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Permalink

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Journal

The Journal of experimental medicine, 187(10)

ISSN

0022-1007

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Publication Date

1998-05-01

DOI

10.1084/jem.187.10.1681

Peer reviewed

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

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Summary

Natural killer (NK) cells have been implicated in early immune responses against certain viruses, including cytomegalovirus (CMV). CMV causes downregulation of class I major histocompatibility complex (MHC) expression in infected cells; however, it has been proposed that a class I MHC homolog encoded by CMV, UL18, may act as a surrogate ligand to prevent NK cell lysis of CMV-infected cells. In this study, we examined the role of UL18 in NK cell recognition and lysis using fibroblasts infected with either wild-type or UL18 knockout CMV virus, and by using cell lines transfected with the UL18 gene. In both systems, the expression of UL18 resulted in the enhanced killing of target cells. We also show that the enhanced killing is due to both UL18-dependent and -independent mechanisms, and that the killer cell inhibitory receptors (KIRs) and CD94/NKG2A inhibitory receptors for MHC class I do not play a role in affecting susceptibility of CMV-infected fibroblasts to NK cell-mediated cytotoxicity.

Key words: cytomegalovirus • class I major histocompatibility complex • UL18 • natural killer cell • cytotoxicity

Human cytomegalovirus (hCMV)¹ is an extremely widespread infectious agent. Healthy individuals acquiring hCMV postnatally are usually asymptomatic, although the virus persists in the host for life (1). Both T and NK cells play a critical role in controlling the initial infection and the disease that follows viral reactivation in immunocompromised individuals (2, 3). The importance of NK cells is highlighted by the fact that patients with an NK cell deficiency are extremely susceptible to hCMV infection and its associated diseases (4). In addition, NK cells also play an important role in the control of mouse CMV (5). Strain-dependent mouse CMV resistance or susceptibility has been mapped to the NKC complex (NKC) region of murine chromosome 6 (6). The NKC region contains

genes involved in modulating murine NK cell functions and codes for molecules that can trigger (NKR-P1) or inhibit (Ly49) NK cell-mediated cytotoxicity (7). Murine Ly49 molecules and their human functional counterparts, the KIRs (killer cell inhibitory receptors) and CD94/NKG2A receptors, inhibit NK cell cytotoxicity after cognate interaction with class I MHC molecules (8). Since many viruses downregulate host cell class I MHC expression upon infection, the hypothesis that NK cell inhibitory receptors serve as a physiological means to monitor for viral infection is compelling.

Both human (9) and mouse CMV (10) encode glycoproteins with homology to class I MHC heavy chains, designated UL18 (human) and M144 (mouse), respectively. It has been suggested that these molecules serve as surrogates for class I MHC molecules to engage inhibitory NK cell class I MHC receptors. In support of this hypothesis, Farrell et al. (10) have shown that mouse CMV lacking the M144 gene is more virulent *in vivo*. Transfection studies using the human CMV UL18 gene have implicated this protein

¹Abbreviations used in this paper: β_2 M, β_2 -microglobulin; CHO, Chinese hamster ovary; cIg, control Ig; hCMV, human cytomegalovirus; HFF, human foreskin fibroblasts; ICAM-1, intracellular adhesion molecule 1; KIR, killer cell inhibitory receptor; MOI, multiplicity of infection.

in the protection of B lymphoblastoid cells from lysis by NK cells expressing CD94/NKG2A receptor (11). In addition, Cosman and coworkers (12, 13) have recently demonstrated that UL18 is specifically recognized by LIR-1, a membrane of the Ig receptor superfamily, which is predominantly expressed on monocytes, B cells, and a minor subset of NK cells (12, 13). In this study we examined the roles of endogenous class I MHC and an hCMV encoded class I homolog (UL18) in modulating NK cell-mediated cytotoxicity during hCMV infection.

Materials and Methods

Cell lines, NK Cell Lines, and Clones. 293EBV is a human kidney cell line expressing the EBNA-1 nuclear antigen (Invitrogen, Carlsbad, CA). COS-7, Chinese hamster ovary (CHO)-K1, 293EBV, and human foreskin fibroblast (HFF) cell lines were maintained in RPMI (MediaTech, Herndon, VA) supplemented with 10% FCS (GIBCO BRL, Bethesda, MD). NK cells were cloned and cultured as previously described (14, 15). HFF cell lines were prepared and cultured as previously described (16).

