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Allelic MHC Class I Chain Related B (MICB) Molecules Affect the Binding to the Human Cytomegalovirus (HCMV) Unique Long 16 (UL16) Protein: Implications for Immune Surveillance

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Unique long 16 (UL16) is a viral glycoprotein produced in a host cell infected with human cytomegalovirus (HCMV). It down regulates surface expression of MICB, one of the NKG2D ligands, by forming stable intracellular complexes and retained in the endoplasmic reticulum. Down expression of MICB renders cells less susceptible to NK cell lysis via the NKG2D receptor. Diverse UL16 sequences were identified from different strains of HCMV. MICB is known to be polymorphic. It is not known whether these polymorphisms affect the interactions between these molecules leading to alteration of the immune surveillance of HCMV. The soluble Fc fusion variant UL16 proteins from four laboratory and clinical isolates (AD169, Toledo, PH, and TR) were produced. Four allelic MICB alleles (008, 003, 004, and 00502) were cloned and stable cell lines expressing these MICB alleles were produced. The binding activities of variant UL16 to allelic MICB proteins were determined by flow cytometry. The variants of UL16 proteins did not affect the binding activities to allelic MICB proteins. However, diverse MICB alleles differentially bound UL16. We found that MICB*008 which contains methionine and asparagine at the amino acid positions 98 and 113, respectively, in the alpha 2 domain showed decreased binding activities to UL16 when compared to MICB*003, 004, and MICB*00502 containing isoleucine and aspartic acid, respectively. This finding may imply that MICB*008 is a protective allele and involved in the immune surveillance of HCMV infected patients.

Keywords: UL16 glycoprotein, HCMV, NKG2D, MICB polymorphism

Introduction

Human cytomegalovirus (HCMV) is a virus in the *Herpesviridae* family, subfamily *Betaherpesvirinae*, genus *Cytomegalovirus*, species *human herpesvirus 5*. Infection with HCMV is normally controllable by the immune system. However, in some cases HCMV infections can be associated with severe and even fatal disease. It can be an opportunistic pathogen which is the cause of morbidity and mortality in immune-compromised patients. The immune response to HCMV infection involves both humoral and cellular immunities but the major function is the cellular immune response which comprises a complex interplay between host immune effector cells and HCMV infected cells. Natural killer (NK) cells are considered as important effector cells in early HCMV surveillance. Activity of NK cells is tightly regulated through signals derived from their stimulatory and inhibitory receptors by generating positive and negative signals. Viral infection can make target cells susceptible to NK cell lysis by induction of ligands to activate NK cell receptors, by down-regulation of ligands for inhibitory NK cell receptors and by induction of cytokines that affect NK cell signaling (reviewed in Carayannopoulos and Yokoyama, 2004). There are several activating receptors on the surface of NK cells, including NKG2D (Houchins *et al*., 1991) which recognizes a diverse group of cell-surface ligands that are distantly related to MHC class I molecules i.e. the MHC class I chain-related molecules (MIC) (Bauer *et al*., 1999; Steinle *et al*., 2001) and the UL16-binding proteins (ULBP) or retinoic acid early transcript 1 (RAET1) in humans (Cosman *et al*., 2001; Chalupny *et al*., 2003). They are poorly expressed on most normal cells but the expressions are upregulated upon stress induction, tumor transformation, and viral infections including HCMV (Groh *et al*., 1999; Chalupny *et al*., 2003). However, these immune responses do not result in complete elimination of the virus. CMV has developed several strategies to evade innate and adaptive immune responses (reviewed in Tortorella *et al*., 2000). One of the mechanisms that HCMV uses to escape NK cell responses is the production of UL16 glycoprotein to block NKG2D ligand expression (Dunn *et al*., 2003; Wu *et al*., 2003). UL16 is a 50 kDa glycoprotein, which is not required for viral growth *in vitro* (Kaye *et al*., 1992). UL16 binds to ULBP-1, ULBP-2, and MICB but not to ULBP-3, MICA (Cosman *et al*., 2001) and ULBP-4 (Chalupny *et al*., 2003). The soluble form of UL16 blocks the interaction between NKG2D and its ligands leading to inhibition of target killing via NKG2D-ligand interaction (Cosman *et al*., 2001; Kubin *et al*., 2001). UL16 was reported to down regulate

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MICB expression by accumulating and co-localizing with MICB forming stable intracellular complexes in the ER/cis-golgi compartment (Dunn *et al.*, 2003; Wu *et al.*, 2003).

