF2a	TAP Interference	[104, 105]
		EBV	BILF1	Endocytosis/Degradation	[106]
		HHV-6	U21 analogues	Lysosomal Degradation	[117]
		HHV-7	U21	Lysosomal Degradation	[113-115]
		VZV	ORF66	Internal Retention	[121, 122]

SURFACE DOWN-MODULATION OF MICA IN MALIGNANT GLIOMA CELLS BY HERPES SIMPLEX VIRUS

by

CARL I. ODOM, DAVID C. GASTON, JAMES M. MARKERT, KEVIN A. CASSADY

In preparation for *Journal of Virology*

Format adapted for thesis

Surface Down-Modulation of MICA in Malignant Glioma Cells by Herpes Simplex Virus

Carl I. Odom[1], David C. Gaston[1], James M. Markert, M.D.[1,2,4], and Kevin A. Cassady, M.D.#[1,3,4]

[1]University of Alabama School of Medicine, University of Alabama at Birmingham, 1600 6th Ave S., CHB 118C, Birmingham, AL 35233-1701
[2]Division of Neurosurgery, Department of Surgery, University of Alabama at Birmingham, 1530 3rd Ave S., FOT 1060, Birmingham, AL 35294-3410
[3]Division of Infectious Disease, Department of Pediatrics, University of Alabama at Birmingham, 1600 7th Ave. South, CHB 118, Birmingham, AL 35233-1701
[4]Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham 1530 3rd Ave. South, Birmingham, AL 35294-3410

kcassady@peds.uab.edu, corresponding author

Abstract word count: 255
Text word count: 3,955

Abstract

Human herpes viruses cause diseases ranging from encephalitis to malignancies. These viruses also manipulate immune responses to evade detection. Cytomegalovirus and Kaposi's sarcoma herpes virus down-modulate MHC class I polypeptide-related sequence A (MICA), a natural killer (NK) cell activating ligand. The mechanisms of down-modulation for these viruses are well studied. HSV-1 has recently been shown to down-modulate surface MICA in HeLa cells, but the exact mechanism is unknown. We hypothesize that HSV-1 infection down-modulates MICA from the surface of HeLa and malignant glioma cells in order to prevent NK cell activation, which in turn could affect the immune mechanisms of oncolytic virus therapy. Flow cytometery and immunofluorescence performed with intact and permeabilized HeLa and U373 glioma cells measured surface and total MICA during HSV-1 infection. MICA mRNA levels have been shown to decrease prior to surface protein levels. To test the contribution of the HSV-1 ribonuclease Virion Host Shut-off (VHS) protein to MICA mRNA degradation and protein down-modulation, RT-qPCR and flow cytometery were performed with HSV-1 and a VHS deleted HSV recombinant. To test the protein specificity of MICA surface down-modulation, flow cytometery experiments measuring transferrin receptor were compared to those for MICA. Results demonstrate surface and total MICA protein down-modulation during HSV-1 infection and suggest degradation and not sequestering as previously proposed. Levels of MICA mRNA in the presence of VHS compared to levels upon deletion suggest that VHS contributes to MICA mRNA degradation but is not the primary mechanism of down-modulation. Measurements of transferrin receptor during infection suggest that down-modulation is MICA specific.

Introduction

The family of herpresvirdae is comprised of viruses that share the characteristics of linear double stranded DNA genomes, membrane envelopes, and ability to undergo latent and lytic lifecycles. As a group they cause significant morbidity and mortality with their acute and chronic infections. These viruses also share the ability to evade the immune system by a variety of mechanisms. All herpes viruses have mechanisms for down-modulating MHC-I, a ubiquitous surface protein involved in antigen presentation to CD8 cytotoxic T cells, an important component of the adaptive immune system (26). In order to evade detection by the innate immune system during infection, herpes viruses have also evolved mechanisms manipulating the regulation of natural killer (NK) cells. The involvement of the innate immune response against these viruses becomes apparent in the context of NK cell activation during herpes infection (10, 11) and recurrent herpes infections in patients lacking NK cells (4).

The regulation of NK cell responses against infection or oncogenesis is based in part on the balance of signals from activating or inhibitory receptors on the NK cell surface. Net NK cell response is dependent upon the ligands for these receptors encountered on surrounding cells (5, 12). As an example, MHC-I also acts as a ligand for a number of NK cell inhibitory receptors in addition to antigen presentation to T cells (13). One of the NK cell activating receptors is the Natural Killer Group 2 Member D (NKG2D) receptor which interacts with numerous ligands, one being MHC-class-I-polypeptide-related sequence A (MICA) (14, 16). MICA and other ligands for NK activating receptors are stress proteins expressed during times of cellular infection or oncogenesis to initiate cytotoxicity (21). In response to this, many viruses have evolved mechanisms specifically decreasing presentation of MICA and other ligands to prevent

NK cell activation. The U_L142 protein of human cytomegalovirus (CMV) is an immunoevasin that binds to MICA and retains it within the golgi network (2, 6). Kaposi's sarcoma herpes virus (KSHV) expresses K5, which mediates ubiquitination and retention of MICA to prevent surface expression (24, 25). The mechanisms by which CMV and KSHV prevent MICA presentation and NK cell activation are well studied. There is recent evidence of herpes simplex virus type-1 (HSV-1) decreasing surface MICA upon infection, but the exact mechanism of down-modulation is still unknown (19).

HSV-1 is an α-herpesvirus that causes cutaneous/ocular lesions and viral encephalitis with a high risk of mortality (3, 7, 18). There are few studies examining the behavior of NK cell ligands during HSV-1 infection and conflicting results concerning which ligands and gene products are involved in altered susceptibility to NK cells. It has been demonstrated that HSV-1 infected human fibroblasts are more susceptible to NK cell lysis, and it was originally proposed that this occurred via the natural cytotoxicity receptors (NCRs) and is dependent upon the immediate-early gene ICP0 at a low multiplicity of infection (MOI) (8). However, at a higher MOI there are ICP0 independent mechanisms observed. A decrease in surface MICA on HeLa cells during HSV-1 infection has been observed with no accompanying changes in the other NK cell activating ligands ULBP 1-3 or MICB (19). This decrease was proposed to be caused by viral late genes independent of ICP0 and was observed only for surface protein and not for total protein in permeabilized cells (19).

In this article, we describe a possible mechanism for the down-modulation of MICA during HSV-1 infection. We hypothesize that HSV-1 decreases surface MICA through a post-translational mechanism, thus lowering the amount available for transport to the surface. Our studies also show that Virion Host Shut-off (VHS) protein

significantly decreases the quantity of MICA mRNA upon HSV infection but does not affect MICA surface down-modulation. Transferrin receptor is another transmembrane surface protein that does not associate with lipid rafts unlike MICA (9), and thus has been used previously as a control for the cell surface location and expression comparison to NK cell ligands (20). Comparison of MICA expression to that of transferrin receptor during HSV-1 infection suggests that MICA surface down-modulation is protein specific. Our studies performed within malignant glioma cells also demonstrate a potential for circumventing the current limitations of oncolytic virus therapy against glioblastoma multiforme (GBM).

