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Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142

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Abstract

Human cytomegalovirus (HCMV) employs a variety of strategies to modify or evade the host immune response, and natural killer (NK) cells play a crucial role in controlling cytomegalovirus infections in mice and humans. Activation of NK cells through the receptor NKG2D/DAP10 leads to killing of NKG2D ligand-expressing cells. We have previously shown that HCMV is able to down-regulate the surface expression of some NKG2D ligands, ULBP1, ULBP2, and MICB via the viral glycoprotein UL16. Here, we show that the viral gene product UL142 is able to down-regulate another NKG2D ligand, MICA, leading to protection from NK cytotoxicity. UL142 is not able to affect surface expression of all MICA alleles, however, which may reflect selective pressure on the host to thwart viral immune evasion, further supporting an important role for the MICA–NKG2D interaction in immune surveillance.

Keywords: NK cell; Cytotoxicity; HCMV; MICA; UL142

Human cytomegalovirus (HCMV) is well adapted to life in its host. Primary infection typically occurs in childhood and is followed by life-long asymptomatic persistence of the virus with intermittent low level shedding of infectious viral particles. However, HCMV can replicate to relatively high levels in several different organs, frequently resulting in morbidity and mortality in people whose immune systems are immature, such as very young children or in immunocompromised individuals, such as transplant recipients or patients with AIDS [1]. The virus has a number of immune evasion strategies that it utilizes to evade detection by the host immune system. For example, the HCMV genome encodes four membrane glycoproteins US2, US3, US6, and US11, that function to inhibit the MHC class I antigen presentation pathway by independent mechanisms. These include prevention of cell surface expression of MHC class I by retention in the ER, increased degradation of MHC class I, and prevention of the transport of peptides into the ER by the TAP transporter, thus impairing presentation of viral antigens to $CD8^+$ host T cells [2–4]. US2 and US3 are also able to block MHC class II-mediated presentation to $CD4^+$ T cells [5–7].

Decreased antigen presentation by virally infected cells provides protection from T cell-mediated recognition. However, decreased expression of MHC class I might render these cells more sensitive to lysis by NK cells, due to decreased engagement of NK cell MHC class I-specific inhibitory receptors. The important role of NK cells in controlling cytomegalovirus infection in both humans and mice has been documented [8,9], and a number of HCMV NK cell evasion mechanisms have already been described. The HCMV-encoded protein UL18 has been shown to bind the inhibitory receptor LIR-1 [10]. A peptide homologous to an MHC class I leader sequence derived from UL40 has been shown to stabilize HLA-E. This results in up-regulation of HLA-E expression on HCMV-infected cells and HLA-E engagement of the inhibitory, heterodimeric receptor, CD94/NKG2A, expressed on NK cells [11,12].

Activation of NK cells through NKG2D leads to cytotoxicity and cytokine production. Surface expression of

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ligands for the activating receptor, NKG2D, which include the MHC class I-related chains A and B (MICA and MICB) [13,14] and the ULBPs [15,16], are initially induced upon HCMV infection [17,18]. However, the HCMV-encoded glycoprotein,UL16, sequesters three of these ligands, MICB, ULBP1, and ULBP2, in the endoplasmic reticulum, thereby impeding their cell surface expression and preventing NK cell recognition [19–21]. Expression of other NKG2D ligands, such as ULBP3, ULBP4, and MICA, is not affected by UL16. These ligands may be targeted by other HCMV proteins. Indeed, recent studies have shown that the full-length form of MICA is down-regulated following HCMV infection but that a truncated allele of MICA is not [22].