Constructs. The UL18 open reading frame was subcloned into the EBV episomal expression vectors pREP10 and pCEP4 (Invitrogen). The cDNA for CD32 and CD94 were cloned into pBlneo. The hCMV gB (UL55) cDNA in the pRcCMV vector (Invitrogen) was a gift from Dr. L. Pereira (University of California San Francisco, San Francisco, CA).

Transient Transfection. 293EBV were plated at 60–80% confluence and transfected with pREP10 UL18 using Lipofectamine (GIBCO BRL). 48 h after transfection, cells were trypsinized and stained with anti-UL18 mAb (10C7), followed by PE-conjugated goat anti-mouse IgG. Viable UL18 positive cells were sorted and cultured for 48 h before use in cytotoxicity assays. COS-7 and CHO-K1 were transfected with pCEP4 UL18 and sorted as above. Flow cytometry was performed as previously described (17).

Antibodies. 10C7 (mouse IgG1) was generated by immunizing mice with soluble, partially deglycosylated UL18. The mAbs against KIRs were: DX9 (KIR3DL1); DX27 (KIR2DL2, KIR2DL3, and KIR2DS2); DX30 (KIR3DL1, KIR3DL2); DX31 (KIR3DL2); and HP-3E4 (KIR2DL1, KIR2DS1, and KIR2DS4). DX22 mAb is against CD94. Anti-class I mAbs (DX15, DX16, and DX17) have been previously described (18). Anti-CMV UL55 (gB) was purchased from the Goodwin Institute (Goodwin Institute, Plantation, FL). Anti-hCMV IE mAb was purchased from Chemicon (Temecula, CA). All other mAbs were provided by Becton Dickinson Immunocytometry Systems (San Jose, CA).

Cytotoxicity Assays. Cell-mediated cytotoxicity was assessed using 4-h ^{51}Cr -release assays. In these assays, effector cells at various concentrations were incubated with 5×10^3 target cells in U-bottomed 96-well microtiter plates at 37°C. Percentage of lysis was determined as previously described (18). Spontaneous ^{51}Cr release was <10% of total counts. Only cells with >80% viability were used for labeling. The spontaneous release of 293EBV UL18 cells and 293EBV controls were similar.

Western Blot. Infected HFFs were lysed in 0.5 ml of lysis buffer (Tris buffered saline, 50 mM Tris, 150 mM NaCl, pH 8.0, with 1% NP-40). Lysates were resolved by SDS-PAGE on 12% gels. Western blots were performed using 10C7 anti-UL18 or anti-hCMV IE mAbs, followed by horseradish peroxidase-conjugated second antibodies as previously described (19).

hCMV Infection. AD169 and $\Delta 18$ (20) viruses were titered and propagated in HFFs as previously described (16). HFFs were infected at a multiplicity of infection (MOI) of 3–5. After adsorption of the virus for 1 h at 37°C, the inoculum was removed and medium containing 10% FCS was added. Viral stocks were titered using classical cytopathic effect as an end-point.

Results and Discussion

The Role of Endogenous Class I in Modulating NK Cell Killing of Infected and Uninfected HFFs. Fibroblasts downregulate class I MHC expression upon hCMV infection (21). In Fig. 1 A, we show that class I MHC is downregulated 10-fold in hCMV-infected HFFs, but a significant level of class I MHC is still present on the cell surface. This downregulation was seen by 24 h and was maintained for the duration of the studies (5 d, data not shown). The ‘missing self’ hypothesis stipulates that the loss of class I MHC expression confers susceptibility to NK cell killing (22). The expectation from this hypothesis is that infected cells would become more susceptible to NK cell-mediated lysis. To address this question, we performed NK cell cytotoxicity assays using hCMV-infected and mock-infected cells. In repeated experiments, NK cells efficiently lysed hCMV-infected HFFs, but not mock-infected cells (Fig. 1 B). This inability to efficiently kill mock-infected HFFs may be due to their high level of class I MHC expression. If NK cells were restrained from killing uninfected HFFs by a class I MHC-dependent mechanism, blocking the KIRs and/or CD94/NKG2A receptors on the effector cells or class I MHC on the fibroblasts should reverse the protection. Experiments performed with anti-class I or a cocktail of mAbs against CD94 and KIRs did not induce killing (Fig. 1 C). This inability to kill was evident using a variety of NK clones and lines (data not shown). The KIR and CD94/NKG2A receptors expressed by these NK cells were functional against B lymphoblastoid target cells transfected with relevant class I MHC genes, with the target cell protection being reversed using anti-class I MHC or anti-CD94 + anti-KIR mAbs. Therefore, class I MHC expression alone by HFFs does not prevent NK cell-mediated killing. It can be argued that mock-infected HFFs lack positive signals needed to trigger NK cells, and that class I MHC may be functional in the context of infected cells, which have up-regulated ligands for the ‘triggering’ receptors. To address this, we repeated these experiments using hCMV-infected HFFs. Again, blocking class I MHC, KIRs, or CD94 neither augmented nor attenuated NK cell-mediated cytotoxicity (Fig. 1 D).

