Human cytomegalovirus (HCMV) is known to evade extrinsic pro-apoptotic pathways not only by downregulating cell surface expression of the death receptors TNFR1, TRAIL receptor 1 (TNFRSF10A) and TRAIL receptor 2 (TNFRSF10B), but also by impeding downstream signalling events. Fas (CD95/APO-1/TNFRSF6) also plays a prominent role in apoptotic clearance of virus-infected cells, so its fate in HCMV-infected cells needs to be addressed. Here, we show that cell surface expression of Fas was suppressed in HCMV-infected fibroblasts from 24 h onwards through the late phase of productive infection, and was dependent on de novo virus-encoded gene expression but not virus DNA replication. Significant levels of the fully glycosylated (endoglycosidase-H-resistant) Fas were retained within HCMV-infected cells throughout the infection within intracellular membranous structures. HCMV infection provided cells with a high level of protection against Fas-mediated apoptosis. Downregulation of Fas was observed with HCMV strains AD169, FIX, Merlin and TB40.

Fas is another member of the tumour necrosis factor receptor superfamily (TNFRSF), recognized as playing a major role in controlling viral infections (Itoh et al., 1991; Trauth et al., 1989; Yonehara et al., 1989). While Fas is expressed on most cell types, its cognate ligand (FasL) is restricted to activated T, NK and dendritic cells (Nagata, 1999; Nagata & Golstein, 1995). The upregulation of FasL and TRAIL on HCMV-infected dendritic cells promotes direct killing of activated T lymphocytes, an action that may preferentially delete HCMV-specific T cells (Raftery et al., 2001). Moreover, the activation of FasL on HCMV-infected retinal pigment epithelial cells may subvert neutrophil function in HCMV retinitis (Chiu et al., 2001; Cinatl et al., 2000). Although HCMV may exploit FasL to dampen immune responses, FasL has the potential to kill HCMV-infected cells. FasL acts by inducing a conformational change in Fas, leading to recruitment of FADD and procaspase-8, and assembly of the death-inducing signalling complex (DISC) (Kischkel et al., 1995;
Fig. 1. Modulation of Fas cell surface expression in cells infected with HCMV. (a) HFFF-hTERTs were infected with HCMV strain Merlin (m.o.i. 10) or mock-infected, and analysed at indicated time points by flow cytometry for cell surface Fas expression [mAb142 (R&D Systems), n≥3]. (b) HFFFs were infected with HCMV strain Merlin (m.o.i. 10, 72 h) in the presence (i) or absence (ii) of 100 μM ganciclovir and analysed by flow cytometry for cell surface Fas expression (n=3). (c) HFFF-hTERTs
HCMV downregulates Fas

Scott et al., 2009). Caspase-8 released from DISC induces cleavage of downstream substrates including effector caspases 3 and 7, resulting in proteolysis of critical cellular components and culminating in apoptosis (Barnhart et al., 2003; Salvesen & Dixit, 1997). While UL36 inhibits caspase-8, the fate of Fas during HCMV infection is unclear (Chaudhuri et al., 1999).

Human foetal foreskin fibroblasts (HFF-hTERTs) (McSharry et al., 2001) were therefore infected with HCMV strain Merlin and cell surface expression of Fas tracked over the course of infection. Fas was unaffected by HCMV infection until 24 h p.i.; the cell surface downregulation detected at this time point persisted through the late phase of infection (48 and 72 h) (Fig. 1a). Consistent with Fas downregulation occurring with early kinetics, cells treated with the viral DNA replication inhibitor ganciclovir showed comparable levels of Fas downregulation, demonstrating that viral DNA replication is not required for this function (72 h p.i., Fig. 1b). Latent carriage of HCMV has been shown to protect CD34+ progenitor cells from FasL-mediated apoptosis through increased cIL10 secretion (Poole et al., 2011). However, transfer of supernatants from Merlin-infected cells did not result in substantial downregulation of Fas at the cell surface, indicating that this function is not carried out by a soluble factor (Fig. 1c). In addition, virus inactivated by γ-irradiation did not modulate Fas expression, thus suggesting the function is attributable to a de novo expressed virus-encoded function rather than input virions (Fig. 1c). MHC class-I was included as an infection control; downregulation of classical MHC class-I expression is achieved by four HCMV genes (US2, US3, US6, US11) that are expressed with immediate early and early kinetics (Ahn et al., 1996; Hengel et al., 1996; Hesse et al., 2013; Lehner & Cresswell, 1996; Park et al., 2002). Downregulation of Fas and MHC class-I exhibit similar kinetics (Fig. 1).

