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Modulation of natural killer cells by human cytomegalovirus

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Abstract

Human cytomegalovirus (HCMV) causes lifelong, persistent infections and its survival is under intense, continuous selective pressure from the immune system. A key aspect of HCMV's capacity for survival lies in immune avoidance. In this context, cells undergoing productive infection exhibit remarkable resistance to natural killer (NK) cell-mediated cytotoxicity *in vitro*. To date, six genes encoding proteins (UL16, UL18, UL40, UL83, UL141 and UL142) and one encoding a microRNA (miR-UL112) have been identified as capable of suppressing NK cell recognition. Even though HCMV infection efficiently activates expression of ligands for the NK cell activating receptor NKG2D, at least three functions (UL16, UL142 and miR-UL112) act in concert to suppress presentation of these ligands on the cell surface. Although HCMV downregulates expression of endogenous MHC-I, it encodes an MHC-I homologue (UL18) and also upregulates the expression of cellular HLA-E through the action of UL40. The disruption of normal intercellular connections exposes ligands for NK cell activating receptors on the cell surface, notably CD155. HCMV overcomes this vulnerability by encoding a function (UL141) that acts post-translationally to suppress cell surface expression of CD155. The mechanisms by which HCMV systematically evades (or, more properly, modulates) NK cell recognition constitutes an area of growing understanding that is enhancing our appreciation of the basic mechanisms of NK cell function in humans.

Keywords

Cytomegalovirus; NK cells

Individuals with defects in natural killer (NK) cell function are fortunately rare, but often exhibit enhanced susceptibility to herpesvirus infection, and to human cytomegalovirus (HCMV) in particular (Biron et al., 1989; Gazit et al., 2004). Since NK cells are crucial in controlling cytomegalovirus (CMV) infections in both the human and the murine systems, there is a compelling need to appreciate fully the interactions of virus with these immune cells. NK cells constitute a heterogeneous population of cells that express a wide range of

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activating and inhibitory receptors on their surface. Many NK cell inhibitory receptors, including the leukocyte Ig-like receptor 1 (LIR1 or ILT2) and the killer inhibitory receptors (KIRs), recognize endogenous classical MHC-I molecules; thus, normal self-recognition acts to suppress killing by NK cells. HCMV encodes four functions, each of which acts efficiently to downregulate endogenous MHC-I and thus impair antigen presentation (Table 1). Consequently, it was anticipated that downregulation of MHC-I by HCMV would render infected cells vulnerable to NK cell attack. However, the results of *in vitro* assays of NK cell function run counter to this expectation. Human fibroblasts (HFs) infected with the high passage HCMV strain AD169 were not highly susceptible to NK cell killing, and furthermore the same cells infected with a low passage strain were extremely resistant to NK cell attack (Fig. 1; Cerboni et al., 2000; Wang et al., 2002). The remarkable resistance of HCMV-infected cells to NK cells is absolutely dependent on an impressive array of immune evasion functions encoded by the virus. Recent studies on CMV NK cell modulatory functions have had a dramatic impact on our basic understanding of HCMV pathogenesis and, in addition, have provided fundamental insights into the mechanisms regulating human NK cell recognition. This short review will focus on surveying the best-characterized HCMV NK cell modulatory functions.