The Role of a Virus-encoded Class I MHC Homolog in NK Cell Killing. hCMV encodes a glycoprotein with homology to class I MHC (9). This molecule is able to form a complex with β_2 -microglobulin ($\beta_2\text{M}$) (23) and contains a peptide-binding groove (24). KIR recognition of class I MHC requires the formation of a trimeric complex comprising class I MHC, $\beta_2\text{M}$, and peptide (25, 26). It was therefore hypothesized that UL18 would interact with NK cell class I receptors to inhibit killing of CMV-infected

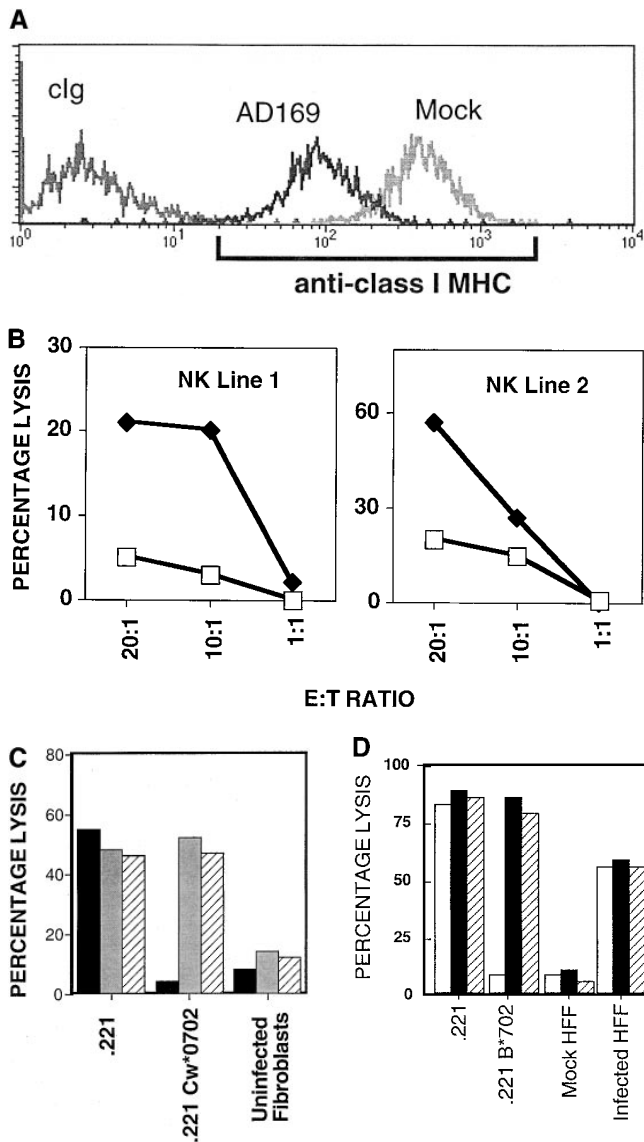


Figure 1. (A) Downregulation of class I MHC in hCMV-infected HFFs. AD169 or mock-infected HFFs were stained with anti-class I (DX17) or control Ig (cIg), followed by PE-conjugated goat anti-mouse Ig. HFFs were infected with AD169 at an MOI of 3, and cells were stained at 24 h after infection. (B) NK cell cytotoxicity assay against hCMV or mock-infected HFF cells. hCMV or mock-infected HFFs were used as targets in a 4-h ^{51}Cr -release assay. HFFs were infected at an MOI of 3 and used at 48 h after infection. Mock-infected, *white squares*; AD169, *black diamonds*. (C) Blocking class I MHC, KIR, or CD94 does not induce NK cell killing of uninfected HFFs. Normal HFFs were incubated in the presence of NK cells and with cIg or a mixture of mAb against class I MHC (DX15, DX16, and DX17 at 20 $\mu\text{g}/\text{ml}$) or a mixture of anti-KIR mAbs (DX27, DX30, DX31, and HP-3E4) and anti-CD94 mAb (DX22), each at 20 $\mu\text{g}/\text{ml}$. cIg, *black bars*; anti-KIR/CD94, *gray bars*; anti-class I, *hatched bars*. (D) AD169 or mock-infected HFFs were incubated in the presence of NK cells with cIg or mAb directed at class I MHC (DX15, DX16, and DX17 at 20 $\mu\text{g}/\text{ml}$) or a cocktail of mAbs against KIR and CD94. Cytotoxicity assays were performed at E/T ratios of 5:1. As controls, 721.221 class I HLA transfectants expressing HLA-B*0702 or HLA-Cw*0702 were analyzed, using NK clones expressing the relevant CD94/NKG2A or KIR, respectively. cIg, *white bars*; anti-KIR/CD94, *black bars*; anti-class I, *hatched bars*.