There are two groups of HCMV strains which are derived from the laboratory or isolated from clinical samples. The HCMV strain, AD169, is usually used in laboratory studies to develop vaccine candidates and in clinical trials. This strain is different from strains isolated from clinical samples in terms of genomic structure and biology. Previous studies demonstrated that there was a large DNA sequence difference between laboratory and clinical strains of HCMV (Cha *et al.*, 1996). In addition, clinical strains were proposed to have strong NK resistance whereas the same target cells infected with laboratory strains were more susceptible to NK cell lysis (Cerboni *et al.*, 2000). The mechanism to explain different NK cell susceptibility between cells infected with these viral strains is unclear. UL16 variants from different HCMV strains may contribute to this observation. The cellular NKG2D ligands, particularly MICB, are also polymorphic (Leelayuwat *et al.*, 1994; Romphruk *et al.*, 2009) with at least 31 alleles reported (http://hla.alleles.org). In addition, the SNPs of MICB have been reported to associate with cytomegalovirus serologically positive schizophrenia patients (Shirts *et al.*, 2007). Thus, polymorphism of MICB may affect the binding to UL16 and could be involved in the immune-surveillance of HCMV. In this study, we examined the binding effect of variant UL16 viral proteins from different strains of HCMV to allelic MICB proteins.

Materials and Methods

Cell lines

293T cells (human renal epithelial cell line) and COS cells (African green monkey SV40-transformed kidney fibroblast cell line) were maintained in a 5% CO_2 atmosphere at 37°C in 10% FCS/RPMI-1640 and 10% FCS/DMEM, respectively (Gibco; Invitrogen, USA) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin.

Construction of plasmids carrying MICB alleles and UL16-Fc variants

The original plasmid DNA encoding UL16-Fc fusion gene was kindly provided by Dr. Hugh Reyburn, Department of Pathology, University of Cambridge, Cambridge, United Kingdom. The sequence of this UL16-Fc construct was found to be identical to the sequence of UL16 from AD169 (Vales-Gomez *et al.*, 2003). This clone was used as a template to produce the other three UL16-Fc variants including Merlin, Toledo, and PH by using the PCR site-directed mutagenesis technique based upon the sequence variations found in the published data (Murphy *et al.*, 2003) and the database of NCBI. The AD169 and Merlin strains represented laboratory strains, whereas PH and Toledo represented clinical strains (Murphy *et al.*, 2003; Dolan *et al.*, 2004). The PCR site-directed mutagenesis technique was performed as previously described (Jumnainsong *et al.*, 2008). In brief, the primers were designed according to the amino acid substitutions shown in Table 1. After PCR products were amplified, PCR reactions were treated with DpnI (Promega, USA) and then transformed into competent cells (MC1061, *Escherichia coli*). The plasmid DNAs encoding UL16-Fc variants were validated by sequencing to confirm that no inappropriate mutations had occurred. The constructed UL16-Fc variants were transfected to COS cells by the DEAE-Dextran method to produce soluble UL16-Fc proteins. These proteins were purified from cultured supernatant by protein A affinity chromatography. For allelic MICB constructions, the original plasmid DNA (pcDNA 3 TOPO vector, Invitrogen, USA) encoding MICB*00502 was used as a template to produce MICB*003, 004, and 008 by site directed mutagenesis. The primers were designed according to the amino acid substitutions shown in Table 2. The protocol for mutagenesis was the same as above but DpnI treated PCR products were transformed into competent cells, Top10, *E. coli*. The plasmid DNAs encoding MICB alleles were validated by DNA sequencing and transfected to 293T cells by FuGENE 6 (Roche, Germany) to produce stable transfectants expressing MICB alleles by G418 (Invitrogen) selection and sorted by flow cytometry.