1. UL18

UL18 was identified as an MHC-I homologue during the original sequencing of the HCMV laboratory strain AD169 genome (Beck and Barrell, 1988). The encoded protein (gpUL18) contains 13 potential N-linked glycosylation sites, and two forms are expressed on the cell surface, one a 69 kDa endoglycosidase H (EndoH)-sensitive species and the other a fully processed 110–160 kDa EndoH-resistant protein (Griffin et al., 2005; Kim et al., 2004). Like classical MHC-I molecules, gpUL18 is expressed as a trimeric complex with β 2-microglobulin and peptide (Browne et al., 1990). The NK cell inhibitory receptor LIR1/ILT2 was found to bind soluble gpUL18 with 1000-fold higher affinity than HLA-I molecules (Cosman et al., 1997; Willcox et al., 2003). Although gpUL18 expression in a class I negative cell line (721.221 cells) elicited protection against killing by NK cell lines (Reyburn et al., 1997), an independent study showed that gpUL18-expressing cells were killed more efficiently by NK cells (Leong et al., 1998). In a recent study, we used CD107 mobilization assays to measure NK cell activation (degranulation) directly. With multiple donors, targets expressing gpUL18 were observed to inhibit LIR1⁺ NK cells but to stimulate LIR-1⁻ NK cells (Prod'homme et al., 2007). This study was consistent with a direct interaction between gpUL18 and LIR-1 suppressing NK cell function, whilst also implying an additional LIR-1-independent interaction in which gpUL18 expression promotes NK cell recognition.

2. UL40

Attention focused on the glycoprotein encoded by UL40 when a nine amino acid sequence (VMAPRTLIL) in its leader peptide was identified as an exact match to an HLA-E-binding peptide (Tomasec et al., 2000; Ulbrecht et al., 2000). HLA-E is a non-classical MHC-I molecule that binds a restricted set of peptides derived from the leader sequences of classical MHC-I molecules and HLA-G. Following peptide binding, HLA-E is transported to the cell surface where it is recognized by the NK cell inhibitory receptor complex CD94/NKG2A. HLA-E thus acts to suppress NK cell-mediated cytotoxicity. During infection, the HCMV US6 protein blocks transport of HLA-E-binding peptides to the ER by inhibiting the transporter associated with antigen processing (TAP), and thus inhibits HLA-E cell surface expression. To counter this vulnerability, UL40 induces protection against NK cell attack in a simple and elegant fashion (Tomasec et al., 2000), in which the UL40-derived peptide upregulates the cell surface expression of HLA-E independent of TAP (Fig. 2). Deletion of

UL40 from HCMV renders infected cells substantially more vulnerable to NK cell-mediated cytotoxicity (Fig. 1a; Wang et al., 2002).

3. UL16

NKG2D is a powerful activating receptor expressed on NK cells, interferon-producing killer dendritic cells, $\alpha\beta$ T cells and $\gamma\delta$ T cells. NKG2D is unusual in recognizing a wide array of ligands (NKG2DLs) on target cells, including MICA, MICB, ULBP1-4 and RAET1G (reviewed in Eagle and Trowsdale, 2007). Expression of NKG2DLs is reported to be activated in response to stress, such as a virus infection, and data are accumulating to demonstrate that adenovirus early and HCMV immediate early (IE1 and IE2) proteins are particularly potent activators (Routes et al., 2005; Tomasec et al., 2007; Venkataraman et al., 2007). The cellular UL16-binding protein (ULBP) family was identified and named on the basis of the affinity of ULBP1 and ULBP2 for the HCMV UL16 glycoprotein (gpUL16). gpUL16 is a potent NK cell evasion function that acts by preventing cell surface expression of MICB, ULBP1 and ULBP2 via direct binding and sequestration in the ER. gpUL16 suppresses NK cell recognition by impeding cell surface expression of these NKG2DLs (Cosman et al., 1997; Kubin et al., 2001; Spreu et al., 2006; Welte et al., 2003).

4. UL83

UL83 encodes an abundant HCMV tegument protein (pp65), which is a major target for the MHC-I restricted CTL response and acts immediately following virion uptake to suppress induction of multiple interferon-responsive and proinflammatory chemokine transcripts (Table 1; Browne and Shenk, 2003). pp65 has also been shown to bind directly to the NK cell activating receptor NKp30, in order to suppress transmission of an activating signal through CD3 ζ and thus impede NK cell activation (Arnon et al., 2005). This situation is unusual in that it involves an HCMV protein that is neither secreted nor expressed on the infected cell surface exerting a direct effect on an NK effector cell. The function of pp65 in NK cell suppression may require its release by lysis of HCMV-infected cells or some other as yet uncharacterized mechanism.