cells. We examined this by infecting HFFs with wild-type AD169 CMV virus or $\Delta 18$ (a mutant virus with the UL18 gene deleted). The level of class I MHC downregulation by the two viruses was comparable (Fig. 2 A). A 69-kD UL18 protein was detectable by 24 h in AD169-infected HFFs by Western blot analysis, but not in $\Delta 18$ -infected HFF lysates (Fig. 2 B). Although UL18 protein was readily detected by Western blot analysis in HFF infected with AD169 virus, analysis by flow cytometry suggested that very little UL18 was expressed on the cell surface (data not shown). The amounts of hCMV IE protein (Fig. 2 B) and class I MHC (data not shown) in lysates prepared from $\Delta 18$ -infected HFFs were comparable to those detected in HFF infected with AD169 wild-type CMV. Furthermore, the titer of AD169 and $\Delta 18$ viruses were comparable, as assessed by plaque formation assays (data not shown) and the HFFs were homogeneously infected as assessed by the uniform downregulation of class I in all the cells (Fig. 2 A). Many NK cell lines and clones killed AD169-infected cells somewhat better than they did $\Delta 18$ -infected cells (Fig. 2 C). However, HFFs infected with AD169 or $\Delta 18$ showed enhanced killing when compared with mock infected cells (Fig. 2 C). Therefore, UL18 confers a slight enhancement of susceptibility to NK cell killing, although UL18-independent mechanism(s) also exists.

To examine directly the ability of UL18 to confer increased susceptibility to NK cell killing in the absence of viral infection, we transfected 293EBV cells with a UL18 expression vector. Fig. 2 D shows the expression of UL18 on the surface of transfectants using a UL18-specific mAb. In a series of experiments, 293EBV UL18 cells consistently showed enhanced lysis compared with 293EBV (Fig. 2 E). In addition, UL18-dependent enhancement of NK cell killing could also be demonstrated in transfected COS-7 and CHO-K1 cells (data not shown) using several NK clones and lines. These clones included those expressing functional KIRs and CD94 (Table 1). Inhibition of NK cell killing by UL18-expressing target cells was never observed in any of these experiments. The enhanced lysis of 293EBV cells transfected with UL18 was not affected by mAbs against CD94 or KIR (data not shown), although it should be noted that not all KIR isoforms are recognized by the available mAbs. The enhanced killing of 293EBV UL18 transfectants versus 293EBV was not due to changes in the level of class I MHC or adhesion molecules (data not shown). As controls, cells were transfected with expression constructs encoding CD32, CD94, an hCMV surface glycoprotein (gB), or vector only; none of these transfectants showed enhanced susceptibility to NK cell lysis (data not shown).