Expression level of stable transfectants expressing MICB alleles

Empty vector transfected cells and stable transfectants expressing MICB alleles were blocked with PBS-2% FCS and 10% NGS (normal goat serum, Sigma-Aldrich, USA) for 20 min on ice after which the cells were washed once in FACS buffer (PBS-2% FCS). The primary antibodies, 2 μg of anti-MICA (2G8) and anti-MICA/B (6B7) antibodies (Wongsena *et al.*, 2008) and the isotype antibodies, mouse IgG2 (Sigma-Aldrich), were applied and incubated for 30 min on ice after which the excess reaction was washed twice in FACS buffer. The secondary antibody, FITC goat anti-mouse IgG (BD Biosciences, USA) was added and incubated for 30 min on ice. The excess reaction was washed twice in FACS buffer after which the 7-AAD (BD Biosciences) was added to exclude dead cells from analysis. The reaction of 7-AAD was left standing in the room temperature for a minute followed by

Table 1. Amino acid substitutions of four HCMV strains

HCMV strains	Amino acid position in UL16				
	49	129	148	155	177
AD169	H	M	T	Y	S
Merlin	H	M	M	Y	S
Toledo	R	I	T	Y	S
PH	R	M	T	H	S

Table 2. Amino acid substitutions of MICA/B alleles

MICB*	Alpha1		Alpha2		Alpha3
	52	57	98	113	189
00502	D	K	I	D	T
003	D	K	I	D	I
004	N	K	I	D	T
008	D	E	M	N	T
MICA*					
00701	D	E	I	N	T