During a search for novel HCMV proteins involved in immune evasion, we examined UL14 and UL142 to determine if either of them was capable of down-regulating expression of MICA. Amino acid sequence analyses of UL14 and UL142 predict that they are both putative type I membrane glycoproteins. UL14 was of interest due to its location in the HCMV genome [23] where it is adjacent to genes encoding UL16 and UL18, both known immunomodulators. UL142 is of particular interest as a member of the UL18 family, which contain a conserved MHC class I domain [24,25] and because it is found adjacent to the gene encoding UL144, a TNFR homologue that binds the inhibitory receptor, BTLA [26]. In addition, UL142 is found in clinical isolates but not in laboratory-attenuated HCMV strains [23,27], and fibroblasts infected with these clinical isolates of HCMV have been shown to be more resistant to NK lysis than those infected with the laboratory adapted strain AD169 [28,29]. Since, clinical isolates of the virus have been shown to have a larger genome [30], it is possible that genes such as UL142, which are found in clinical isolates but not in the AD169 strain, might encode NK cell-specific inhibitory genes.

Here, we show that the viral gene product UL142 is able to down-regulate the cell surface expression of full-length MICA, leading to protection from NK cytotoxicity. UL142 does not cause down-regulation of a truncated and prevalent allele of MICA, MICA *008. This allele has a frameshift mutation in the transmembrane region, which results in a truncated transmembrane region and the lack of a cytoplasmic tail. These results suggest that this mutant allele could have been evolutionarily selected for resistance to this HCMV immune evasion mechanism.

Materials and methods

Cell lines and purification of cells. EL4, a murine thymoma cell line (ATCC TIB-39), was grown in RPMI-1640 supplemented with 5% fetal calf serum (FCS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 0.055 mM 2-mercaptoethanol. EL4 cells expressing MICA were generated and grown as described [19]. HeLa cells, a cervical adenocarcinoma line (ATCC CCL-2.1), were grown in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. Human NK cells were isolated from peripheral blood mononuclear cells (PBMC). PBMC were isolated from Leuko Packs by Ficoll centrifugation. NK cells were isolated using the Miltenyi NK Cell Isolation Kit II and the AutoMACS (Miltenyi Biotec) according to manufacturer's specifications. Freshly isolated human NK cells were stimulated in media overnight at 10^6 cells/ml with 50 ng/ml recombinant human IL-15 (Amgen) before use in cytotoxic assays.

Flow cytometric analysis. The following monoclonal antibodies were used for flow cytometric analysis: M90, anti-hCD40-L used as a mouse IgG1 isotype control; M673, mouse IgG1 anti-MICA (Amgen), Phycoerythrin (PE)-conjugated rat anti-mouse CD8α (BD Pharmingen), or PE-conjugated rat anti-mouse CD45R/B220 as a negative control (BD Pharmingen).

Cells (5×10^5) were incubated in 100 μl staining buffer with 1 μg antibody for 30 min on ice. Specific binding of unlabeled antibodies was detected with an APC-conjugated F(ab′)2 fragment goat anti-mouse IgG (Jackson Immunoresearch). After staining, cells were analyzed on a Becton–Dickinson FACScan or sorted on a Vantage Mo Flo.

Retroviral vector construction and transduction. The retroviral vector containing the cDNA encoding full-length MICA was constructed and cells were transduced as described [15,19].

Lentiviral vector construction. The UL14 and UL142 sequences were PCR amplified from cDNA extracted from clinical isolates of HCMV obtained from Cristina Cerboni [28]. UL14 was amplified from cDNA extracted from patient isolate 109 (UL14.2H—GenBank Accession No. DQ457001). UL142 was isolated from cDNA extracted from patient isolates 13B (UL142.6A—GenBank Accession No. DQ453517) and 109 (UL142.7A—GenBank Accession No. DQ453518). The primers used to amplify UL14 (forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCACCATGGGCGGTGGTCGG-3′; reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTCCCGCCGCTGCTC-3′) and UL142 (forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGGCACCATGCGGATTGAATGG-3′; reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTGACCGCGCCATAC-3′) included attB1 and attB2 sites for Gateway transfer into Gateway-adapted lentiviral vectors. The sequence of the genes was verified by sequence analysis.