Hence, the hypothesis that UL18 replaces class I MHC molecules to prevent NK cell lysis does not seem to apply to hCMV-infected HFFs and transfected epithelial and ovary cells (293, COS-7, and CHO-K1). It was recently reported that UL18 inhibits NK cell lysis of 721.221 B lymphoblastoid targets, mediated through CD94 (11). In repeated studies, we never observed inhibition of NK cell

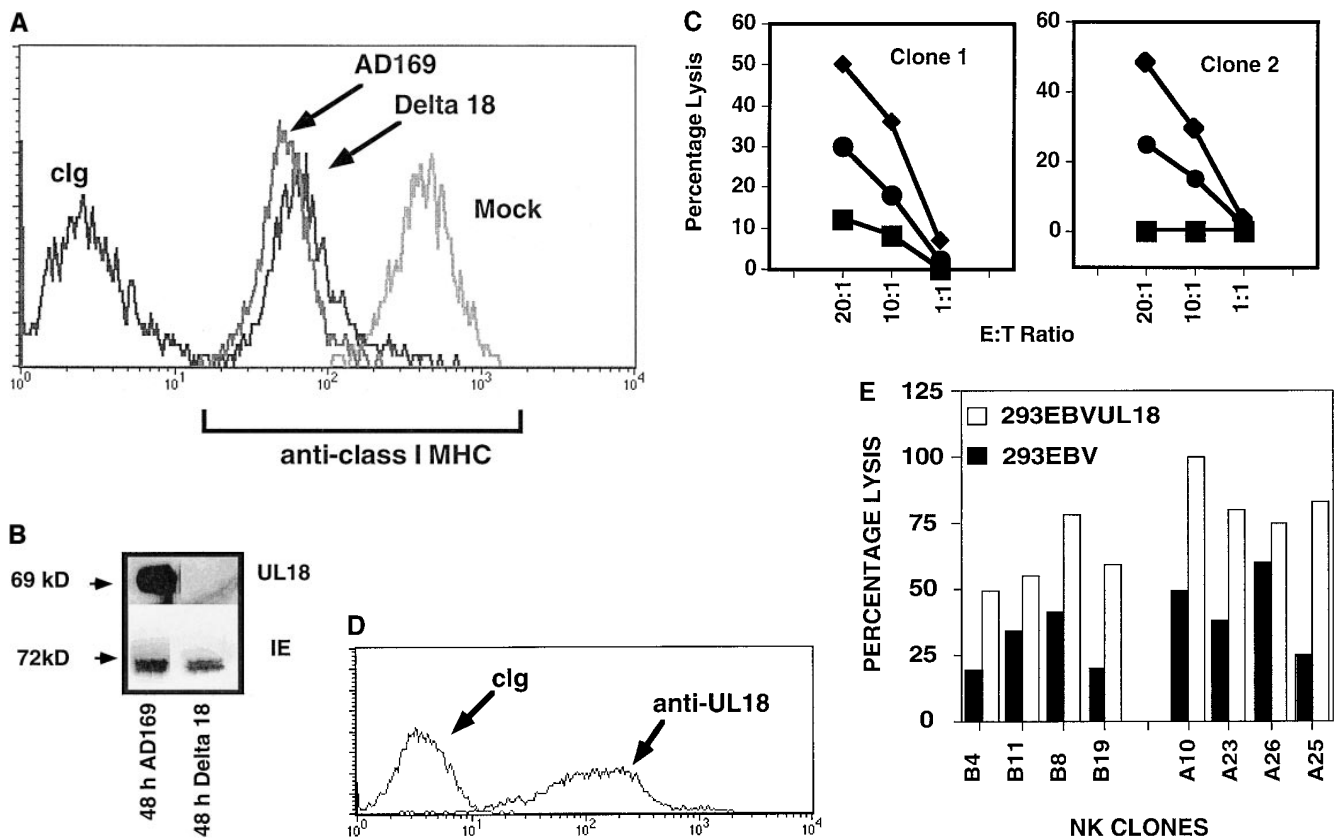


Figure 2. (A) Downregulation of class I MHC after $\Delta 18$ and AD169 hCMV infection of HFFs. HFFs were infected with $\Delta 18$ or AD169 at an MOI of 5. At 48 h after infection cells were stained with anti-class I MHC or clg, followed by PE-conjugated goat anti-mouse second step. (B) Expression of UL18 in AD169 but not $\Delta 18$ cell lysates. Lysates were prepared from HFF-infected with $\Delta 18$ or AD169 at 48 h after infection and blotted with anti-UL18 or anti-hCMV IE mAb, followed by horseradish peroxidase-conjugated second step. (C) AD169-infected HFFs were lysed more efficiently than were $\Delta 18$ -infected HFFs. HFFs were infected with AD169 or $\Delta 18$ at an MOI of 5, and used as targets at 48 h after infection in 4-h ^{51}Cr -release assays. Assays were performed at an E/T ratio of 20:1, 10:1, and 1:1. Standard deviation between triplicates was $<10\%$. $\Delta 18$, black circles; AD169, black diamonds; mock-infected, black squares. (D) Surface UL18 expression on 293EBV transfectants. 293EBV cells were transfected with pREP10 UL18. Cells were cultured for 48 h before use in cytotoxicity assays. Histograms of sorted UL18-positive cells. Cells were stained with anti-UL18 mAb 10C7 followed by PE-conjugated goat anti-mouse Ig. (E) 293EBV transfectant expressing UL18 were lysed at an enhanced level compared with parental controls. UL18-transfected 293EBV cells were used as targets in NK cell cytotoxicity assays. Experiments were performed at an E:T ratio of 5:1. All transfectants other than vector only were positively sorted using mAb directed against the protein encoded by the transfected cDNA. Cells were cultured for 48 h and used in 4-h cytotoxicity assays as described above.