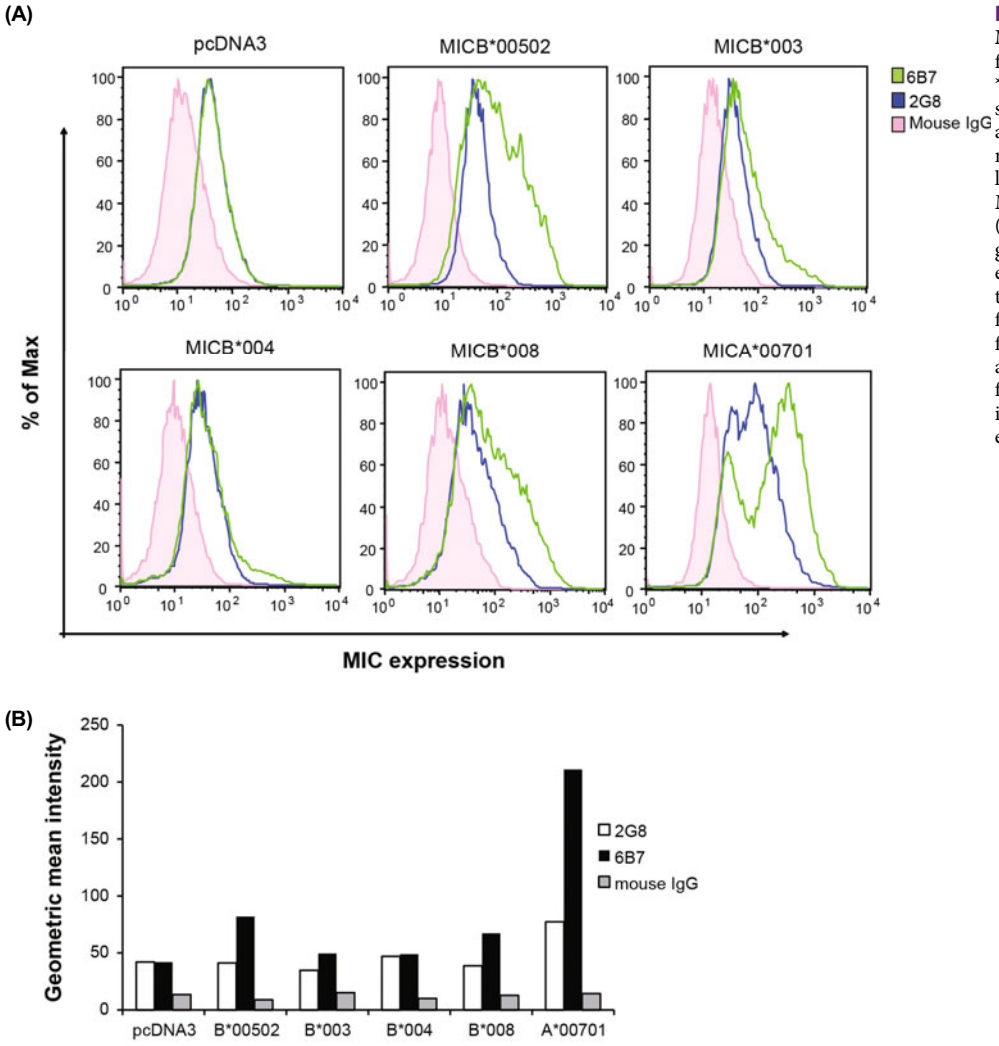

Fig. 1. Differential expression level of MICB stable transfectants. Stable transfected 293T cells with MICB*00502, *003, *004, *008, and MICA*00701 were stained with anti-MICA (2G8, blue line), anti-MICA/B (6B7, green line) and mouse IgG isotype control (solid pink line) and analyzed by FACSCalibur (A). Mocked transfectants with pcDNA3.1 (pcDNA3) were used as control. The geometric mean fluorescent intensity of each transfectant was determined from the Flowjo software to present the differential expressions of MICA/B transfectants (B). 293T cells express MIC as a baseline presented by mocked transfectant (pcDNA3). The figure presented is a representative of three independent experiments.

washing and analyzing by the FACSCalibur (Becton Dickinson, USA).

UL16-Fc protein binding assay

The affinity binding of MICB and UL16 was performed by staining the transfected cells expressing allelic MICB proteins with UL16-Fc soluble proteins and then analyzed by flow cytometry. The MICB tranfectants were blocked with MOBS-2% FCS and 10% NGS for 20 min on ice followed by washing with FACS buffer (MOBS-2% FCS). 2 μg of variant UL16-Fc proteins, the commercial UL16-Fc (Immunex, USA) and human Fc (Sigma-Aldrich) were added and incubated for 1 h on ice after which the excess reaction was washed twice in FACS buffer. The secondary antibody, anti-human Fc biotin (Sigma-Aldrich) was added and incubated on ice for 30 min. APC streptavidin (BD Biosciences) was applied and incubated for 30 min on ice. The reaction was washed once with FACS buffer after which the 7-AAD was added and incubated for 1 min. The stained cells were washed twice in the FACS buffer and analyzed by the FACSCalibur. The geometric mean intensity was determined by the Flowjo software (Tree Star, USA).

Results

MICB stably expressing 293T cells

MICB*003, 004, 00502, and 008 are commonly found in various populations such as Northeastern Thais, Spanish, and Caucasian (Hughes *et al*., 2005; Fernandez-Morera *et al*., 2008; Jumnainsong *et al*., 2008). Thus, plasmids encoding MICB*003, 004, 00502, and 008 were generated and transfected to 293T cells as described in the methods. MICA*00701 was used as a negative control. Table 2 shows the amino acid substitutions of these MICB alleles. Nucleotide polymorphisms are distributed in all three extracellular domains (α1, 2, and 3). The stable transfectants of these MICB alleles were evaluated for MICB expression levels by flow cytometry with anti-MICA antibody (2G8) and anti-MICA/B antibody (6B7) (Fig. 1A). All transfectants expressed MICB on the cell surface but at different levels. The expression level of MICB*00502 represented by geometric mean intensity was higher than MICB*008, 004, and 003, respectively (Fig. 1B).