These cDNAs were subcloned into the pDONR 201 entry vector via the Gateway reaction according to manufacturer's specifications (Invitrogen). From here they were subcloned into the pLV415G, a Gateway-adapted lentiviral vector (Amgen), which contains the cDNA encoding mouse CD8α as a reporter gene. The lentiviral vector backbone has been previously described [31], and was modified to incorporate alternative promoter and reporter gene elements as well as to be compatible with the commercially available Gateway cloning system (Invitrogen). Briefly, the pLV415G expression constructs utilize the EF1-α promoter to express an IRES-muCD8α reporter gene. In addition, an *att*R1/*att*R2 Gateway Conversion Element (Invitrogen) was subcloned into the *Not*I site between the RRE and Ψ sites of the vector backbone to facilitate subsequent incorporation of various cDNA expression cassettes.

For construction of UL14- and UL142-GFP fusions, UL14 and UL142 sequences were amplified from plasmids containing the full-length cDNAs. Primers used to amplify UL14 (forward primer 5′-TATGTCGACCTCGGCACCATGGGCGGTGGTCGG-3′; reverse primer 5′-TATGAGATCTCCCTCCCGCCGCTGCTC-3′) and UL142 (forward primer 5′-TATGTCGACCTCGGCACCATGCGGATTGAA-3′; reverse primer 5′-TATGGGATCCCCCTGACCGCGCCATAC-3′) incorporated a *Sal*I site 5′ and a *Bam*HI site 3′. These fragments were inserted into the pENTR eGFP (Amgen) plasmid creating GFP fusions. The cDNA encoding the UL14- or UL142-GFP fusions was transferred to the pLV418G lentiviral vector (Amgen) via the gateway reaction. The pLV418G vector is a derivative of the pLV415G vector described above, which contains IRES-zeocin resistance gene (Invivogen) instead of an IRES-muCD8α reporter gene.

Generation of lentiviral vectors. Lentiviral vector stocks were generated by co-transfection of HEK293 cells with the pLP1, pLP2, and pLP/VSVG lentiviral vector packaging constructs (Invitrogen) along with individual lentivector expression constructs using LipofectAMINE 2000 according to manufacturer's protocols (Invitrogen). Crude culture supernatants were harvested and concentrated ~100-fold by ultracentrifugation at 100,000*g*

for 2 h prior to aliquoting in PBS + 0.5% BSA for storage at −80 °C. Vector stocks were assayed for titer on 293 cells, and titers of 10^7–10^9 TU/ml were typically attained.

Generation of transduced cell lines. Cells were infected via spinfection. Briefly, 10^5 cells were placed in a single well of a 24-well plate in 0.5 ml media plus 4 μg polybrene and virus at an M.O.I of 30–100. Cells and virus were spun at room temperature for 45 min at 18,000 rpm. Following spinfection, the cells were washed and resuspended in fresh media. UL14- and UL142-expressing cells were selected by sorting cells expressing the murine CD8α reporter gene or GFP by flow cytometry.

Isolation of MICA alleles. cDNAs were synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 50 ng mRNA extracted from the mid MICA+ EL4 cells, or HeLa cells using the FastTrack 2.0 Kit (Invitrogen) according to manufacturer's specifications. Double stranded MICA cDNA was amplified by PCR using primers (forward primer 5′-TATATGGGGCTGGGCCCG-3′; reverse primer 5′-TATCTAGGTGCCCTCAGTGGA-3′) and then TA-cloned into the pCR4-TOPO vector (Invitrogen). MICA sequence was verified by sequence analysis.

Cytotoxicity assay. The ^{51}Cr release cytotoxicity assay was performed as previously described [15]. Proper shielding procedures for ^{51}Cr were used (according to manufacturer's specifications).

Results

UL142 is highly polymorphic in the HCMV genome

DNA from HCMV clinical isolates [28] was amplified by PCR using UL14- and UL142-specific primers. The UL14 sequence amplified from patient isolate 109 contained only two predicted amino acid differences from the prototypic AD169 strain. The predicted UL14 protein contains a signal sequence and transmembrane domain. The UL142 sequences amplified from patient isolates 13B and 109 were different in sequence from reported UL142 sequences and from each other and were given the names UL142.6A and UL142.7A, respectively. There is considerable polymorphism in the UL142 sequence among clinical isolates (Fig. 1). The UL142.6A sequence also contained three amino acid insertions not found in any of the UL142 sequences reported to date. The predicted UL142 protein contains a signal sequence and transmembrane domain.