killing against UL18 expressing targets using clones with functional CD94 or KIR. In view of this discrepancy, several aspects of the previous report should be highlighted. First, in the prior study UL18 was transfected into 721.221 targets; however, transfectants were isolated on the basis of surface $\beta_2\text{M}$ expression, not UL18. Second, we have failed to generate stable UL18 transfectants in 721.221 or in 15 other human or mouse lines, because it seems that prolonged expression (>2 wk) of UL18 results in cell death. We could only generate UL18 transfectants in high efficiency transient transfection systems such as 293, COS-7, and CHO-K1. Notably, 721.221 cells express low levels of HLA-E (27) and -F (28). Since Reyburn et al. (11) sorted UL18-transfected 721.221 on the basis of $\beta_2\text{M}$ surface expression, rather than UL18, cells expressing HLA-E or -F may have been inadvertently enriched and could be responsible for the protection against NK cell lysis. In fact, a

Table 1. Phenotype of NK Cell Lines and Clones

Clones	KIRs	CD94
A10	DX9 ⁻ , DX24 ⁺	CD94
A23	DX9 ⁻ , DX24 ⁺	CD94
A26	DX9 ⁺ , DX24 ⁺	ND
A25	DX9 ⁺ , DX24 ⁺	ND
BR	KIR ⁻	CD94/NKG2A
B8	KIR ⁻	CD94/NKG2A
B11	KIR ⁻	CD94/NKG2A
B19	KIR ⁻	CD94/NKG2A

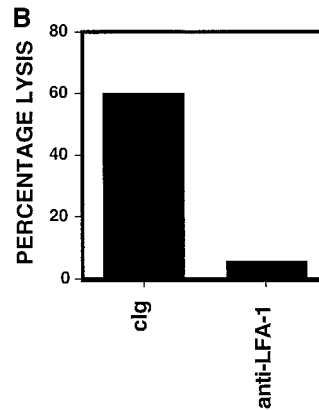
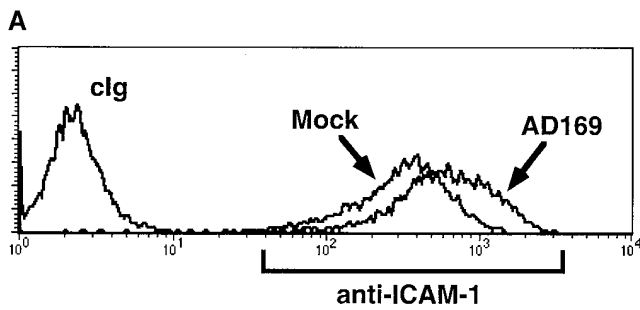


Figure 3. (A) Upregulation of ICAM-1 on hCMV-infected cells. AD169 or mock-infected HFFs were stained (24 h after infection) with FITC-conjugated anti-ICAM-1 (LB2 mAb) or FITC-conjugated clg. Mean fluorescence intensity of ICAM-1 increased from 351 to 705. (B) Enhanced cytotoxicity against hCMV-infected HFFs was reversed with anti-LFA-1 β (CD18). hCMV or mock-infected HFFs were incubated with clg or anti-LFA-1 β (20 μ g/ml). 4-h 51 Cr-release assays were performed at an E/T ratio of 5:1.