5. miR-UL112

In addition to proteins, HCMV has been shown to express an array of small, non-coding microRNAs (miRNAs; Dunn et al., 2005; Grey et al., 2005; Pfeffer et al., 2005). Since miRNAs are associated with both gene silencing and stress responses (reviewed in Leung and Sharp, 2007), it seems appropriate that their regulatory mechanism has been requisitioned by HCMV to modulate immune recognition. One miRNA (miR-UL112) was predicted to target a sequence in the 3' untranslated sequence of the MICB transcript, and, when expressed in isolation, was shown specifically to suppress cell surface expression of MICB. Deletion of the miR-UL112 function from the genomes of HCMV strain AD169 or TB40/E enabled restoration of surface MICB expression and was associated with enhanced sensitivity to NK cell recognition (Stern-Ginossar et al., 2007).

6. UL/b' sequence encodes NK cell modulatory functions

HCMV has the largest genome of any characterized human virus (236 kbp) and is predicted to contain approximately 165 protein-coding genes, of which only ~45 are essential for growth in fibroblasts (Dolan et al., 2004; Dunn et al., 2003). The widely used laboratory strains Towne and AD169 have evidently suffered a number of genetic changes during extensive passage *in vitro*, the most extensive being deletion of a sequence (UL/b') of 13 and 15 kbp, respectively, from the right end of the UL region. These strains are less effective in promoting protection against NK cell attack than low passage isolates, and this has been

associated with loss of UL/b' since insertion of UL/b' from the low passage strain Toledo into strain Towne restored protection against NK cell attack (Fig. 1b; Tomasec et al., 2005). We therefore anticipated that genes in UL/b' may encode additional NK cell evasion functions. In approaching this experimentally, we were aware that NK cell evasion and other immunomodulatory genes are not generally required during *in vitro* culture and may be lost by mutation during passage. The fact that even low passage strains may contain mutations (e.g. strain Toledo has suffered inversion of a sequence that includes part of UL/b') prompted us to characterize an HCMV strain that more closely represents wild type virus.

Strain Merlin was isolated on HFs from a congenitally infected child's urine sample kindly provided by Cardiff Diagnostic Virology Laboratory. It was characterized at passage 3 as having excellent *in vitro* growth and storage properties. It was shown to be comprised of a single genotype before being sequenced and annotated (Fig. 3; Dolan et al., 2004). From extensive sequence comparisons, strain Merlin is believed to be genetically intact except for a defined point mutation in UL128 that promotes growth in fibroblasts. Strain Merlin is designated a prototypical HCMV genome at GenBank (AY446894) and RefSeq (NC 006273), is available from both the ATCC and the National Collection of Pathogenic Viruses (UK), and its genome has been cloned as a bacterial artificial chromosome and sequenced (Stanton, unpublished data). We consider that the strain Merlin genome represents a reliable source of authentic HCMV sequences for analysing gene functions.

Strain Merlin UL/b' was predicted to contain 19 genes, the protein-coding regions of which are detailed in Fig. 3. To investigate potential contributions to NK cell immune evasion, all 19 putative open reading frames have been expressed using a replication-deficient human adenovirus (RDAd) vector. RDAd vector technology uniquely allows for efficient infection of primary HFs, allowing NK cell assays to be performed both in an HCMV-permissive target and in an autologous background. To complete the generation of this expression library, it proved necessary to develop a high throughput adenovirus cloning vector compatible with expression of 'toxic' gene products (designated AdZ; Stanton, unpublished). This expression library is being used to screen the UL/b' genes for novel NK cell modulatory functions. Preliminary analysis has already been instrumental in identifying UL141 and UL142 as novel NK cell modulatory functions.