Materials and Methods

Cell lines and Virus Strains. The HeLa cell prototypical epithelial tumor line and U-373MG astroglioma cell lines were used in virus infection assays. These lines along with U-251MG, U-87MG, U-118MG, and D54MG glioma cell lines were additionally used in western blot and sequencing assays. The cells were maintained in Dulbecco's Modified Eagle's Medium mixed 50: 50 with Ham's Nutrient Mixture F-12 (DMEM/F12) (Cellgro, Corning) with 7% Fetal Bovine Serum (FBS) for growth and 1% during infection. The 293-T cell line was used for transfection assays and was maintained in MEM with 7% Fetal Bovine Serum. All cell lines were maintained in 37°C and 5% CO_2 humidified atmosphere incubators.

Virus strains and recombinants used include the wild type HSV-1(F) virus, R2621 (HSV-1 ΔUL41), M2001 (HSV-1 :: GFP), and C101 (HSV-1 $\Delta\gamma_1$34.5 :: Green Fluorescent Protein). Viruses were maintained in milk stocks, and infections were carried out in the previously indicated media for 2 hours and then refreshed growth media for the indicated time post-infection.

Validation of anti-MICA Antibodies. Recombinant human MICA (clone BC016929) in vector pCMV-Sport6 was purchased (MHS1010-58438, Open Biosystems). Primers for Myc-tag expression (forward: GATCTTCCTCTGAGATAAGCTTCTGATC; reverse: CTAGAAGGAGACTCTATTCGAAGACTAG) were purchased (Eurofin MGW Operon), diluted to 6 μM, and annealed in NEBuffer #2 (New England Biolabs) by bringing reactions to 100°C for 7 minutes and then letting cool slowly overnight. The recombinant hMICA was restriction enzyme digested at a unique BglII site (New England Biolabs) and dephosphorylated using Calf Intestine Phosphorylase (Invitrogen).

The annealed oligos were diluted to 10 nM and added to 10 ng of digested vector for a 1:3 ratio at 2 hours with T4 ligase (Invitrogen). Ligated products were used to transform XL-blue competent bacteria by electroporesis, grown on a 37°C shaker for 3 hours, and then streaked at 1, 10, and 100 μL on 100 μg/mL carbenicillin resistant plates for incubation overnight. Colonies were selected, used to inoculate 100 carbenicillin μg/mL LB, and incubated overnight. Candidate plasmids were then isolated using DNA mini-prep kits (Qiagen) and validated by sequencing at the Heflin Genetics Sequencing Core. These myc-tagged recombinant MICA candidates were also validated by 293-T cell transfection (Mirus TransIT-LT1 Transfection reagent) followed by permeabilization and dual staining immunofluorescence with anti-hMICA, anti-hMICA/B, and anti-Myc antibodies.

Reverse Transcriptase Quantitative PCR. U373 cells or HeLa cells were seeded in 24 well plates at $1.5x10^5$ cells per well or in 12 well plates at $3.0x10^5$ cells per well and allowed to adhere overnight. Samples were then infected at multiplicity of infection (MOI) of 5 PFU/cell with either HSV-1(F), R2621, or mock infection for 6 hpi. Total RNA was isolated from cell samples using TRIZOL reagent and RNeasy kit reagents and columns (Qiagen). RNA was quantified by Nanodrop photospectroscopy (General Electric). Reverse transcription was performed using Goscript reverse transcriptase system (Promega) with random hexamer primers. RT-qPCR primers for MICA (forward: AGGAGCTCCCAGCATTTCTA; reverse: CTTCATGGCATCTTCCTTCA), MICB (forward: GGGATTTCAGCCTCTGATGT; reverse: GGTCAGGAAACAGAGGGAAA), GAPDH (forward: GTCGGAGTCAACGGATTTG; reverse: TGGGTGGAATCATATTGGAA), and 18s rRNA (forward: CCGATTGGATGGTTTAGTGA; reverse:

GGTTCACCTACGGAAACCTT) were designed and purchased (Eurofin MGW Operon). Prior to use, all primers were diluted to 3 M. Reactions were carried out using a SYBR green master mix (Applied Biosystems) according to instructions. PCR and data collection was performed with an ABI 7300 Real-Time PCR system (Applied Biosystems, Inc.) at an extension temperature of 95°C and annealing temperature of 54°C for 40 cycles. As a quantification control, topo-cloning was used to create a MICA DNA standard through the insertion of MICA exon 3-5 PCR amplified from U373 cells into a Topo 2.1 plasmid.

Immunofluorescence and Flow Cytometery. U373 cells or 293-T cells were seeded on glass coverslips in 24 well plates at 7.5 x10^4 cells per well for immunofluorescence (IFC) or in 100mm Petri dishes for FACS and allowed to adhere overnight. Samples were either transfected with appropriate plasmids as previously described or infected with appropriate virus an MOI 0.1 for IFC or MOI 5 PFU/cell for FACS. At 18 hpi, cells were then fixed in 3% paraformaldehyde for immunofluorescence or 2% for FACS for at least 30 minutes and then either permeabilized with Phosphate Buffered Saline (PBS)/0.1% Triton for IFC or PBS/2% FBS/Perm Wash (BD Bioscience) for FACS for 15 minutes. Non-permeabilized samples were incubated in PBS/2% FBS alone. Cells were then blocked with 10% goat IgG 30 minutes for IFC or blocked with 100% goat serum 30 minutes for FACS. Anti-hMICA, anti-hMICA/B, and anti-Myc primary antibodies and appropriate secondary antibodies were applied separately at the indicated dilutions in PBS and incubated at 37°C for 1 hour. After staining, coverslips were mounted onto slides with DAPI and then sealed. Washes with PBS were performed at least 4 times between each step. Images were captured on a stereo fluorescent microscope (Zeiss). Fluorescence-activated cell sorter (FACS) analysis data was

collected with a FACSCalibur flow cytometer (BD Bioscience) and analyzed with FloJo software.

Western blotting. Cells were seeded on either 6 well plates at 6.0 x10^5 cells per well or 24 well plates at 1.5 x10^5 cells per well and allowed to adhere overnight. Samples were either transfected with appropriate plasmids as previously described or infected with appropriate virus an MOI of 5 PFU/cell. At 24 hpi samples were collected with protein lysis buffer, boiled for 5 minutes and allowed to cool on ice. Samples were loaded on 11% SDS-PAGE and run with pre-stained markers at 100V until markers reached the end of the gel. Protein was transferred to a nitrocellulose membrane at 100V for 3 hours. The membrane was blocked with 5% BSA in TBS/0.05% Tween-20 (TBS-T) for 1 hour at room temperature with rocking. Primary antibody was applied at the indicated dilution in TBS-T/5% BSA and incubated at 4°C overnight with rocking. HRP conjugated secondary antibody was applied at the indicated dilution in TBS-T/5% BSA and incubated at room temperature for 1 hour with rocking. Substrate was applied for 5 minutes and the membrane was wrapped in saran wrap before film exposure. Washes with TBS-T were performed at least 4 times between each step at room temperature for 10 minutes each with rocking.