recent study demonstrated that the endogenous HLA-E expressed by 721.221 cells can protect these target cells from lysis by NK cells expressing the inhibitory CD94/NKG2A receptor (29). Although HLA-E molecules bind peptides derived from the leader segments of certain other MHC class I proteins (27), the leader of UL18 does not conform to the preferred peptide bound by HLA-E. Therefore, it is possible that Reyburn et al. (11) simply selected for a variant of 721.221 that expressed higher levels of the endogenous HLA-E protein.

Recent studies by Cosman and coworkers (12, 13) have shown that the human CMV UL18 protein interacts with a membrane receptor designated ILT2 (30) or LIR-1 (12, 13), which is expressed predominantly on monocytes, B cells, and a subset of NK cells. Binding of a UL18-Ig Fc fusion protein to NK cells and monocytes was completely blocked using an anti-ILT2/LIR-1 mAb, suggesting that ILT2/LIR-1 is the predominant, if not exclusive, receptor for UL18 expressed on leukocytes (12). It is possible that ILT2/LIR-1 on NK cells might interact with UL18 on CMV-infected cells, preventing NK cell-mediated cytotoxicity. However, it should be appreciated that ILT2/LIR-1 is expressed on only a minor subset of NK cells (12, 31); therefore, the physiological significance of ILT2/LIR-1 on NK cells during a CMV infection is uncertain. Although prior studies of UL18 and its mouse homolog M144 have focused on a potential role for these proteins in NK cell-mediated immunity (10, 11), an alternative possibility is that these molecules may be more important in affecting monocyte and dendritic cell function during CMV infection. For example, interactions between UL18 and ILT2/LIR-1 on monocytes or dendritic cells during a CMV infection may suppress IL-12 production, which would in turn limit IFN- γ secretion by NK cells and thus alter the early host immune response. This scenario could potentially explain the increased virulence of mouse CMV virus lacking M144 (10). This is of particular interest given

the recent finding that dendritic cells may serve as the reservoir for latent CMV infection (32).

Upregulation of Cell Surface Adhesion Molecules in hCMV-infected HFFs. Since enhanced killing was observed in HFF infected with both AD169 and Δ 18, other molecules in addition to UL18 were also playing a role. To examine what molecules were upregulated after infection, we stained mock-infected and hCMV-infected HFFs with a panel of mAb against cell surface adhesion/costimulatory molecules. The only molecule examined that was consistently upregulated in the infected cells was intracellular adhesion molecule 1 (ICAM-1; CD54) (Fig. 3 A). Blocking of ICAM-1 interaction with its ligand using anti-LFA-1 β (CD18) was able to prevent the killing of infected HFF (Fig. 3 B). These data suggest that ICAM-1 is a crucial component in NK cell-mediated killing of hCMV-infected cells, but does not exclude the existence of other molecules capable of triggering NK cell cytotoxicity.

Based on our observations, the hypothesis that KIRs and CD94/NKG2A class I MHC inhibitory receptors are a physiological surveillance mechanism for viral infection may not be universally applicable. Similarly, we have previously shown that in certain target cells (e.g., Jurkat and K562), the expression of an appropriate class I MHC allele recognized by KIR3DL1 (i.e., HLA-B*5801) was insufficient to prevent killing of these transfected target cells (14).

hCMV has evolved many strategies that allow its highly successful dissemination throughout the population. Although infected individuals are generally clinically asymptomatic (1), further viral transmission still occurs. In cases where the infection is not effectively controlled, death often results, thereby limiting the opportunity for further viral dissemination. Therefore, the UL18-dependent and independent mechanisms may serve to limit the severity of CMV-induced disease by rendering infected cells more susceptible to immune destruction.

We thank the members of the DNAX FACS facility for flow cytometry, and Dr. Lenore Pereira, Debra Liggett, Sasha Lazetic, and Brian Corliss for advice and reagents.

DNAX Research Institute is supported by the Schering Plough Corporation. T.L Chapman is supported by a National Defense Science and Engineering Pre-Doctoral Fellowship.

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Received for publication 3 December 1997 and in revised form 26 February 1998.

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