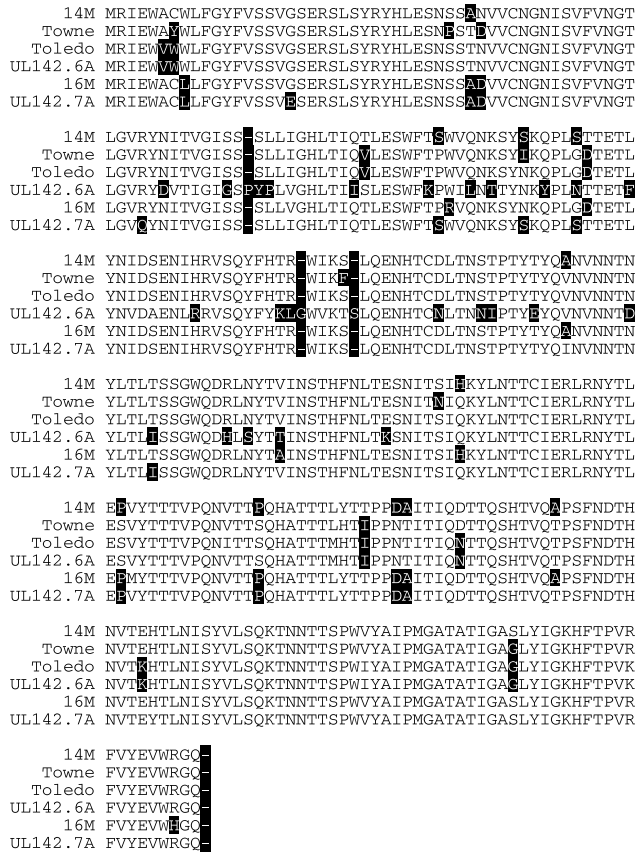

Fig. 1. Amino acid sequence alignment of UL142.6A and UL142.7A. UL142.6A and UL142.7A sequences are aligned with representative UL142 sequences from the laboratory strains Toledo (GenBank Accession No. AY446871) and Towne (GenBank Accession No. AY446869), and patient isolates 14M (GenBank Accession No. AY217042) and 16M (GenBank Accession No. AY941173). Residues boxed in black indicate sequence differences.

Co-expression of UL142 results in decreased surface expression of MICA

In order to examine possible effects of UL14 and UL142 on MICA expression, we subcloned cDNAs encoding these genes into lentiviral vectors containing a co-expressed murine CD8α reporter gene. The UL14-IRES-muCD8α or UL142-IRES-muCD8α lentiviral vector stocks were transduced into a MICA+ EL4 cell line, or into HeLa cells. Populations of MICA+ EL4 cells or HeLa cells expressing full-length UL14 or UL142 were isolated by staining with anti-mouse CD8α-PE and sorting by flow cytometry. Populations of cells with equal levels of surface expression of mouse CD8α (Fig. 2A) were then analyzed for expression of MICA.

MICA+ EL4 cells transduced with the UL14-IRES-muCD8α construct showed no changes in cell surface expression of MICA, whereas surface MICA expression was markedly decreased on cells transduced with either the UL142.6A-IRES-muCD8α or UL142.7A-IRES-muCD8α construct (Fig. 2B). The MICA+ EL4 cells express a full-length form of MICA (GenBank Accession No. AAD52060). Transduction of HeLa cells with UL14 or UL142 constructs had no effect on surface MICA expression (data not shown). HeLa cells are reportedly homozygous for the truncated MICA *008 allele. This allele has previously been reported to be unaffected by HCMV infection, whereas surface expression of other full-length alleles of MICA was down-regulated [22]. In order to confirm that our HeLa cells did express the reported MICA allele, MICA cDNA was amplified from mRNA isolated from MICA+ EL4 cells and HeLa cells using MICA-specific primers. Sequence results confirmed that the MICA+ EL4 cells expressed the full-length allele and HeLa cells express the truncated MICA *008 allele (data not shown).