Generation of variant UL16-Fc fusion proteins

The original UL16-Fc clone encodes a predicted signal pep-

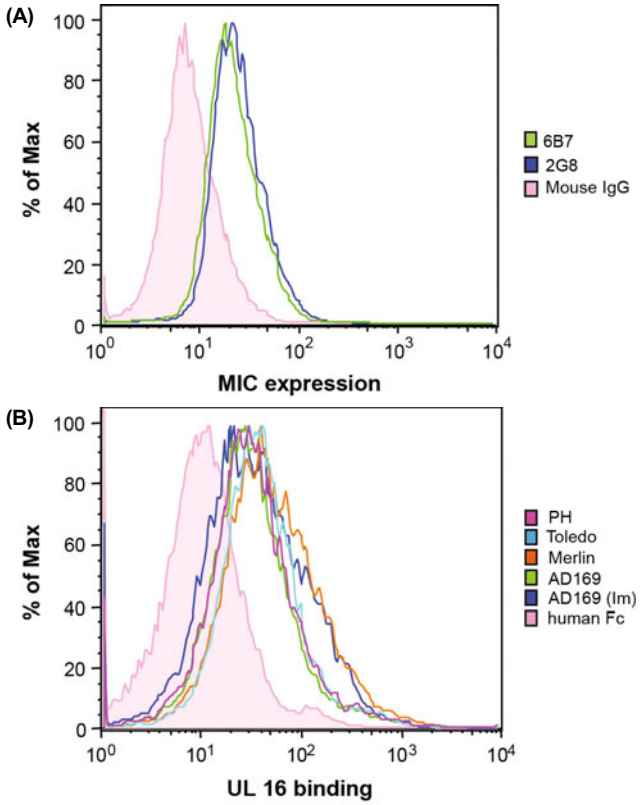

Fig. 2. Comparable binding activities of UL16-Fc from various HCMV strains. 293T cells were stained with anti-MICA (2G8, blue line), anti-MICA/B (6B7, green line), and mouse IgG isotype control (solid pink line) to detect the background expression level of MICA/B (A). Various UL16-Fc proteins (dark pink, bright blue, orange, green, and bright pink representing PH, Toledo, Merlin, AD169, and human Fc as control, respectively) were used to stain 293T cells compared to the commercial UL16-Fc from Immunex (AD169(Im); dark blue line) to confirm that all constructed UL16-Fc proteins were functional (B). Three experiments were performed. The figure shows one experiment as a representative of all experiments.

tide (amino acid -25 to -1) and the extracellular domain of HCMV AD169 strain (amino acid 1–159) in pcDNA3.1 carrying sequence of Fc portion of human IgG1 (Vales-Gomez *et al.*, 2003). Another three UL16 variants (Merlin, PH, and Toledo) were constructed as described. Soluble UL16-Fc fusion proteins were produced from COS transfectants. 293T cells were slightly shifted from isotype control when stained with 6B7 and 2G8 indicating the presence of background MIC expression (Fig. 2A). All produced UL16-Fc fusion proteins bound to 293T cells when were compared to the commercial UL16-Fc protein from Immunex (Fig. 2B). This indicated that all constructed UL16-Fc variants were conformationally active.

Interaction of polymorphic UL16 binding to MICB alleles

Binding activities of four UL16-Fc variants to MICB alleles were analyzed by flow cytometry and presented as the geometric mean intensity (Fig. 3). The four MICB transfectants stained positive for all UL16-Fc proteins when compared to vector transfectant whereas a control, MICA*00701 transfectant, was negative. The geometric mean intensity of UL16-Merlin staining was slightly higher than those of the other strains. In addition, MICB*008 transfectant showed very weak binding to the four variants of UL16-Fc even though the expression level of this allele on the cell surface was higher than MICB*003 transfectant. This was confirmed by staining all the MICB transfectants with the commercial UL16-Fc protein which showed similar results to the constructed UL16 proteins (Fig. 3).

Discussion

Human cytomegalovirus is comprised of two groups, laboratory and clinical isolated strains. A previous study showed that both strains were different in terms of genomic structure and biology especially in different NK cell susceptibility (Cerboni *et al.*, 2000). The mechanism underlining this observation is unclear. The UL16 glycoprotein is an important molecule involved in NK cell susceptibility because the previous studies demonstrated that UL16 could interact with ULPB1, 2 and MICB in the ER/cis-golgi compartment which blocked NKG2D recognition and inhibited NK cell activation signal (Dunn *et al.*, 2003; Wu *et al.*, 2003). In addition, it was reported that in the absence of UL16, NKG2D ligands moved rapidly through the golgi apparatus to the cell surface. Thus, deletion of the UL16 gene was found to enhance the susceptibility of HCMV-infected cells to NK cell lysis (Vales-Gomez *et al.*, 2003). The UL 16 protein is a class I transmembrane glycoprotein containing 159 amino acids. We investigated the binding activities of UL16-Fc fusion proteins of

