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Specific Recognition of the Viral Protein UL18 by CD85j/LIR-1/ILT2 on CD8$^+$ T Cells Mediates the Non-MHC-Restricted Lysis of Human Cytomegalovirus-Infected Cells[1]

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Immune evasion mechanisms of human CMV are known; however, the immune control of infection remains poorly elucidated. We show that interaction between the viral protein UL18 on infected cells and the invariant receptor CD85j/LIR-1/ILT2 expressed on CTL is relevant for the control of infection. Resting and activated CD8$^+$ T cells lysed UL18 expressing cells, whereas cells infected with CMV defective for UL18 were not killed. Lysis was not dependent on CD8$^+$ T cell Ag specificity, MHC-unrestricted and specifically blocked by anti-CD85j and anti-UL18 mAb. Moreover, soluble recombinant UL18Fc immunoprecipitated CD85j from T cells. Activation is mediated by CD85j and its pathway is unrelated to CD3/TCR engagement. UL18 is detected in immunocompromised patients with productive infection and the mechanism used in vivo by human CMV to ensure survival of the immunocompetent host may be mediated by activation signals delivered by infected cells to T lymphocytes via UL18/CD85j interactions. ***The Journal of Immunology,*** **2004, 172: 5629–5637.**

The human CMV (hCMV)[4] has developed strategies to avoid its elimination by both cytolytic T lymphocytes (1, 2) and NK cells (3, 4). These immune escape mechanisms should lead to death of the host and thus impair virus spreading. In contrast, a large proportion of individuals are hCMV infected, but the majority of them prevent disease by maintaining infection in a latent phase (5). Active infection only occurs in subjects with impaired cell-mediated immunity (6, 7). An efficient control of the infection must therefore exist.

In mice, CMV infection is kept under check by the Ly49H-activating receptor expressed on NK cells that is specific for the viral protein m157 (8, 9). However, NK cells do not appear to control entirely the productive phase of murine CMV (mCMV) infection as NK cell depletion is not sufficient to sustain active infection. Instead, a crucial role for the maintenance of systemic mCMV latency appears to be exerted by CD8$^+$ T lymphocytes (10). It is not clear whether T cell control is Ag specific as, despite the high coding ability of the hCMV genome, only few hCMV antigenic peptides are immunodominant and are presented to CD8$^+$ T cells by MHC class I (11). This suggests that a non-MHC-restricted T cell control of hCMV infection is operational.

UL18, a surface glycoprotein coded by the hCMV genome (12), is a viral homologue of MHC class I (13). However, its function is unknown. It has been proposed that UL18 engages the inhibitory NK cell receptor CD94/NKG2A, thus providing an escape mechanism to infected cells (14). However, this finding has been questioned and the result has been attributed to expression of HLA-E, the ligand of CD94/NKG2A (15, 16), on cells transfected with UL18 (3, 17). So far, no evidence of molecular interactions between UL18 and CD94 has been provided. Another study suggests an activating role for UL18 on cells of the immune system rather than an inhibitory one, as cells transfected with the UL18 gene are susceptible to NK cell-mediated lysis in vitro (17).

Expression cloning of the receptor for UL18 has revealed a novel molecule of the Ig superfamily, i.e., CD85j/LIR-1/ILT2 that binds to MHC class I (18, 19). CD85j is a transmembrane molecule with four cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) that mediate transduction of inhibitory signals (20). It is detected on the surface of a proportion of T cells (19, 20) and in the cytoplasm of all T lymphocytes (21). Cytoplasmic expression of CD85j in all CD4$^+$ T cells has been recently confirmed (22). CD85j is functional in all T cells, independently of its surface expression (21), and it down-regulates Ag-specific functions of T lymphocytes (21, 23). Cross-linking of the receptor by specific mAbs exerts a dual effect on cytokine production. IL-10 and

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[4] Abbreviations used in this paper: hCMV, human CMV; hF, human fibroblasts; ITIM, immunoreceptor tyrosine-based inhibition motif; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; mCMV, murine CMV; rh, recombinant human.