Since HCMV exhibits an exceptionally high level of interstrain sequence variation (Dolan et al., 2004), we were interested in determining whether Fas regulation is a conserved function. The level of Fas downregulation was similar in cells infected with HCMV strains Merlin, AD169, FIX and TB40 (Fig. 1d). Comparable results were also obtained using HFF cells and primary dermal fibroblasts (data not shown). Variation in the efficiency of MHC class-I downregulation is attributable to the fact that strains FIX (AUS2, AUS3 and AUS6) and TB40 (AUS3 and AUS6) are derived from BAC clones, and were deleted in the US segment to facilitate genome manipulation (Murphy et al., 2003; Sinzger et al., 2008).

The sensitivity of HCMV-infected cells to Fas-mediated apoptosis was ascertained by measuring the activation of effector caspases 3 and 7. Cells were infected with HCMV strains Merlin or AD169 or mock-infected and treated with FasL or a cross-linking Fas mAb, soluble TR2 or an IgM

![Fig. 2](http://vir.sgmjournals.org)  
Fig. 2. HCMV infection renders cells less sensitive to Fas-mediated apoptosis. HFFs were infected with strain Merlin or AD169 (m.o.i. 10), or mock-infected. At 4 (a) or 60 (b) h p.i., cells were treated with cycloheximide (Sigma) at 10 μg ml⁻¹ concentration and FasL (IBA-Lifesciences), Fas mAb (Beckman-Coulter), sTRAIL-R2 (control for Fas ligand) or IgM isotype control at 500 ng ml⁻¹. Apoptosis was then measured at the indicated time points as caspase 3/7 activation using the Caspase-Glo 3/7 kit (Promega). Results are presented as mean relative light units (RLU) ± SE (n = 4). P-values were calculated using a one-way ANOVA test and a Bonferroni post test.
control antibody. Caspase 3/7 activity was then measured at 16 and 72 h p.i. by its capacity to cleave a luminogenic substrate in the presence of a recombinant luciferase (Fig. 2). At 16 h p.i., prior to Fas downregulation at the cell surface, there was no significant difference in caspase 3/7 activity between mock-infected and HCMV-infected cells in any of the treatment groups (Fig. 2a). However, at 72 h p.i., cells infected with strains Merlin or AD169 became less sensitive to Fas signalling induced by either FasL or Fas mAb (Fig. 2b). In addition, there was no significant difference in the level of protection imparted to cells by strains Merlin and AD169. This is interesting, since the AD169 variant that was used in this experiment carries a single amino acid substitution in the UL36 gene that abolishes the anti-apoptotic function of vICA (Skaletskaya et al., 2001).

HCMV infection therefore renders cells less sensitive to Fas-mediated apoptosis. This function correlates with Fas downregulation from the surface of infected cells, and can occur independently of vICA function.

Fas mRNA levels, as assessed by quantitative reverse transcriptase PCR (qRT-PCR), were not significantly affected by HCMV infection at 24, 48 or 72 h p.i. (Fig. 3a). Nevertheless, levels of Fas in total cell lysates appeared moderately reduced following infection with HCMV strains Merlin, AD169, Fix or TB40 (Fig. 3b). HCMV is known to suppress the cell surface expression of specific proteins (e.g. CD112, CD155, MHC-I, MICB, TR2, ULBP2), often by sequestering them within the cell (Cosman et al., 2001; Jones et al., 1996; Nemčovičová et al., 2013; Prod'homme et al., 2010; Smith et al., 2013; Tomasec et al., 2005). N-linked glycoproteins acquire resistance to endoglycosidase-H (EndoH) during maturation in the Golgi apparatus. Fas was clearly heavily glycosylated, as evidenced by its sensitivity to peptide N-glycosidase-F (PNGaseF), and was resistant to EndoH treatment ± HCMV infection (Fig. 3c). Consequently, HCMV does not appear to retain newly synthesized Fas in pre-Golgi compartments. Immunofluorescence showed Fas to illuminate the surface of infected cells.
of uninfected fibroblasts, in addition to a diffuse cytoplasmic staining pattern (Fig. 3d). In cells infected with HCMV, Fas appeared largely excluded from the plasma membrane; rather, the protein localized to extended membranous perinuclear structures (Fig. 3d).

Fas joins an impressive list of immunomodulatory proteins that HCMV downregulates from the cell surface by post-translational regulation; others include MHC class-I, MICA, MICB, ULBP2, CD155, CD112, TR1, TR2 and TNFR1 (Baillie et al., 2003; Dunn et al., 2003; Nemčovicová et al., 2013; Prod’homme et al., 2010; Smith et al., 2013; Stern-Ginossar et al., 2007; Tomasec et al., 2005). While HMC class-I, CD112 and MICA (C. Fielding, unpublished) are targeted for efficient proteolytic degradation, CD155, TNFR1, TR2 and Fas are maintained at significant levels within infected cells.