De novo Protein Detection. *De novo* MICA protein translated during HSV-1(F) infection was labeled with biotin through Click-iT amino acid labeling and detection (Invitrogen). U373 cells were seeded in T-25 flasks at 1.0 x10^6 cells per flask as previously described and allowed to adhere overnight. Cells were then either mock infected or infected with HSV-1(F) at MOI 5 PFU/cell in infection media for 1.5 hours. The media of infected samples and appropriate mock samples was refreshed with methionine-free DMEM for 30 minutes before an HPG amino acid label was added

directly to the media and allowed to incubate for 4 hours prior to cell lysis. Lysis buffer was prepared with 1% SDS in 50mM Tris-HCl with Protease and Phosphatase inhibitors according to manufacturer's instructions (Complete, Roche) and Benzonase endonuclease 250 U/mL (EMD, Merck). Total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's specifications and an ELx808 Ultra Microplate Reader (Bio-Tek Instruments, Inc.). Equivalent amounts of total protein were then used to carry out the remainder of the Click-iT reaction with azide biotin detection reagent according to manufacturer's specifications with one mock sample containing the HPG component but withheld from the detection component. Portions of the original, unreacted total protein from each sample were kept for comparison to reacted protein as a negative control for biotinylation reaction conditions. After a 20 min reaction period the biotin-labeling reaction mixture was diluted in 1 mL of PBS/1% NP-40. 20 µL of Ultra HBC Streptavidin Agarose Resin (Gold Biotechnology) was added directly to the diluted reaction mixture and incubated at 4°C on a tube rotator for 1 hour. Samples were then centrifuged at 1,000 x g for 5 minutes at 4°C and unbound sample was transferred to clean centrifuge tubes. The remaining resin was washed 5 times with PBS/1% NP-40 at 4°C on a tube rotator for 5 minutes and centrifuged at 1,000 x g for 5 minutes at 4°C. Disruption buffer was then added to the original and resin bound samples and heated at 100°C for 3 minutes. Western blots of each sample were then developed as previously described.

Results

HSV-1 down-modulates surface and total MICA upon infection of glioma cells and HeLa cells. Previously Schepis *et al.* have reported the down-modulation of MICA from the surface of HeLa cells with no change of total MICA in permeabilized cells during HSV-1(F) infection (19). In infected U373 cells there was a down-modulation of ULBP2 and no reported change in MICA protein levels (19). To test for changes in MICA expression on glioma cells, U373 cells infected with wild type HSV-1(F) or mock conditions were stained with a validated (Supplemental Fig. 2) anti-MICA/B monoclonal antibody. MICA was measured by Mean Fluorescence Intensity (MFI) normalized to isotype levels for each experimental condition and observed to be down-modulated from the surface upon infection with HSV-1(F) (Fig. 1a) as compared to mock infected cells. The total MICA was also down-modulated in permeabilized cells upon infection as seen by decreased normalized MFI compared to mock conditions (Fig. 1b). The average normalized MFI measuring surface MICA on U373 cells during HSV-1(F) infection was 29% of mock infection MFI and 35% for total protein levels. The normalized MFI of stained MICA was lower for both non-permeabilized (49.5% of mock) and permeabilized (62.7% of mock) HeLa cells during infection with HSV-1(F) (Fig. 1c and d).

Surface and internal down-modulation of MICA upon infection of U373 cells with HSV-1(F) was visualized by immunofluorescence (Fig. 2). The Green Fluorescent Protein expressing HSV-1(F) recombinants M2001 and C101 were used to indicate infected cells (Fig. 2 a and d). Staining against MICA shows decreased surface MICA on infected cells as compared to uninfected cells in the same field with no aggregations of MICA internally (Fig. 2 b and e).

Virion Host Shut-off protein contributes to MICA mRNA degradation but not to protein down-modulation. A decrease in MICA mRNA levels has previously been reported as early as 3 hpi with HSV and precedes protein down-modulation in HeLa cells (19). Virion Host Shut-off (VHS) protein is an HSV encoded RNase within the viral tegument that degrades host mRNA upon infection. To test if VHS contributes to MICA mRNA degradation RT-qPCR experiments were performed to measure MICA mRNA quantity in U373 cells infected with either the wild type virus or a recombinant deleted of VHS relative to that of mock-infected cells. When VHS is present in HSV-1 infection, MICA mRNA was measured to be between 70-90% less compared to mock-infected U373 cells (Fig. 3). In contrast, MICA mRNA levels have a mean 40% reduction compared to mock-infected cells in the absence of VHS.

To test if VHS degradation of MICA mRNA contributes to MICA protein down-modulation as a potential mechanism, FACS experiments measuring surface and total MICA protein were performed with the VHS deleted virus in parallel to the wild-type virus. The normalized MFI of stained MICA was lower for both surface and total protein in both U373 and HeLa cells infected with the VHS deleted virus compared to mock infected cells (Fig. 1a-d).

Down-modulation of MICA during HSV infection is protein specific. To test whether the down-modulation of MICA during HSV infection is specific or part of a general protein down-modulation, FACS analysis was also performed with the surface protein transferrin receptor during infection as a separate trans-membrane protein not associated with membrane lipid rafts and thus serves as a suitable control to monitor for a general phenomenon affecting trans-membrane proteins at other locations of the cell membrane (9, 20). Infection of U373 cells with either wild type HSV or R2621 increased

the percentage of cells expressing surface transferrin receptor to 50.37% with wild type and 55.13% with R2621 (Fig. 4a) and increased total levels (Fig. 4b).

MICA protein amounts measured by immunoblotting differ from flow cytometery. To measure the relative amounts of MICA protein within cell lysates under mock and HSV infected conditions, immunoblotting was performed in HeLa cells and multiple glioma lines. Total protein was quantified for equivalent gel loading and then probed with validated (Supplemental Fig. 1) anti-MICA antibody. Within each cell line tested, there were no differences in apparent MICA protein amounts between mock conditions and HSV recombinants for the predicted molecular weight measured by the anti-MICA antibody used (Fig. 5). However, multiple bands were also detected at higher molecular weights within each cell line corresponding to about 63 kDa. Within these higher weight bands there is less apparent protein among infected cells compared to mock infected cells. At any molecular weight band there are no differences in MICA protein amounts observed between viruses.

HSV infection does not block production of de novo MICA protein. To test if HSV infection affects MICA protein levels via a transcriptional mechanism that decreases newly formed protein, biotinylation was used to isolate *de novo* protein for anti-MICA immunoblotting. Comparison of bands at the anticipated molecular weight show no difference between mock and infected conditions for either the previously translated non-biotinylated MICA protein or the biotinylated *de novo* MICA protein at 12 hpi (Fig. 6). For bands at higher molecular weights there is less apparent MICA protein for both the non-biotinylated MICA protein and the biotinylated *de novo* MICA protein upon infection compared to mock conditions. Comparison of these samples to control mock

infected cell lysates not subjected to biotinylation reaction shows that streptavidin binding is specific for biotinylated proteins.