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TGF-β that inhibit immune responses are increased, whereas cytokines supporting T cell activation such as IL-2, IFN-γ, and IL-13 are reduced (24). In addition, in Jurkat cells transfected with CD85j, TCR/CD85j cross-linking induces a phosphorylation of CD85j by the $p56^{lck}$ kinase (25). Remarkably, CD85j is not phosphorylated following cross-linking of this receptor alone (25). This suggests that, in the absence of an activating signal able to recruit $p56^{lck}$, CD85j does not transduce inhibitory signals.

Expression of UL18 was investigated in this study and it was detected in tissues from immunocompromised patients with productive hCMV infection, as well as in fibroblasts infected in vitro. Therefore, we searched for a role of UL18 in hCMV infection. We found that binding of UL18 to T lymphocytes via CD85j, in the absence of CD3/TCR involvement, leads to activation and not inhibition of T cells and to the subsequent lysis of hCMV-infected cells. In vitro expanded $CD8^+$ T cell clones as well as resting or activated peripheral blood $CD8^+$ T cells lysed efficiently hCMV-infected and $UL18^+$ cells, irrespective of their Ag-specificity, in a non-MHC-restricted fashion. Lysis results from an interaction between monomorphic proteins, namely CD85j on T lymphocytes and UL18 expressed by infected cells. These data indicate that an invariant receptor expressed on $CD8^+$ T cells mediates the specific recognition of an infectious Ag component, and provide a novel example that extends the characteristics of innate immunity to cells that are mainly involved in adaptive immunity.

Materials and Methods

Abs, cells, and viral strains

The following Abs were used: anti-CD4, anti-CD8, anti-CD16, anti-CD19, anti-CD31, anti-CD44, anti-CD54, anti-CD58, anti-CD69, anti-IL-2, anti-IFN-γ mAb (BD PharMingen, Hamburg, Germany), anti-CD3 (clone OKT3; American Type Culture Collection (ATCC), Manassas, VA), anti-MHC class I mAb (clone A2, produced in our laboratory), anti-CD85j clone HP-F1 (kindly provided by M. Lopez-Botet, Servicio de Immunologia, Hospital Universitario de la Princesa, Madrid, Spain), anti-hCMV UL44 (DAKO, Milan, Italy), and anti-UL18 (clone 10C7; ATCC).

$CD8^+$ T cell clones were produced by PHA stimulation and by specific EBV-Ag selection as described previously (21, 23, 24). HeLa and Jurkat cells were provided by ATCC. Human fibroblasts (hF) were derived from bone marrow, and stromal cells were obtained from iliac crest marrow aspirates from healthy donors enrolled for bone marrow transplantation at the S. Martino Hospital and the G. Gaslini Pediatric Hospital (Genoa, Italy). Donor age ranged between 3 and 50 years and informed consent was obtained; institutional ethical committees approved all procedures. Bone marrow and stromal cell cultures were performed as described (26). Mononuclear cells were plated at 2–5 $\times$ 10^6/100 mm dish in Coon's modified Ham's F-12 medium supplemented with 10% FCS. Half of the plates were cultured in the presence of 1 ng/ml human recombinant fibroblast growth factor-2 (Austral Biologicals, San Ramon, CA). The medium was replaced after 3 days and then twice a week.

$CD8^+$ resting T lymphocytes were obtained from PBMC by negative immunomagnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA), with depletion of $CD4^+$, $CD19^+$, and $CD16^+$ cells. Enrichment was tested by FACS analysis (>95% $CD8^+$) and cells were plated in complete medium supplemented with human AB serum alone (resting $CD8^+$ T cells) or with the addition of recombinant human (rh)IL-2 (100 U/ml) and of immobilized OKT3 mAb (activated $CD8^+$ T cells). After 48 h, cells were harvested and used in a cytotoxicity assay (see below).

The hCMV strain AD169 was purchased from ATCC. Δ*UL18* and Δ*UL16* hCMV viral mutants and vaccinia virus (*VVβ2* and *VV103*) containing genes coding for β_2-microglobulin and UL18, respectively, were selected as described (27) and kindly provided by H. Browne (Division of Virology, Department of Pathology, University of Cambridge, Cambridge, U.K.).