7. UL141

The glycoprotein encoded by UL141 (gpUL141) was defined as an NK cell evasion function from an RDAd screen. In such *in vitro* functional NK cell assays, gpUL141 proved the most powerful and robust HCMV NK cell modulator so far tested, inhibiting 67% of NK cell clones tested in an autologous setting (Tomasec et al., 2005). gpUL141 was shown to act by sequestering CD155 in the ER. CD155, also known as the poliovirus receptor (PVR) or nectin-like molecule 5 (necl-5), is involved in multiple cellular functions including motility, adhesion, transendothelial migration, focal adhesions and endocytosis. Unlike NKG2DLs, CD155 is expressed constitutively in HFs, and the majority is concealed in intercellular heterophilic *trans*-interactions with nectin 3. HCMV infection causes a disruption of normal intercellular interactions (Stanton et al., 2007), and under these conditions CD155 is likely to be exposed on the cell surface, where it can be recognized by the NK cell activating receptors CD226 (DNAM-1) and CD96 (TACTILE). Thus, gpUL141 acts to prevent surface expression of the ligand for DNAM-1 and CD96. The strength and breadth of the gpUL141 function *in vitro* point to CD155 playing a key role in modulating NK cell function.

8. UL142

Interest in UL142 as a potential NK cell evasion gene was prompted both by its location in UL/b' and its sequence similarity to UL18, the MHC-I homologue described above. UL18

and UL142 together constitute the HCMV MHC gene family (Fig. 3; Davison et al., 2003). *In silico* analysis also predicted that UL142 encodes intact MHC-I-related $\alpha 1$ and $\alpha 2$ domains, whilst the $\alpha 3$ domain is truncated (Wills et al., 2005). The encoded protein (gpUL142) contains 17 potential N-linked glycosylation sites, implying that it is heavily glycosylated. When expressed from an RDAd vector, gpUL142 provided efficient protection against NK cell-mediated cytotoxicity in autologous NK cell assays, whilst knock-down of UL142 expression using siRNA enhanced NK cell killing of HCMV-infected targets (Wills et al., 2005). The capacity of gpUL142 to suppress NK cell recognition was more readily observed in certain donors (Prod'homme et al., 2007; Wills et al., 2005). Recently, gpUL142 was demonstrated to downregulate cell surface expression of the NKG2DL MICA, although the mechanism of action is yet to be elucidated (Chalupny et al., 2006). MICA exhibits significant sequence polymorphism. The most dramatic difference is observed in the common MICA*008 allele where the sequence encoding the C-terminal cytoplasmic domain has been lost due a frame-shift mutation. The truncated MICA*008 allele was not downregulated by gpUL142 (Chalupny et al., 2006), raising the intriguing possibility that the virus-encoded immune evasion function may be exerting selective pressure on favor of the MICA*008 allele.

9. Evasion or modulation

Published studies have to date identified seven NK cell 'evasion' functions (Table 2). However, in defining an HCMV gene as an NK cell evasion function, the population of NK cells under consideration is key. Is gpUL18 an immune evasion function? Whilst LIR1⁺ NK cells are inhibited via their direct interaction with gpUL18, there is also an activatory effect on LIR1⁻ NK cells. Likewise, whilst HLA-E is a ligand for the CD94/NKG2A⁺ NK cell inhibitory receptor, it is also potentially a ligand for the activating receptor CD94/NKG2C⁺. Interestingly the frequency of CD94/NKG2C⁺ NK cells is elevated in HCMV seropositive individuals (Guma et al., 2004). However, as yet there is no clear evidence that the UL40-derived peptide can promote killing by CD94/NKG2C⁺ NK cells. We consider it more accurate to consider the genes listed in Table 2 as NK cell modulatory, rather than evasion, functions.

10. Additional functions

The functions listed in Table 2 were identified by their effects on NK cells. Whilst NKG2D is ubiquitously expressed on NK cells, it is also ubiquitously expressed on $\gamma\delta$ and $\alpha\beta$ T cells, where it provides a key role in controlling their function. LIR1 is expressed on a subset of $\alpha\beta$ T cells and various myeloid cells types, whilst DNAM-1 is found on T cells, NK cells, myeloid cells, platelets and a subset of B cells. Recent studies have also indicated that CD8⁺T cells can recognize HLA-E loaded with the UL40-derived peptide, and thus UL40 may potentially render HCMV-infected cells vulnerable to CTL attack (Pietra et al., 2003; Romagnani et al., 2004). Thus, in considering the HCMV functions assigned as NK cell modulatory functions, we are aware that these genes have additional functions involving other effector cell populations.