Discussion

NK cells play a role in the control of viral infections, including those of herpes viruses. The regulation of NK cell response to infection is partially controlled by exposure to ligands on host cells that interact with activating or inhibitory receptors on the NK cell surface. In order to evade NK cell recognition, some members of the herpes family use methods of down-modulating the surface presentation of host cell ligands that could otherwise activate NK cells. Specifically, CMV and KSHV down-modulate the activating ligand MICA by well studied mechanisms. The phenomenon of MICA down-modulation during HSV-1 infection has been previously reported in HeLa cells, but the mechanism by which this occurs has yet to be elucidated.

From the studies reported here we observe down-modulation of MICA from the surface of HeLa cells during HSV infection similar to previous findings. We also observed MICA surface down-modulation on glioma cells, a finding not previously reported. These findings suggest that HSV-1 is able to down-modulate the presentation of MICA as a manner of preventing NK cell activation and counter to HSV-1 mediated MHC-I down-modulation. The replication of surface MICA down-modulation on HeLa cells, which express the truncated MICA*008 allele, also supports the hypothesis that the HSV-1 mechanism is not allele dependent like UL-142 of CMV or K5 of KSHV.

Experiments utilizing flow cytometery with permeabilization suggest that down-modulation occurs not only at the cell surface, but also in the cell interior. This observation is different from findings previously reported for permeabilized HeLa cell

flow cytometery. Because of this difference, we used recombinant human MICA transfection and validated our antibodies in their detection of MICA. The decrease in total MICA within permeabilized infected cells measured by flow cytometery suggests that MICA is being degraded, shed from cells, or altered in half-life during infection rather than being sequestered or arrested in surface transport. This is also confirmed by immunofluorescence in which no intracellular accumulations of MICA protein, which would otherwise suggest internal sequestration within infected cells, are observed.

In addition to a possible mechanism that decreases surface MICA protein or increases surface turnover, the previously reported observation that MICA mRNA levels decrease prior to MICA surface down-modulation suggested that decreases in mRNA could contribute to decreased protein levels. Experiments performed with the VHS deleted recombinant virus, R2621, suggests that VHS contributes significantly to MICA mRNA degradation. However, even in the absence of VHS, down-modulation of surface MICA is still observed by flow cytometery to levels comparable to wild type HSV containing VHS. In the absence of VHS, MICA mRNA levels are still observed to be lower than mock infected cells, which suggest that other factors in viral infection contribute to MICA mRNA degradation.

Studies of transferrin receptor on the surface of neoplastic brain tissue have demonstrated that the baseline expression on excised human glioblastoma multiforme is high and diffuse throughout compared to the lower and focal expression on more benign tumors (17). The diffuse nature of transferrin receptor expression glioblastoma can allow it to be compared to other surface proteins unrelated in function for changes occurring by a common mechanism. Our experiments measuring the surface expression of transferrin receptor during HSV-1 infection for comparison to MICA show that, in contrast to down-

modulation of MICA, transferrin receptor increases in surface expression compared to the baseline expression of mock infected glioma cells. This suggests that surface MICA is not part of a generalized surface protein down-modulation on glioma cells. This up-regulation of transferrin receptor during infection can be explained as part of the stress response induced by viral infection as transferrin receptor expression is affected by hypoxic and inflammatory conditions (22, 23).

Although our data by flow cytometry suggest that MICA protein is degraded or otherwise expelled from infected cells, by western blotting the lack of distinguishable change in the immunodominant protein observed at the expected molecular weight for the MICA molecule between virus and mock-infections indicates that there is no decrease in total protein. If surface levels of MICA are down-modulated during infection, an endocytotic or sequestering mechanism could explain this equivalent amount of protein observed in western blotting. Comparison of the miniscule amounts of de novo MICA protein formed to the original amounts of MICA protein in mock or HSV infected conditions suggest that the half-life of certain MICA protein forms is great enough that down-modulating changes related to infection are obscured by older un-degraded protein.

There is evidence indicating that the higher molecular weight bands observed with our anti-MICA antibody could be more highly glycosylated forms of mature MICA (9) or possibly dimers of MICA that are dependent upon an Asn-8 oligosaccharide given its weight being approximately twice that of the expected weight (15). A return to a lower molecular weight has been reported for MICA treated with PNGase F, a method demonstrating that N-linked glycosylation affects the migration of MICA through polyacrylamide gels (1). Studies are being performed to determine the immunoblotting

patterns of MICA dependent upon glycosylation and other post-translational modifications that are specifically down-modulated by HSV-1.

In summary, evidence supports the HSV-1 mediated down-modulation of MICA from the surface of infected cells, most likely through a post-translational mechanism. This phenomenon has been reproduced on multiple cell lines and is independent of mRNA degradation. MICA surface down-modulation also occurs in the absence of the $\gamma_1$34.5 neurovirulence gene. This could have important consequences for the immune mediated anti-tumor mechanism of oHSV therapy by decreasing NK activation during treatment. Thus, further studies to identify the mechanism by which HSV-1 down-modulates presentation of this NK cell activating ligand are crucial for the design of future oHSV vectors to circumvent this phenomenon and optimize anti-tumor conditions.

Acknowledgments

This publication was supported by NIH P01-CA 071933-13 (KAC, JMM), P20CA151129 (KAC, JMM), NIH training grant 5T32 NS048039 (DCG), and the National Center for Advancing Translational Sciences 5TL1 RR025775-04 (CIO). The authors wish to acknowledge the University of Alabama at Birmingham Heflin Genetics Sequencing Core for sequencing data. The authors acknowledge the assistance of laboratory members Justin Roth, Ph.D., Li Li, M.D., Jennifer Coleman, and Tyrel Smith.