Virus infection

In all of the experiments, fibroblasts were infected at a multiplicity of infection of 10. After adsorption of the virus for 1 h at 37°C, the inoculum was removed and fresh medium was added. In some experiments, HeLa cells were infected with vaccinia virus at a multiplicity of infection of 5. After adsorption of the virus for 1 h at 37°C, the inoculum was removed and fresh medium was added. Infected HeLa cells were used for immunostaining and cytotoxicity experiments 2 days after infection.

Immunohistochemical localization of UL18

hCMV-infected cells in two interstitial pneumonia autopsy specimens and one colon biopsy were analyzed for the expression of UL44 and UL18 by immunohistochemistry. One lung specimen was obtained from a patient (age 20 years) who underwent bone marrow transplantation for the treatment of T cell lymphoma. The second lung specimen was derived from a spontaneous abortion of a 25-wk fetus affected by hCMV infection. The colon biopsy was from a patient (age 53 years) who underwent bone marrow transplantation for the treatment of renal cancer and was affected by ulcerative hCMV infection of the bowel.

Abs for immunohistochemical analyses were anti-UL44 (DAKO) and anti-UL18 (clone 10C7; ATCC). Tissue fragments were fixed with 2% paraformaldehyde and paraffin embedded. Five micrometer thick sections were treated in a microwave oven four times with citrate buffer (pH 6) for 5 min at 960 W. Sections were saturated with 10% BSA in PBS with 0.1% Triton X-100 and incubated overnight at 4°C in a humidified chamber with the specific primary Ab. The reaction was developed after addition of a secondary goat anti-mouse antiserum (Southern Biotechnology Associates, Birmingham, AL) according to the alkaline phosphatase-anti-alkaline phosphatase technique, and stained with Fast Red TR (DAKO) (28). Controls were provided by samples incubated with isotype-matched mAb (anti-CD31) + goat anti-mouse antiserum or with the secondary reagent alone (see *Results*).

Cytotoxicity assays

Cytotoxicity tests were performed as described (21, 23), using as targets hF, the murine mastocytoma cell line P815, and HeLa cells uninfected or after viral infection. Target cells were labeled for 1 h with ^{51}Cr (Amersham Biosciences, Buckinghamshire, U.K.) in 96-well plates. $CD8^+$ T cell clones or freshly isolated $CD8^+$ T lymphocytes were the effector cells at an E:T ratio of 10:1 (with some exceptions, as indicated).

Biochemical analyses

Cell surface proteins were labeled with NHS-LC-biotin (Pierce, Helsingborg, Sweden) as described (29), using cells growing in suspension or adherent (30). Metabolic labeling was performed by incubating cells for 1 h in Met-Cys free culture medium followed by 1 h in [^{35}S]Met-Cys 3.7 MBq/ml (Amersham Biosciences). Cells were lysed with RIPA lysis buffer (10 mM NaH_2PO_4, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, with 150 mM NaCl, 1% Triton X-100, protease inhibitors) (complete mini protease inhibitory cocktail; Roche, Mannheim, Germany) and nuclei were discarded by centrifugation at 400 $\times$ *g*. After two 30-min preclearing cycles with GammaBind protein G-Sepharose beads (Amersham Biosciences), specific absorption was performed by incubating cell extracts with anti-UL18 mAb, HP-F1 mAb, or UL18Fc protein (10 μg/ml respectively) and 20 μl protein G-Sepharose beads. Incubation was extended from 3 h to overnight at 4°C with rotation. Sepharose was thoroughly washed with lysis buffer, and bound material was eluted with SDS-PAGE sample buffer. Immunoprecipitated proteins were fractionated by SDS-electrophoresis in 10% polyacrylamide gels, unless otherwise indicated, under reducing conditions. Biotinylated samples were analyzed by Western blot using HRP-conjugated streptavidin, according to standard procedures. Polyacrylamide gels with metabolically labeled samples were soaked in Amplify (Amersham Biosciences), dried, and autoradiographed. CD85j phosphorylation on tyrosines was triggered and evaluated as described (25).