To measure the effects of UL142 on surface expression of MICA more directly, without using a co-expressed reporter, we constructed UL14- and UL142-GFP fusion

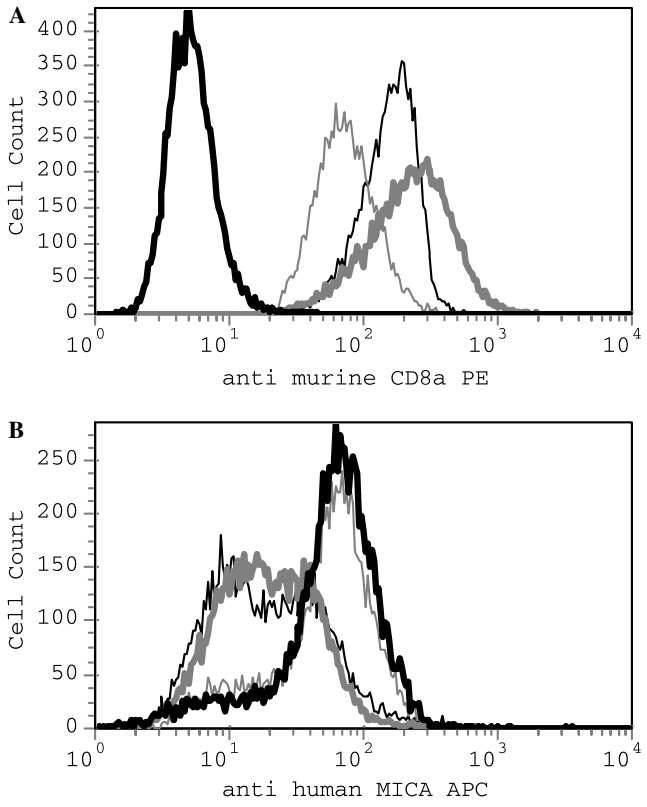

Fig. 2. UL142 expression causes down-regulation of surface MICA expression. Uninfected MICA+ EL4 cells (thick black line), or MICA+ EL4 cells infected with lentiviral constructs expressing UL14 (thin grey line), UL142.6A (thick grey line), or UL142.7A (thin black line) were stained with antibodies specific for the reporter gene, mouse CD8α, and MICA, and analyzed by flow cytometry. (A) Mouse CD8α expression of the cell lines. The MFI of cell lines stained with an isotype-matched control antibody ranged from 2 to 3. (B) MICA expression of the mouse CD8α positive cells. The MFI of cell lines stained with an isotype-matched control antibody ranged from 3 to 7. The results shown are representative of three separate experiments.