References

1. **Andresen, L., S. L. Skovbakke, G. Persson, M. Hagemann-Jensen, K. A. Hansen, H. Jensen, and S. Skov.** 2-deoxy D-glucose prevents cell surface expression of NKG2D ligands through inhibition of N-linked glycosylation. J Immunol **188:**1847-55.
2. **Ashiru, O., N. J. Bennett, L. H. Boyle, M. Thomas, J. Trowsdale, and M. R. Wills.** 2009. NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142. J Virol **83:**12345-54.
3. **Berardi, A., L. Lugli, C. Rossi, C. L. Maria, I. Guidotti, C. Gallo, and F. Ferrari.** 2011. Neonatal herpes simplex virus. J Matern Fetal Neonatal Med **24 Suppl 1:**88-90.
4. **Biron, C. A., K. S. Byron, and J. L. Sullivan.** 1989. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med **320:**1731-5.
5. **Bryceson, Y. T., S. C. Chiang, S. Darmanin, C. Fauriat, H. Schlums, J. Theorell, and S. M. Wood.** 2011. Molecular mechanisms of natural killer cell activation. J Innate Immun **3:**216-26.
6. **Chalupny, N. J., A. Rein-Weston, S. Dosch, and D. Cosman.** 2006. Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. Biochem Biophys Res Commun **346:**175-81.
7. **Chisholm, C., and L. Lopez.** 2011. Cutaneous infections caused by Herpesviridae: a review. Arch Pathol Lab Med **135:**1357-62.
8. **Chisholm, S. E., K. Howard, M. V. Gomez, and H. T. Reyburn.** 2007. Expression of ICP0 is sufficient to trigger natural killer cell recognition of herpes simplex virus-infected cells by natural cytotoxicity receptors. J Infect Dis **195:**1160-8.
9. **Eleme, K., S. B. Taner, B. Onfelt, L. M. Collinson, F. E. McCann, N. J. Chalupny, D. Cosman, C. Hopkins, A. I. Magee, and D. M. Davis.** 2004. Cell surface organization of stress-inducible proteins ULBP and MICA that stimulate human NK cells and T cells via NKG2D. J Exp Med **199:**1005-10.
10. **Fitzgerald-Bocarsly, P., D. M. Howell, L. Pettera, S. Tehrani, and C. Lopez.** 1991. Immediate-early gene expression is sufficient for induction of natural killer cell-mediated lysis of herpes simplex virus type 1-infected fibroblasts. J Virol **65:**3151-60.
11. **Fitzgerald, P. A., M. Mendelsohn, and C. Lopez.** 1985. Human natural killer cells limit replication of herpes simplex virus type 1 in vitro. J Immunol **134:**2666-72.
12. **Joyce, M. G., and P. D. Sun.** 2011. The structural basis of ligand recognition by natural killer cell receptors. J Biomed Biotechnol **2011:**203628.
13. **Lanier, L. L.** 2005. NK cell recognition. Annu Rev Immunol **23:**225-74.
14. **Li, P., D. L. Morris, B. E. Willcox, A. Steinle, T. Spies, and R. K. Strong.** 2001. Complex structure of the activating immunoreceptor NKG2D and its MHC class I-like ligand MICA. Nat Immunol **2:**443-51.
15. **Li, P., S. T. Willie, S. Bauer, D. L. Morris, T. Spies, and R. K. Strong.** 1999. Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand. Immunity **10:**577-84.
16. **Raulet, D. H.** 2003. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol **3:**781-90.
17. **Recht, L., C. O. Torres, T. W. Smith, V. Raso, T. W. Griffin.** 1990. Transferrin receptor in normal and neoplastic brain tissue: implications for brain-tumor immunotherapy. J Neurosurg **72:**941-945
18. **Rozenberg, F., C. Deback, and H. Agut.** 2011. Herpes simplex encephalitis : from virus to therapy. Infect Disord Drug Targets **11:**235-50.
19. **Schepis, D., M. D'Amato, M. Studahl, T. Bergstrom, K. Karre, and L. Berg.** 2009. Herpes simplex virus infection downmodulates NKG2D ligand expression. Scand J Immunol **69:**429-36.
20. **Schneider, C. L., and A. W. Hudson.** 2011. The human herpesvirus-7 (HHV-7) U21 immunoevasin subverts NK-mediated cytoxicity through modulation of MICA and MICB. PLoS Pathog **7:**e1002362.
21. **Stern-Ginossar, N., and O. Mandelboim.** 2009. An integrated view of the regulation of NKG2D ligands. Immunology **128:**1-6.
22. **Tacchini, L., D. Fusar Poli, A. Bernelli-Zazzera, and G. Cairo.** 2002. Transferrin receptor gene expression and transferrin-bound iron uptake are increased during postischemic rat liver reperfusion. Hepatology **36:**103-11.

23. **Tacchini, L., E. Gammella, C. De Ponti, S. Recalcati, and G. Cairo.** 2008. Role of HIF-1 and NF-kappaB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals. J Biol Chem **283:**20674-86.
24. **Thomas, M., J. M. Boname, S. Field, S. Nejentsev, M. Salio, V. Cerundolo, M. Wills, and P. J. Lehner.** 2008. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. Proc Natl Acad Sci U S A **105:**1656-61.
25. **Thomas, M., M. Wills, and P. J. Lehner.** 2008. Natural killer cell evasion by an E3 ubiquitin ligase from Kaposi's sarcoma-associated herpesvirus. Biochem Soc Trans **36:**459-63.
26. **Wiertz, E. J., R. Devlin, H. L. Collins, and M. E. Ressing.** 2007. Herpesvirus interference with major histocompatibility complex class II-restricted T-cell activation. J Virol **81:**4389-96.