HCMV infection induces resistance to Fas-mediated apoptosis, yet the extent to which this can be attributed to cell surface suppression of Fas will ultimately require the identification of the HCMV gene(s) responsible. Despite systematic screening of an expression library encoding the canonical HCMV genes, the function responsible has yet to be mapped (Seirafian, 2013). In this context, multiple HCMV genes can be expected to impact Fas signalling. The UL36 and UL37 gene products efficiently inhibit Fas-mediated apoptosis by inhibiting caspase-8 activation and cytochrome c release, respectively (Arnoult et al., 2004; Goldmacher et al., 1999; Skaletskaya et al., 2001). Moreover, IE2 is known to upregulate c-FLIP, a protease-deficient procaspase-8 homologue (Chiou et al., 2006), whilst the tegument protein UL45 suppresses Fas-mediated killing in the context of HCMV infection by an uncharacterized mechanism (Patrone et al., 2003). These functions operate at or downstream of the DISC, and are thus likely to impact on both TRAIL and Fas-mediated signalling to similar degrees. In addition, since UL141 downregulation of TR2 had a marked impact on TRAIL-mediated cell death (Smith et al., 2013), it is likely that HCMV downregulation of Fas is also an important component of HCMV immune evasion.

Autoimmune lymphoproliferative syndrome (ALPS) is a rare disorder characterized by abnormal lymphocyte survival resulting from a defect in Fas function. A study of two brothers with ALPS experiencing HCMV disease following neonatal exposure documented the development of disseminated infections that were eventually controlled (Arkwright et al., 2000). That Fas-mediated apoptosis is not critical for the control of HCMV disease is consistent with the virus having evolved effective countermeasures to evade Fas-mediated killing. The immune-evasion functions of HCMV are a realistic target for therapeutic intervention.

**Acknowledgements**

This work was supported by funds from the Wellcome Trust (WT090323ZA) and MRC (G1000236). We are grateful to Victor Goldmacher for advice on setting up the apoptosis assay.

**References**

Ahn, K., Angulo, A., Ghazal, P., Peterson, P. A., Yang, Y. & Früh, K. (1996). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. Proc Natl Acad Sci U S A 93, 10990–10995.

Arkwright, P. D., Rieux-Lauracat, F., Le Deist, F., Stevens, R. F., Angus, B. & Cant, A. J. (2000). Cytomegalovirus infection in infants with autoimmune lymphoproliferative syndrome (ALPS). Clin Exp Immunol 121, 353–357.

Arnoult, D., Bartle, L. M., Skaletskaya, A., Poncet, D., Zamzami, N., Park, P. U., Sharpe, J., Youle, R. J. & Goldmacher, V. S. (2004). Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. Proc Natl Acad Sci U S A 101, 7988–7993.

Babić, M., Krmpotić, A. & Jonić, S. (2011). All is fair in virus-host interactions: NK cells and cytomegalovirus. Trends Mol Med 17, 677–685.

Baillie, J., Sahlender, D. A. & Sinclair, J. H. (2003). Human cytomegalovirus infection inhibits tumor necrosis factor α (TNF-α) signaling by targeting the 55-kilodalton TNF-α receptor. J Virol 77, 7007–7016.

Barnhart, B. C., Alappat, E. C. & Peter, M. E. (2003). The CD95 type I/type II model. Semin Immunol 15, 185–193.

Chaudhuri, A. R., St Jeor, S. & Maciejewski, J. P. (1999). Apoptosis induced by human cytomegalovirus infection can be enhanced by cytokines to limit the spread of virus. Exp Hematol 27, 1194–1203.

Chiou, S.-H., Liu, J.-H., Hsu, W.-M., Chen, S. S.-L., Chang, S.-Y., Juan, L.-J., Lin, J.-C., Yang, Y.-T., Wong, W.-W. & other authors (2001). Up-regulation of Fas ligand expression by human cytomegalovirus immediate-early gene product 2: a novel mechanism in cytomegalovirus-induced apoptosis in human retina. J Immunol 167, 4098–4103.

Chiou, S.-H., Yang, Y.-P., Lin, J.-C., Hsu, C.-H., Jhang, H.-C., Yang, Y.-T., Lee, C.-H., Ho, L. L., Hsu, W.-M. & other authors (2006). The immediate early 2 protein of human cytomegalovirus (HCMV) mediates the apoptotic control in HCMV retinitis through up-regulation of the cellular FLICE-inhibitory protein expression. J Immunol 177, 6199–6206.