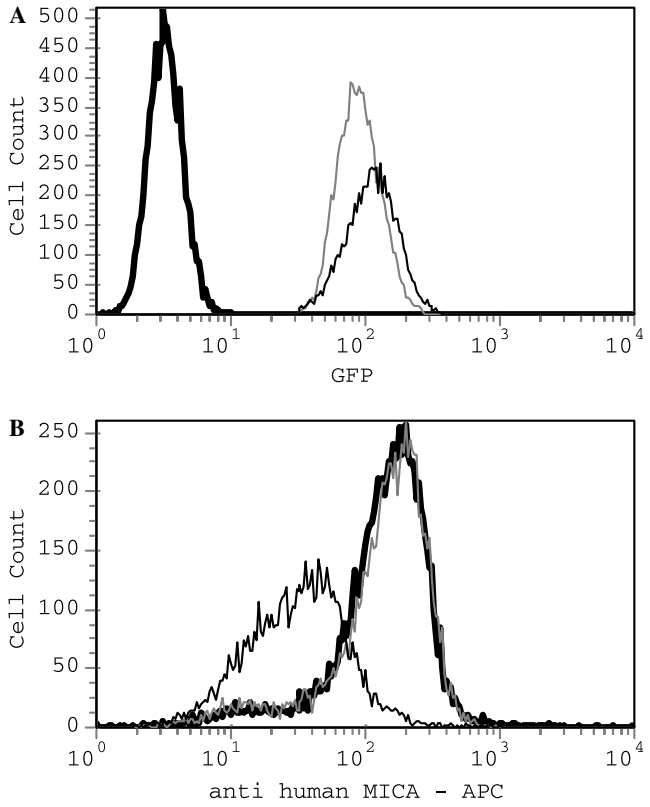

Fig. 3. Expression of GFP-tagged UL142 causes down-regulation of surface MICA expression. Uninfected MICA+ EL4 cells (thick black line), or MICA+ EL4 cells infected with lentiviral constructs expressing UL14-GFP (thin grey line) or UL142.7A-GFP (thin black line) were stained with antibodies specific for MICA analyzed by flow cytometry for expression of GFP and MICA. (A) GFP expression of the cell lines. (B) MICA expression of the GFP positive cell lines. The MFI of cell lines stained with an isotype-matched control antibody ranged from 5 to 9. The results shown are representative of two separate experiments.

proteins. These fusion constructs were subcloned into lentiviral vectors and then transduced into MICA+ EL4 cells and HeLa cells. Cells expressing GFP were sorted via flow cytometry. Populations of cells with high expression of GFP were then analyzed for expression of MICA (Fig. 3A). MICA+ EL4 cells transduced with the UL14-GFP construct showed no changes in cell surface expression of MICA, while surface MICA expression was significantly decreased on cells transduced with UL142.7A-GFP (Fig. 3B), demonstrating that the UL142.7A-GFP fusion was still able to down-regulate surface expression of MICA. Transduction of HeLa cells with UL14-GFP or UL142-GFP constructs had no effect on surface MICA expression (Fig. 4A and B).

UL142 expression increases resistance to MICA-mediated cytotoxicity

The preceding experiments established that UL142 was able to interact directly or indirectly with MICA, altering its trafficking within the cell and consequently decreasing its surface expression. To determine how this might affect recognition of cells by NKG2D-expressing immune effector cells, UL142 expressing MICA+ EL4 cells were examined for their ability to act as targets for NK cytotoxicity. MICA+ EL4 cells are sensitive targets, whereas the parental EL4 cells are not killed efficiently (Fig. 5). MICA+ EL4 cells transduced with UL142.6A-IRES-muCD8α or UL142.7A-IRES-muCDα and sorted for mouse CD8α expression were killed much less efficiently than the non-transduced MICA+ EL4 cells. MICA+ EL4 cells transduced with UL14-IRES-muCD8α, which does not affect surface expression of MICA, were killed as efficiently as the non-transduced MICA+ EL4 cells (Fig. 5). The greater sensitivity to lysis of the UL142.7A expressing targets compared to the UL142.6A expressing targets may reflect the slightly higher level of MICA expression on the UL142.7A expressing targets used in this experiment. The populations of transduced and sorted cells used in Fig. 5 had been grown for two months since the analysis shown in Fig. 2. During this time there had been some drift in MICA surface expression. Immediately prior to use in the killing assay, the mean fluorescence intensity (MFI)