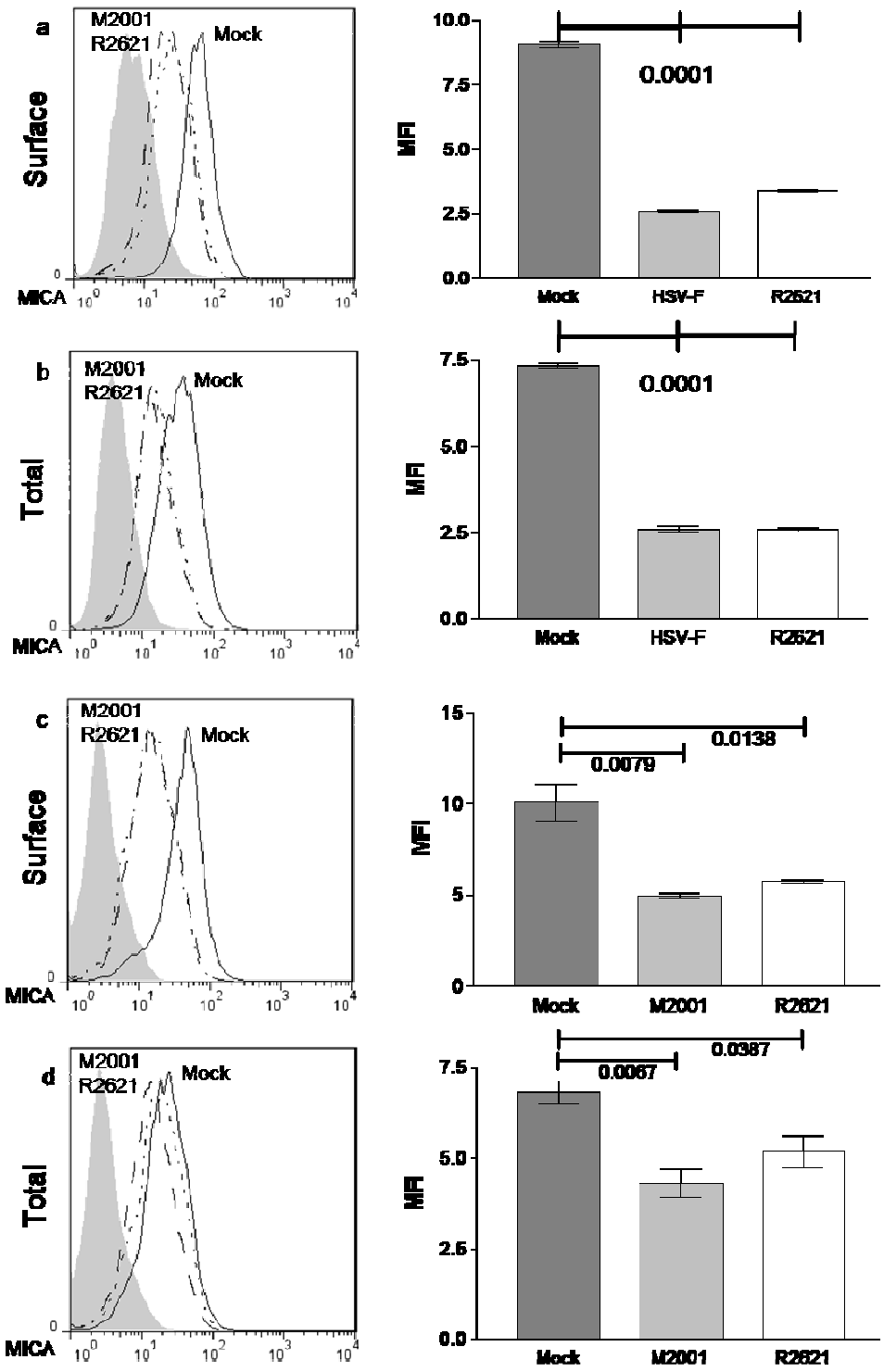


Fig 1 HSV down-modulates surface and total MICA protein on glioma and HeLa cells. U373 (a,b) and HeLa (c,d) cells were infected with HSV-F (dashed line, light gray) or R2621 (dotted line, white) at MOI 5 or mock infected (solid line, dark gray). At 18 hpi cells were fixed and stained for MICA. Cells were either non-permeabilized to stain surface MICA only (a,c) or permeabilized with saponin to stain total MICA (b,d). Mean fluorescent intensity for MICA was normalized to isotype levels for each condition.

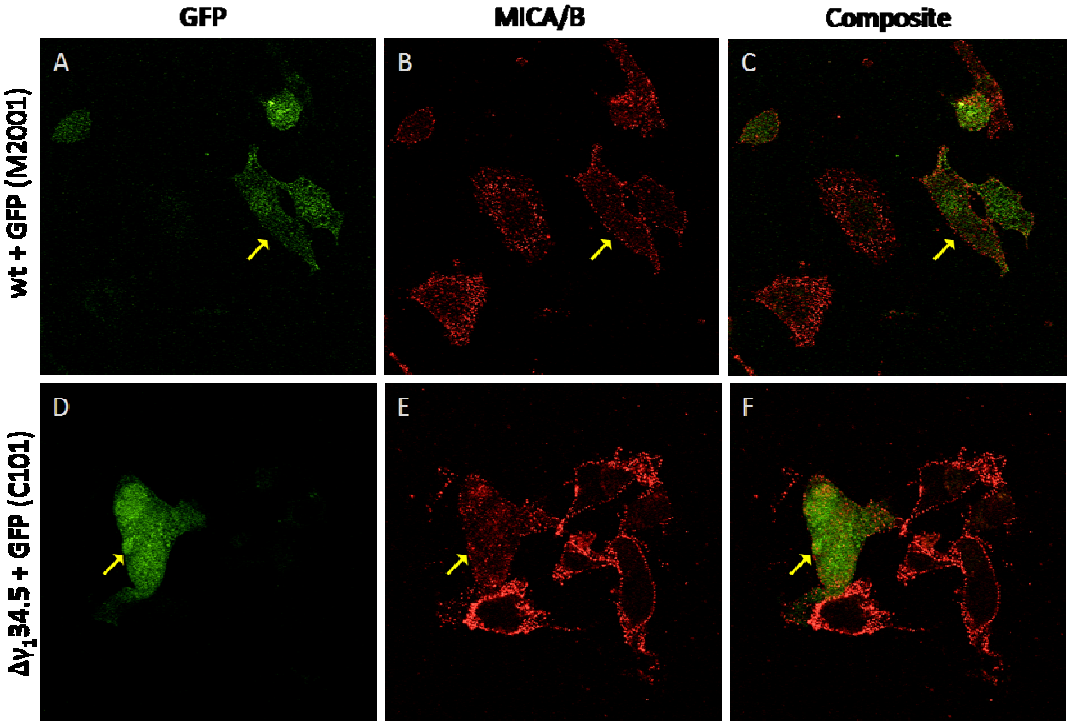


Fig 2 HSV-1 down-modulates MICA without evidence of intracellular sequestration. U373 cells infected with M2001 (a-c) or C101 (d-f). At 18 hpi cells were fixed, permeabilized, and stained for MICA/B (b,c,e,f). GFP is expressed by infected cells (arrow) for comparison of MICA on and within uninfected cells in the same field (b,e). Confocal, 400x magnification.

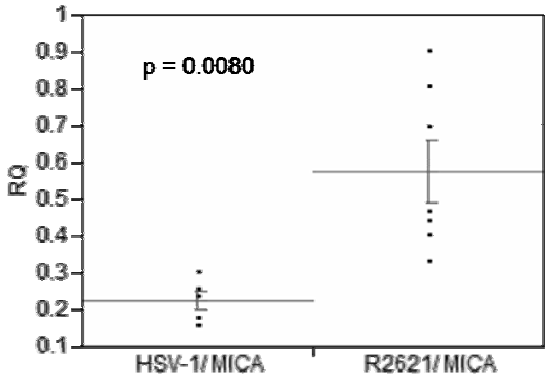


Fig 3 Virion Host Shut-off contributes to MICA mRNA degradation. U373 cells infected with HSV-F (left) or R2621 (right) at MOI 5 or mock infected. At 6 hpi cells were lysed and mRNA was collected. PCR was performed on total mRNA using random hexamers. Primers for MICA and 18s rRNA were used in qPCR with SYBR green detection to obtain quantities of MICA mRNA relative to 18s rRNA. RQ is the ratio of MICA during infection to MICA during mock infection.

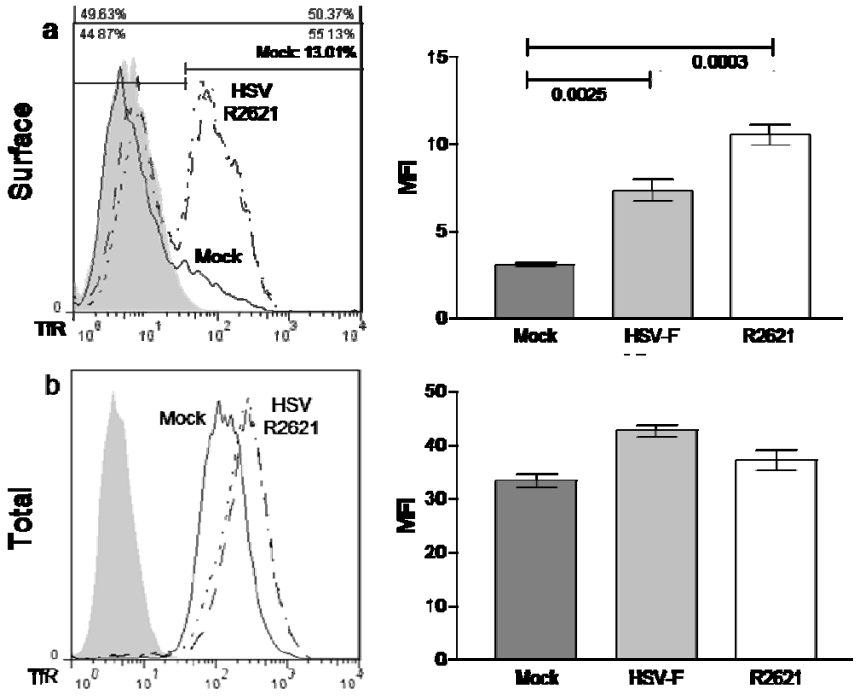


Fig 4 U373 cells infected with HSV-F (dashed line) or R2621 (dotted line) at MOI 5 or mock infected (solid line). At 18 hpi cells were fixed and stained for TFN-R. Cells were either non-permeabilized to stain surface TfR only (a) or permeabilized with saponin to stain total TfR (b).