Cinatl, J., Blaheta, R., Bittoova, M., Scholz, M., Margraf, S., Vogel, J.-U., Cinatl, J. & Doerr, H. W. (2000). Decreased neutrophil adhesion mediated by virus-induced up-regulation of Fas ligand independent of neutrophil apoptosis. J Immunol 165, 4405–4413.

Cosman, D., Möllberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M. & Chaplyn, N. J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14, 123–133.

Dolan, A., Cunningham, C., Hector, R. D., Hassan-Walker, A. F., Lee, L., Addison, C., Dargan, D. J., McGeoch, D. J., Gatherer, D. & other authors (2004). Genetic content of wild-type human cytomegalovirus. J Gen Virol 85, 1301–1312.

Dunn, C., Chaplyn, N. J., Sutherland, C. L., Dosch, S., Sivakumar, P. V., Johnson, D. C. & Cosman, D. (2003). Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med 197, 1427–1439.

Ebermann, L., Ruzsics, Z., Guzmán, C. A., van Rooijen, N., Casalegno-Garduño, R., Koszinowski, U. & Cičin-Sain, L. (2012). Block of death-receptor apoptosis protects mice cytomegalovirus...
from macrophages and is a determinant of virulence in immunodeficient hosts. PLoS Pathog 8, e1003062.

Früh, K., Malouli, D., Oxford, K. L. & Barry, P. A. (2013). Non-human-primate models of cytomegalovirus infection, prevention, and therapy. In Cytomegaloviruses: from Molecular Pathogenesis to Intervention, pp. 463–496. Edited by M. J. Reddeshae. Wyomondham, Norfolk: Caister Academic Press.

Goldmacher, V. S., Bartle, L. M., Skaletskaya, A., Dionne, C. A., Kedersha, N. L., Vater, C. A., Han, J. W., Lutz, R. J., Watanabe, S. & other authors (1999). A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A 96, 12536–12541.

Hengel, H., Flohr, T., Hämmerling, G. J., Koszinowski, U. H. & Momburg, F. (1996). Human cytomegalovirus inhibits peptide translocation into the endoplasmic reticulum for MHC class I assembly. J Gen Virol 77, 2287–2296.

Hesse, J., Ameres, S., Besold, K., Krauter, S., Moosmann, A. & Plachter, B. (2013). Suppression of CD8+ T-cell recognition in the immediate-early phase of human cytomegalovirus infection. J Gen Virol 94, 376–386.

Itóh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. & Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66, 233–243.

Jones, T. R., Wiertz, E. J., Sun, L., Fish, K. N., Nelson, J. A. & Ploegh, H. L. (1996). Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. Proc Natl Acad Sci U S A 93, 11327–11333.

Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P. H. & Peter, M. E. (1999). Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J 14, 5579–5588.

Le, V. T. K., Trilling, M. & Hengel, H. (2011). The cytomegaloviral protein pUL138 acts as potentiator of tumor necrosis factor (TNF) receptor 1 surface density to enhance UL1'-encoded modulation of TNF-α signaling. J Virol 85, 13260–13270.

Lehner, P. J. & Cresswell, P. (1996). Processing and delivery of peptides presented by MHC class I molecules. Curr Opin Immunol 8, 59–67.

McSharry, B. P., Jones, C. J., Skinner, J. W., Kipling, D. & Wilkinson, G. W. (2001). Human telomerase reverse transcriptase-immortalized MRC-5 and HCA2 human fibroblasts are fully permissive for human cytomegalovirus. J Virol 82, 855–863.

Montag, C., Wagner, J., Gruska, I. & Hagemeier, C. (2006). Human cytomegalovirus blocks tumor necrosis factor alpha- and interleukin-1β-mediated NF-κB signaling. J Virol 80, 11686–11698.

Montag, C., Wagner, J. A., Gruska, I., Vetter, B., Wiebusch, L. & Hagemeier, C. (2011). The latency-associated UL138 gene product of human cytomegalovirus sensitizes cells to tumor necrosis factor α (TNF-α) signaling by upregulating TNF-α receptor 1 cell surface expression. J Virol 85, 11409–11421.

Murphy, E., Yu, D., Grimwood, J., Schmutz, J., Dickson, M., Jarvis, M. A., Hahn, G., Nelson, J. A., Myers, R. M. & Shenk, T. E. (2003). Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc Natl Acad Sci U S A 100, 14976–14981.