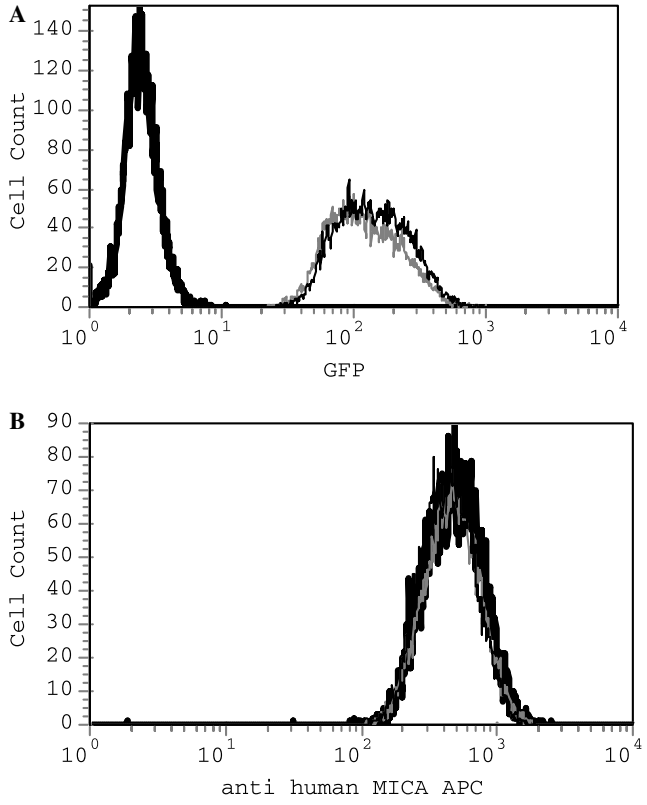

Fig. 4. Expression of GFP-tagged UL142 does not cause down-regulation of surface MICA expression on HeLa cells. Uninfected HeLa cells (thick black line), or HeLa cells infected with lentiviral constructs expressing UL14-GFP (thin grey line) or UL142.7A-GFP (thin black line) were stained with antibodies specific for MICA analyzed by flow cytometry for expression of GFP and MICA. (A) GFP expression of the cell lines. (B) MICA expression of the GFP positive cell lines. The MFI of cell lines stained with an isotype-matched control antibody was 4. The results shown are representative of two separate experiments.

of MICA surface staining for the cell lines was as follows: MFI = 148 for UL142.7A expressing targets; MFI = 58 for UL142.6A expressing targets; MFI = 238 for UL14 expressing targets; MFI = 240 for uninfected MICA + targets; and MFI = 4–10 for all targets stained with an isotype matched control antibody.

Discussion

HCMV has developed several strategies to evade the host immune response. Some of these strategies involve down-regulating the surface expression of proteins involved in triggering an anti-viral response, such as MHC class I antigens [2] and the NKG2D ligands ULBP1, ULBP2, and MICB [19–21]. UL16, the HCMV glycoprotein responsible for the down-regulation of these NKG2D ligands, is not able to down-regulate the remaining NKG2D ligands ULBP3, ULBP4, and MICA [19 and Chalupny unpublished data]. Three separate gene products in MCMV, m145, m152, and m155, have been shown to sequester the mouse NKG2D ligands MULT-1, Rae-1, and H60, respectively, [32–35]. Thus, it is reasonable to

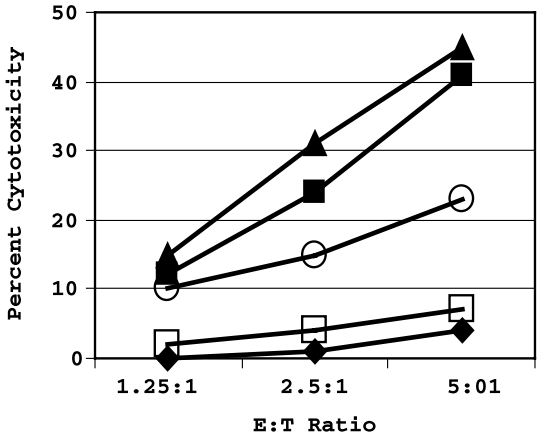

Fig. 5. Expression of UL142 decreases surface expression of MICA, leading to decreased susceptibility to lysis by human NK cells. Parental EL4 cells (black diamonds), uninfected MICA+ EL4 cells (black squares), or MICA+ EL4 cells expressing UL14 (black triangles), UL142.6A (open squares) or UL142.7A (open circles) were tested as targets in a 3-h ^{51}Cr cytotoxicity assay at the indicated E:T ratios. NK cells isolated from human donors and cultured overnight in 50 ng/ml recombinant human IL-15 were applied as effectors. Individual data points are calculated from the averages of triplicate samples. The results shown are representative of three separate experiments.

predict that HCMV might utilize other gene products to down-regulate surface expression of those NKG2D ligands not affected by UL16. Our results demonstrate that the HCMV gene product UL142 is responsible for down-regulation of the surface expression of some alleles of MICA. Decreased surface expression of MICA results in decreased sensitivity to NK-mediated lysis, suggesting that this might constitute another mechanism of viral immune evasion.