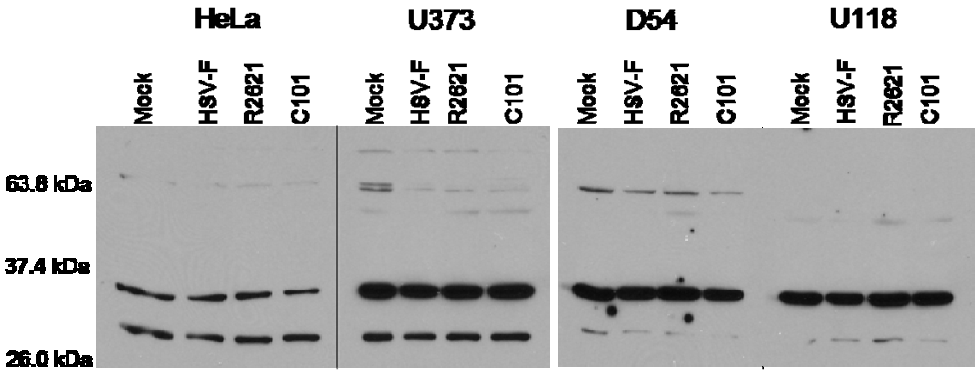


Fig 5 MICA protein levels upon infection with wild-type HSV and recombinants. The indicated cell lines were seeded, allowed to adhere overnight, and infected with the indicated viruses at MOI 5. At 18 hpi cells were lysed and total protein was quantified for equivalent gel loading. Nitrocellulose membranes with the total protein were stained with Epitomics rabbit anti-MICA at 4°C overnight. 15 second exposure.

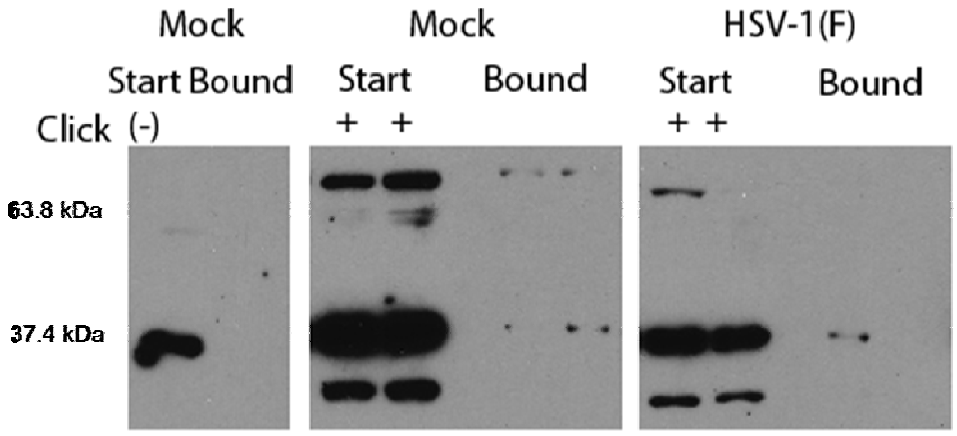
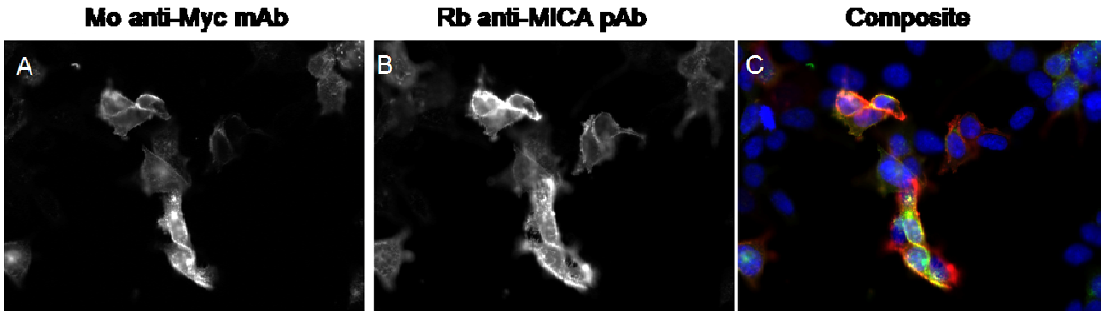
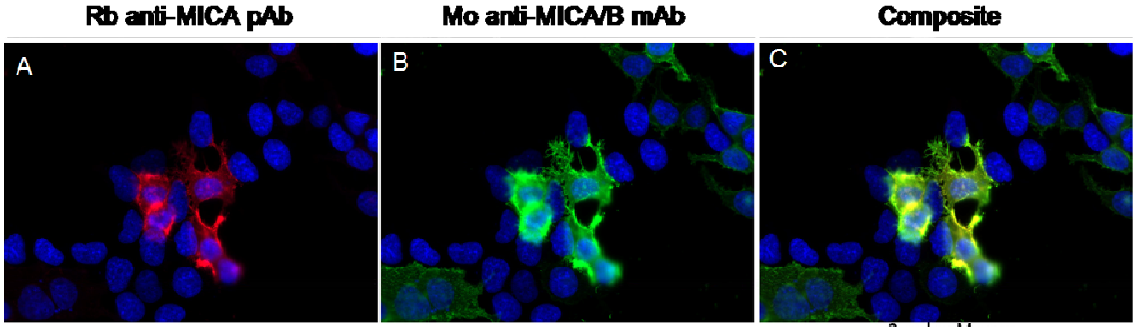


Fig 6 Isolation of de novo MICA. U373 cells either infected with HSV-1 at MOI 5 or mock conditions and lysed at 12 and 18 hpi. Prior to lysis, incorporation of an amino acid ortholog into de novo proteins was performed and followed by biotinylation reactions. Biotinylated de novo proteins were isolated by reaction with streptavidin agarose beads. All proteins were quantified, equivalently loaded, and separated by SDS-page gel and stained for MICA.



Supplemental Fig 1 Rabbit anti-MICA pAb recognizes recombinant human MICA. 293GT cells were transfected with recombinant hMICA containing a Myc-tag amino acid sequence. At 18 hpt cells were fixed and dual-stained for Myc using Cell Signaling 9611 mouse anti-Myc mAb (a, green) and MICA using Epitomics rabbit anti-MICA pAb (b, red). Nuclei were stained blue with DAPI. Three color merge (c) shows transposition of MICA and Myc staining by both antibodies. 100x magnification.