Protein identification by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) peptide mass fingerprinting was performed as follows. Bands of interest were excised from the gel, cut in ~1 $\times$ 1 mm pieces, transferred to a 0.5-ml Eppendorf tube, rinsed with water and reduced, alkylated, and digested overnight with bovine trypsin as described elsewhere (31). Briefly, 1 μl of the digestion supernatant containing the generated tryptic peptides was loaded onto the MALDI target using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix. MALDI mass spectroscopy measurements were performed on a Voyager-DE STR (Applied Biosystems, Framingham, MA) TOF mass spectrometer and processed via the Data Explorer software (32). Proteins were unambiguously identified by searching against a comprehensive nonredundant sequence database using the program ProFound (27).

Transfectants and Fc fusion protein

The region of ILT2 gene coding for the CD85j receptor was amplified by RT-PCR, cloned in the pRc/CMV vector (Invitrogen, San Diego, CA), and

sequenced. This construct (pCMV/ILT2) was subsequently mutated to obtain an ILT2 mutant lacking all four ITIM motifs. To this end, a stop codon was generated by mutation of the TAT triplet coding for Y533 to TAA (pCMV/ILT2-STOP) using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The construct was sequenced to assess the presence of the mutation. The constructs obtained and the empty vector (pCMV) were transfected into Jurkat cells (J-pCMV) by electroporation using a multiporator (Eppendorf, Milan, Italy). Stable transfectants were obtained by selection with 500 μg/ml Geneticin (Invitrogen). Jurkat cells transfected with pCMV/ILT2 (J-ILT2) and with pCMV/ILT2-STOP (J-STOP) were cloned and a positive clone for each transfectant was selected.

The UL18Fc recombinant protein was obtained by cloning the hCMV UL18 gene-coding region, from AA.16 to AA.318, into the pcDNAspIg2.0 vector that contains the CD33 leader sequence and the genomic Fc portion (CH2 and CH3) of human IgG_1. Transient expression of UL18Fc in suspension-adapted HEK293-EBV-encoded nuclear Ag cells was determined as described (33). The UL18Fc protein was purified from cell-free supernatants by chromatography on Streamline protein A (Amersham Biosciences).

Results

hCMV-infected cells express the UL18 protein in vivo and in vitro

To investigate the role of UL18 in hCMV infection, two lung autopsy specimens from patients with interstitial pneumonia and lethal productive hCMV infection were analyzed for the expression of this protein in vivo. Both samples displayed multiple foci of cytomegalic cells that stained strongly for the hCMV Ag UL44, an early DNA-binding protein (data not shown), and for UL18 (Fig. 1, *a–c*). In addition, UL18 was detected in a colon biopsy specimen from a patient with ulcerative intestinal infection after allogeneic bone marrow transplantation (Fig. 1*d*). Of note, in this patient, in addition to cytomegalic interstitial cells present in the submucosa, UL18 was also expressed in the cytoplasm of endothelial cells (Fig. 1, *e* and *f*). An isotype matched mAb, anti-CD31, used as control yielded a negative result. These observations indicate that cells infected by wild-type hCMV express UL18 in vivo.

To evaluate the expression of the viral protein on the surface of infected cells and the role of UL18 in immune recognition, we used an in vitro model where hF were infected with the hCMV strain AD169. Infection was monitored by expression of the UL44 Ag. In accordance with previous observations on mRNA kinetics (34), UL18 was detected on the surface of fibroblasts 3–4 days after infection and reached its highest level on day 6 (Fig. 2, *a* and *b*).

$CD8^+$ T cells lyse hCMV-infected fibroblasts in a non-MHC-restricted fashion

Infected fibroblasts were the target cells in cytotoxicity assays using allogeneic (Fig. 2*a*) or autologous (Fig. 2*b*) $CD8^+$ T cell clones as effectors. The specificity of the clones is unrelated to hCMV Ags as clones AK2 and BE1, derived from two distinct donors, were specific for autologous B-EBV cell lines, whereas clones PG 1.14 and PG 10B5 were obtained following PHA stimulation. In both instances, T cell clones lysed fibroblasts significantly, starting 4 days after infection, and the peak of lysis was concomitant with the highest level of UL18 surface expression, 6 days after infection. No lysis occurred when noninfected fibroblasts were the target cells.