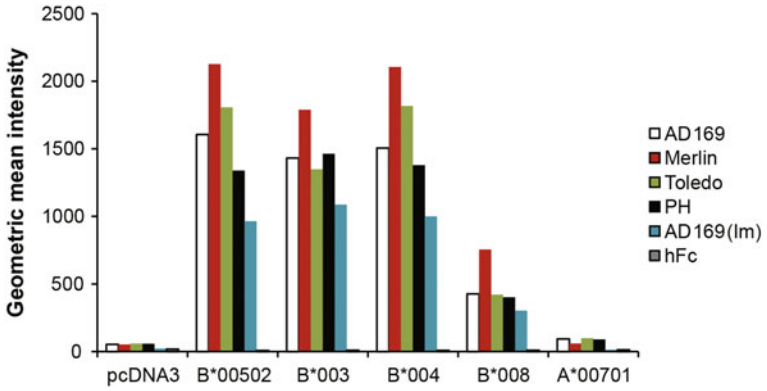

Fig. 3. Binding of UL16-Fc variants to diverse MICB alleles. Allelic MIC stable transfectants were stained with UL16-Fc from different HCMV strains and the commercial UL16-Fc (AD169Im) detected by anti-human Fc biotin and APC streptavidin. Human Fc (hFc) was used as control. The binding activities were presented as the geometric mean values indicating the intensity of UL16-Fc binding among the diverse MICB alleles. The empty vector tranfected cells (pcDNA3) were used as the background of UL16 binding and MICA*00701 was presented as the negative of UL16 binding activity. The experiment was repeated three times and similar results were obtained. The figure is a representative of the experiments.

AD169 and Merlin which are the laboratory strains versus Toledo and PH which are the clinical strains to different MICB alleles based upon the values of geometric mean intensity by flow cytometry. All UL16-Fc fusion proteins contained the extracellular domains of UL16 fused to the amino region of human Fc of IgG1. Although the binding of HCMV UL16-Merlin were slightly higher than those of other strains, no significant differences were observed among UL16 from 4 HCMV strains in binding to MICB stable transfectants. This result indicated that the variations of UL16 tested had no effect on MICB binding and may not affect different NK cell susceptibility of laboratory and clinical HCMV strains regarding NKG2D mediated lysis of NK cells.

For all four MICB stable transfectants, various levels of expression of MIC were detected on the surface. Interestingly, the expression level of MICB*003 and MICB*004 transfectants were low but bound strongly to UL16-Fc. It is possible that this is either the effect of stable transfectant selection process or alleles affecting the expression level (Li *et al*., 2000). Alternatively, this could be the effect of selective allelic activities of the MIC antibodies. All of these possibilities await further investigations. Nevertheless, polymorphisms of MICB affecting binding activities to the UL16 proteins were demonstrated in this study. The transfectant expressing MICB*008 showed lower binding activity to UL16 when compared to other alleles, although the expression level of MICB proteins was similar to that of MICB*00502 and higher than those of MICB*004 and 003. This suggests that MICB*008 may be less efficient to be down regulated by UL16. This means that it can be expressed on the cell surface of infected cells and can induce the activation signal of immune cells to eliminate the infected cells via the NKG2D receptor. Thus, it may be a protective allele for HCMV. Ideally, the study of this effect on NK cell killing should confirm the speculation.

To identify which amino acid substitutions are potentially important in UL16 binding, the amino acid substitutions were compared among the MICB alleles. Interestingly, MICB*008 contains methionine (M) and asparagine (N) at the amino acid positions 98 and 113 in the alpha 2 domain and glutamic acid (E) at the position 57 in alpha 1 domain, whereas the other alleles contain isoleucine (I), aspartic acid (D), and lysine (K), respectively. However, glutamic acid and asparagine at the amino acid positions 57 and 113 were also found in MICB*002 which was reported to bind to UL16 (Cosman *et al*., 2001). In addition, selective MICB recognition by UL16 was affected by helical structures of the MICB alpha 2 domain (Spreu *et al*., 2006). The crystal structure of UL16-MICB complex from previous publication also demonstrated that isoleucine at position 98 of MICB is one of the positions that interacts with UL16 protein (Muller *et al*., 2010). Thus, it suggests that the amino acid at position 98 in the alpha 2 domain of MICB may have a functional role in binding to UL16. However, the functional role of amino acids at the position 113 in the alpha 2 domain and 57 in alpha 1 domain of MICB could not be excluded because the previous study did not show the comparative level of UL16 binding to MICB*002. Unfortunately, we did not include this allele in our study.

In conclusion, our results demonstrated an interaction of UL16 from different HCMV strains with the polymorphic MICB proteins. UL16 variants have no significantly different binding activities to MICB. However, the polymorphism of MICB did affect UL16 binding activities. This finding may imply that MICB*008 is a protective allele for HCMV infections because of very weak binding to UL16 that leads to higher expression of MICB on the cell surface of infected cells than other alleles and can induce the activation signal of immune cells to eliminate the infected cells via the NKG2D receptor. Our study is the first report on the functional polymorphism of MICB proteins on differential UL16 binding that may be involved in immune surveillance of HCMV.

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