HCMV NK cell modulatory functions have been identified predominantly from computer predictions or by good fortune. To date, only a small proportion of the HCMV genome has been screened systematically. Compelling evidence exists that HCMV encodes additional, unmapped genes capable of suppressing or stimulating NK cell recognition. RDAd have proven to be an exceptionally powerful technology to map and characterize NK cell modulatory functions, and this process should now accelerate with the development of enhanced vector systems. We anticipate that CMV research will continue to provide valuable insights into the fundamental mechanisms regulating NK cell function.

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Fig. 1.

Infection with low passage HCMV strains provides maximum protection against NK cell attack. (a) HFIs infected with high passage strain AD169 show significant protection from NK cell lysis, compared with uninfected targets or targets infected with an AD169 UL40 deletion mutant. Cells infected with strain Toledo or other low passage isolates (not shown) were completely resistant to NK cell attack. (b) The ability to induce complete resistance to NK cell lysis could be restored by inserting the strain Toledo UL b' sequence into high passage strain Towne to produce recombinant Tx4 (kindly provided by E. Mocarski). Modified from Tomasec et al. (2005).

Fig. 2.

Upregulation of HLA-E by HCMV gpUL40. HLA-E exhibits only minor allelic variation and binds a conserved nonameric peptide derived from the leader sequence of HLA-A, -B, -C and -G in a TAP-dependent manner. The HCMV US6 protein binds TAP to inhibit peptide transport. Consequently, US6 inhibits both MHC-I antigen presentation and surface expression of HLA-E. However, the leader sequence of gpUL40 also encodes an HLA-E-binding peptide that is released to the ER independently of TAP. UL40 thus upregulates cell surface expression of HLA-E, a recognized ligand for the NK cell inhibitory receptor CD94/NKG2A.

Fig. 3.

Gene map of the HCMV strain Merlin genome. Inverted repeat regions are shown in a thicker format than the two unique regions. Protein-coding regions are indicated by coloured arrows grouped according to the key, with gene nomenclature below. Introns are shown as narrow white bars. Genes whose names commence with RL, TRS and IRS are given in full, but the UL and US prefixes have been omitted from UL1-UL150 (12–194 kbp) and US1-US34A (199–231 kbp). Colours differentiate between genes on the basis of conservation among the *Alpha*, *Beta*- and *Gammaherpesvirinae* (core genes) or between the *Beta*- and *Gammaherpesvirinae* (sub-core genes), with subsets of the remaining non-core genes grouped into gene families. The location of the UL/b' region (UL148 to UL150) is highlighted by thick horizontal lines. Modified from Dolan et al. (2004) by permission of the Society for General Microbiology.

Table 1

HCMV genes implicated in immune modulation

Downregulation of MHC-I	US2, US3, US6, US11
Downregulation of MHC-II	US2, US3
NK cell evasion	See Table 2
Chemokine receptor	US28, UL33, UL78
TNFR homologue	UL144
IL-10 homologue	UL111A
Virokine	UL146, UL147
RANTES-binding	UL22A
Inhibitors of apoptosis	UL123 (IE1), UL122 (IE2), UL36, UL37
Interferon	IRS1, TRS1, UL83

Table 2

HCMV genes implicated in NK cell modulation

HCMV gene	Comment	Acts on inhibitory receptor
UL18	MHC-I homologue	LIR1 (ILT2)
UL40	Upregulates HLA-E	CD94/NKG2A
HCMV gene	Comment	Acts on activating receptor
UL16	Sequesters MICB, ULBP1-2	NKG2D
UL83	Direct binding	NKp30
miR-UL112	Suppresses MICB	NKG2D
UL141	Sequesters CD155	DNAM-1, CD96
UL142	Acts on MICA	NKG2D