Supplemental Fig 2 Mouse anti-MICA/B mAb recognizes recombinant human MICA. 293GT cells were transfected with recombinant hMICA containing a Myc-tag amino acid sequence. At 18 hpt cells were fixed and dual-stained for MICA using Epitomics rabbit anti-MICA pAb (a, red) and eBioscience mouse anti-MICA/B mAb (b, green) at equivalent concentrations. Nuclei were stained blue with DAPI. Three color merge (c) shows transposition of MICA staining by both antibodies. 200x magnification.

CONCLUSION

As can be seen in our review article *Herpesviridae Methods of Natural Killer Cell Evasion*, the mechanisms by which herpesviruses escape adaptive and innate immune system detection are numerous and have functional consequences for viruses and tumor cells. Specifically considering MICA and NKG2D, the discrepancy in amount of knowledge and number of studies examining modulation is vast between HSV-1 and other herpes viruses. Studies of MICA behavior during HSV-1 infection have thus far confirmed that surface protein is decreased upon infection, however initial studies examining the mechanism remain contradictory.

In previously published results there was surface down-modulation of MICA without any decrease in total protein levels thus suggesting that the mechanism was endocytosis or internal retention (7). In the prepared manuscript *Surface Down-modulation of MICA in Malignant Glioma Cells by Herpes Simplex Virus*, we also observed down-modulation of surface MICA by FACS not only on HeLa cells but also glioma cells. We have evidence to dismiss mRNA degradation and a general transmembrane protein down-modulation as trivial mechanisms. We additionally observed down-modulation of total protein levels by the same experimental methods which suggested protein degradation, shedding of soluble MICA, decreased production of mature MICA, or shortening of protein half-life as possible mechanisms. Yet, when we examined this phenomenon by a different experimental method, western blotting, we observed no change in MICA protein levels at the predicted molecular weight during

HSV-1 infection that would suggest endocytosis or retention. However, we have also observed higher molecular weight protein bands that demonstrate decreased protein levels in HSV-1 infected conditions.

Future experiments are aimed at resolving the discrepancy in observations between FACS and western blotting data. This is being done by testing if the anti-MICA antibodies utilized in our experiments are recognizing different forms of MICA protein which are selectively down-modulated by HSV-1. To determine if the native form of the protein is detected to a different extent than the denatured form caused by standard western blotting techniques, native protein western blotting techniques, which limit the amount of protein denaturing and reduction through SDS and BME, will be utilized. Deglycosylation studies utilizing PNGase F, Endo H, and O-glycosylase will also be performed to determine if antibody detection or HSV-1 down-modulation is dependent upon glycosylated forms of the protein. These will be performed on both endogenous U373 MICA and transfected recombinant MICA. To study the effect of HSV-1 infection on the mRNA quantity of transferrin receptor for comparison to MICA, RT-qPCR experiments will be performed utilizing primers amplifying transferrin receptor cDNA. Flow cytometery data demonstrating decreased surface MICA upon infection with recombinant viruses deleted of the $\gamma_1$34.5 neurovirulence gene will be refined for publication quality and inclusion in the original research manuscript.

Pulse-chase experiments through the biotinylation and isolation of *de novo* MICA protein followed by western blot detection will also be performed in mock infected and HSV-1 infected U373 cells to determine the baseline half-life of MICA protein and observe alterations in half-life caused by HSV-1 infection. It is anticipated that the half-life of MICA protein is longer than the timeframe of down-modulation observed in past

experiments. It is also anticipated that HSV-1 alters this half-life to increase the natural degradation of the protein.

Initial optimization experiments testing the functional consequences of MICA-specific down-modulation are also being performed. These are utilizing the NK92 natural killer cell line as effectors, U373 cells under mock and HSV-1 infected cells as experimental targets, and K562 cells as control targets. NK cell mediated cytotoxicity will be measured by LDH spectrophotometery at increasing geometric ratios of effectors to targets. To obtain a scale of MICA specific cytotoxicity, MICA negative controls obtained by either transfection with CMV UL-142 or anti-MICA antibody blocking will be used along with heat shocked, oxidatively stressed, or heat shock protein transfected cells for MICA positive controls. Because previous literature has demonstrated no change in other NKG2D activating ligands during infection, anti-ligand antibody blocking will not initially be required but can be performed. It is anticipated that HSV-1 mediated down-modulation of surface MICA will decrease NK cell cytotoxicity of targets at levels comparable to the MICA negative controls.

The anticipated effect of this phenomenon on the anti-tumor efficacy of oHSV therapy is summarized at the conclusion of the manuscript in preparation as being negative by decreasing NK cell activation and subsequent immune-mediated tumor clearance. The HSV-1 mechanism of MICA down-modulation must be addressed for better design of recombinant viruses either unable to down-modulate MICA or increase MICA and NK cell recognition and activation. Therapy with such oncolytic viruses could significantly improve patient survival as an adjuvant to current treatments.

REFERENCES

1. **Eisele, G., J. Wischhusen, M. Mittelbronn, R. Meyermann, I. Waldhauer, A. Steinle, M. Weller, and M. A. Friese.** 2006. TGF-beta and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. Brain **129:**2416-25.
2. **Friese, M. A., M. Platten, S. Z. Lutz, U. Naumann, S. Aulwurm, F. Bischof, H. J. Buhring, J. Dichgans, H. G. Rammensee, A. Steinle, and M. Weller.** 2003. MICA/NKG2D-mediated immunogene therapy of experimental gliomas. Cancer Res **63:**8996-9006.
3. **Lawrence, Y. R., M. V. Mishra, M. Werner-Wasik, D. W. Andrews, T. N. Showalter, J. Glass, X. Shen, Z. Symon, and A. P. Dicker.** Improving prognosis of glioblastoma in the 21st century: Who has benefited most? Cancer.
4. **Markert, J. M., P. G. Liechty, W. Wang, S. Gaston, E. Braz, M. Karrasch, L. B. Nabors, M. Markiewicz, A. D. Lakeman, C. A. Palmer, J. N. Parker, R. J. Whitley, and G. Y. Gillespie.** 2009. Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. Mol Ther **17:**199-207.
5. **Markert, J. M., M. D. Medlock, S. D. Rabkin, G. Y. Gillespie, T. Todo, W. D. Hunter, C. A. Palmer, F. Feigenbaum, C. Tornatore, F. Tufaro, and R. L. Martuza.** 2000. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. Gene Ther **7:**867-74.
6. **Parker, J. N., S. Meleth, K. B. Hughes, G. Y. Gillespie, R. J. Whitley, and J. M. Markert.** 2005. Enhanced inhibition of syngeneic murine tumors by combinatorial therapy with genetically engineered HSV-1 expressing CCL2 and IL-12. Cancer Gene Ther **12:**359-68.
7. **Schepis, D., M. D'Amato, M. Studahl, T. Bergstrom, K. Karre, and L. Berg.** 2009. Herpes simplex virus infection downmodulates NKG2D ligand expression. Scand J Immunol **69:**429-36.