To assess the ability of cytolytic T lymphocytes to lyse additional allogeneic hCMV-infected fibroblasts, clones BE1 and AK2 were used as effectors in a cytotoxicity assay against allogeneic hF, from several donors, 6 days after hCMV infection. Comparable levels of cytotoxicity were observed consistently when fibroblasts were infected with hCMV (data not shown). These data indicate that target cell lysis is non-MHC-restricted.

Next, the ability of 49 cytotoxic T cell clones, derived from 12 donors, to lyse allogeneic fibroblasts 6 days after hCMV infection was evaluated. Possibly none of these clones was specific for hCMV Ags, as 39 of them were produced following PHA-stimulation and 10 clones were specific for autologous B-EBV cells. This is reinforced by the finding that no lysis occurs on day 2 postinfection. Lysis of fibroblasts, 6 days after infection, was significant (>20%) for 41 T cell clones in comparison with lysis of noninfected fibroblasts (Fig. 2*c*). Fibroblasts were derived from seven donors and all exhibited similar levels of susceptibility to lysis.

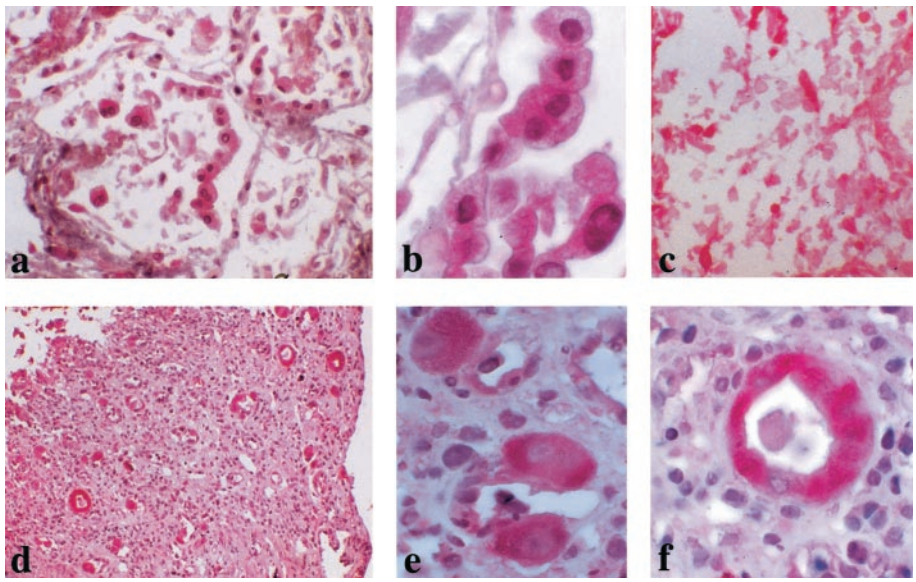


FIGURE 1. UL18 is detected in tissues from patients infected by wild-type hCMV strains. *a*, Lung autopsy specimen from a patient (age 20 years) who underwent bone marrow transplantation for T cell lymphoma. The patient developed interstitial hCMV pneumonia following an immunosuppressive regimen. UL18 was localized using the specific mAb 10C7 and the alkaline phosphatase-anti-alkaline phosphatase technique, and counterstained with Fast Red TR. *b*, A higher magnification showing several $UL18^+$ cells. *c*, Localization of UL18 in a lung autopsy specimen derived from a spontaneous abortion of a 25-wk-old fetus with severe hCMV infection. *d*, Detection of UL18 in a colon biopsy from a patient (age 53 years) who underwent bone marrow transplantation for renal cancer and developed ulcerative intestinal hCMV infection. *e* and *f*, Higher magnifications of the case depicted in *d*. UL18 is detected in cytomegalic cells close to blood vessels (*e*) and in endothelial cells (*f*).