A recent report by Zou et al. [22] suggests the existence of a second mechanism of MICA down-regulation by HCMV. They report that infection of cells with the AD169 strain of HCMV results in down-regulation of surface expression of full-length forms of MICA but not of the truncated form, MICA *008. The AD169 genome lacks the ULb' region [30], in which the UL142 gene lies [23]. Therefore, the decreased surface expression observed after AD169 infection must be mediated by a gene product other than UL142. HCMV strains such as Toledo, which contain the UL142 gene, would be predicted to be capable of down-regulating surface expression of MICA through both mechanisms.

Wills et al. [27] have shown that a panel of 31 NK cell clones, derived from three donors, were inhibited by Toledo-infected fibroblasts. Only eight of the clones, however, were inhibited by AD169-infected fibroblasts. Twelve of the clones were not inhibited by UL142 expressed in isolation by the fibroblasts. These discrepancies may be due to ability of the Toledo strain to employ both the mechanism of MICA down-regulation used by AD169 as well as UL142, perhaps leading to a more complete decrease in MICA surface expression. NK clones that are highly effective killers might require a more complete down-regulation of MICA in order to be inhibited. In addition, it is not

known which MICA alleles were expressed by the primary human fibroblasts used in this study. These fibroblasts were established from different donors and, therefore, may express distinct alleles of MICA. Our results demonstrate that not all MICA alleles can be down-regulated by UL142. Zou et al. [22] likewise show that the mechanism of MICA down-regulation employed by the AD169 strain is unable to affect all MICA alleles. In addition, allelic variants of MICA have been shown to differ substantially in their binding affinities for NKG2D [36], which may affect the strength of an NKG2D-mediated NK cell response.

The mechanism by which UL142 down-regulates MICA expression is not yet understood. Co-immunoprecipitation studies with MICA+/UL142.7A+ EL4 cells failed to demonstrate any direct association between MICA and UL142 (data not shown). It is possible that UL142 exerts its effect on MICA via another protein or that the association between MICA and UL142 is weak and does not survive typical immunoprecipitation conditions. It is also possible that our Western blotting reagents are not sensitive enough to detect small amounts of co-immunoprecipitated MICA in cell lysates. Conversely, MICA may be degraded by a UL142-mediated mechanism. Zou et al. [22] also failed to detect MICA in Western blots of cells, in which surface MICA expression had been down-regulated after HCMV infection. Further studies will be necessary to resolve these questions. Similar to MICA, the UL142 gene is also quite polymorphic. It is possible that some alleles of UL142 are more efficient at down-regulating MICA expression than others.

The reason HCMV employs multiple mechanisms of MICA down-regulation may be due to the important role of MICA as a danger signal capable of enhancing immune responses against pathogens through its interaction with NKG2D on NK cells or cytotoxic T cells. MIC cell surface expression has been shown to be up-regulated by stress signals such as heat shock [37], HCMV infection [17], and mycobacterial infection [38,39]. Increased MICA expression has also been implicated in autoimmune conditions. Groh et al. [40] have demonstrated aberrant expression of MICs on proliferating synoviocytes in patients with rheumatoid arthritis. MICA expression is up-regulated at the surface of gut epithelial cells in celiac disease, providing a target for NKG2D-positive intraepithelial lymphocytes [41,42]. Levels of MICA expression on these cells appear to correlate with the severity of disease.

HCMV has evolved several mechanisms to evade host immune surveillance via the MIC-NKG2D pathway; including down-regulation of surface MICB by UL16 [19–21] as well as two methods of down-regulation of surface expression of some MICA alleles [22], one of which is mediated by UL142. Selective pressure on the host to thwart viral immune evasion may have resulted in the selection of alleles such as MICA *008, which are not affected by the currently identified viral strategies. This possibility is intriguing given that MICA *008 is the most frequently expressed allele in many populations [43,44]. The work described here provides evidence that UL142 is a gene involved in HCMV immune evasion, which may be accomplished via the inhibition of NK cell killing of virus-infected cells.

Acknowledgments

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