Nagata, S. (1999). Fas ligand-induced apoptosis. Annu Rev Genet 33, 29–55.

Nagata, S. & Golstein, P. (1995). The Fas death factor. Science 267, 1449–1456.

Nemčovičová, I., Benedict, C. A. & Zajonc, D. M. (2013). Structure of human cytomegalovirus UL141 binding to TRAIL-R2 reveals novel, non-canonical death receptor interactions. PLoS Pathog 9, e1003224.

Park, B., Oh, H., Lee, S., Song, Y., Shin, J., Sung, Y. C., Hwang, S.-Y. & Ahn, K. (2002). The MHC class I homolog of human cytomegalovirus is resistant to down-regulation mediated by the unique short region protein (US)2, US3, US6, and US11 gene products. J Immunol 168, 3464–3469.

Patrone, M., Percivalle, E., Secchi, M., Fiorina, L., Pedrali-Noy, G., Zoppé, M., Baldanti, F., Hahn, G., Koszinowski, U. H. & other authors (2003). The human cytomegalovirus UL45 gene product is a late, virion-associated protein and influences virus growth at low multiplicities of infection. J Gen Virol 84, 3359–3370.

Poole, E., McGregor Dallas, S. R., Colston, J., Joseph, R. S. V. & Sinclair, J. (2011). Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in CD34+ progenitors. J Gen Virol 92, 1539–1549.

Prod'homme, V., Sugrue, D. M., Stanton, R. J., Nomoto, A., Davies, J., Rickards, C. R., Cochran, D., Moore, M., Wilkinson, G. W. & Tomasec, P. (2010). Human cytomegalovirus UL141 promotes efficient downregulation of the natural killer cell activating ligand CD112. J Gen Virol 91, 2034–2039.

Raftery, M. J., Schwab, M., Eibert, S. M., Samstag, Y., Walczak, H. & Schönnich, G. (2001). Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. Immunity 15, 997–1009.

Salvesen, G. S. & Dixit, V. M. (1997). Caspases: intracellular signaling by proteolysis. Cell 91, 443–446.

Scott, F. L., Stec, B., Pop, C., Dobaczewska, M. K., Lee, J. J., Monosov, E., Robinson, H., Salvesen, G. S., Schwarzenbacher, R. & Riedl, S. J. (2009). The Fas-FADD death domain complex structure unravels signalling by receptor clustering. Nature 457, 1019–1022.

Seirafian, S. (2013). An analysis of human cytomegalovirus gene usage. PhD thesis, Cardiff University.

Sinzer, C., Hahn, G., Digel, M., Katona, R., Sampaio, K. L., Messerle, M., Hengel, H., Koszinowski, U., Brune, W. & Adler, B. (2008). Cloning and sequencing of a highly productive, endotheliotropic cytomegalovirus. J Virol 82, 359–368.

Skaletskaya, A., Bartle, L. M., Chittenden, T., McCormick, A. L., Mocarski, E. S. & Goldmacher, V. S. (2001). A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A 98, 7829–7834.

Smith, W., Tomasec, P., Aicheler, R., Loewendorf, A., Nemčovičová, I., Wang, E. C., Stanton, R. J., Macauley, M., Norris, P. & other authors (2013). Human cytomegalovirus glycoprotein UL141 targets the TRAIL death receptors to thwart host innate antiviral defenses. Cell Host Microbe 13, 324–335.

Stern-Ginossar, N., Elefant, N., Zimmermann, A., Wolf, D. G., Saleh, N., Biton, M., Horwitz, E., Prokocimer, Z., Prichard, M. & other authors (2007). Host immune system gene targeting by a viral miRNA. Science 317, 376–381.

Tomasec, P., Wang, E. C., Davison, A. J., Vojtesek, B., Armstrong, M., Griffin, C., McSharry, B. P., Norris, R. J., Llewellyn-Lacey, S. & other authors (2005). Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. Nat Immunol 6, 181–188.

Trauth, B. C., Kias, C., Peters, A. M., Matzku, S., Möller, P., Falk, W., Debatin, K.-M. & Kramer, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245, 301–305.
Vidal, S., Krmpotić, A., Pyzik, M. & Jonjić, S. (2013). Innate immunity to cytomegalovirus in the murine model. In Cytomegaloviruses: from Molecular Pathogenesis to Intervention, pp. 192–214. Edited by M. J. Reddehase. Wymondham, Norfolk: Caister Academic Press.

Yonehara, S., Ishii, A. & Yonehara, M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-down-regulated with the receptor of tumor necrosis factor. J Exp Med 169, 1747–1756.