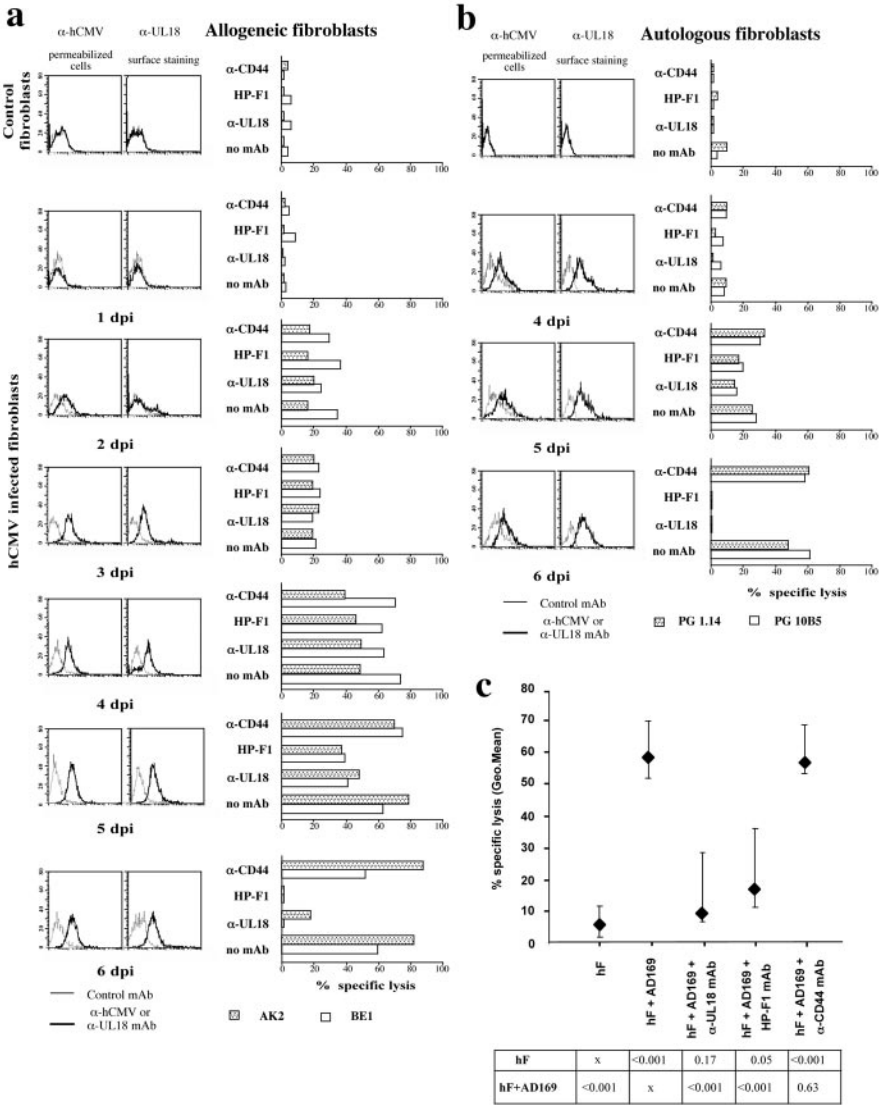


FIGURE 2. hF infected with hCMV are lysed by $CD8^+$ T cells in a non-MHC-restricted fashion. *a*, hF infected with the hCMV strain AD169 express surface UL18 from day 3 after infection (dpi, days postinfection). To assess infection, the anti-hCMV Ag UL44 was detected using an immunofluorescence assay with permeabilized cells. Cytolytic assays were performed using two T cell clones, BE1 and AK2, allogeneic for fibroblasts, at an E:T ratio of 10:1. In some experiments mAb specific for CD85j (HP-F1) and for UL18, or isotype-related irrelevant mAb, such as anti-CD44, were included in the assay. *b*, Two T cell clones derived from the same donor that provided fibroblasts were used in the cytolytic assay. Clones were generated from PBMC by limiting dilution, in the presence of 1% PHA. *c*, Forty-one $CD8^+$ clones (from 12 subjects) that, in preliminary experiments, lysed >20% hF infected with the hCMV strain AD169, were used as cytolytic effector cells. Targets were hF and hF 6 days after infection with AD169 without addition of mAb, or in the presence of mAb specific for CD85j (HP-F1), for UL18, or for an irrelevant mAb (anti-CD44). Data are expressed as geometric means from individual clones (◆) and dashes indicate the upper and lower 95% confidence interval. Wilcoxon test has been used for statistical analysis.

To determine whether this was due to interaction between CD85j and UL18, mAb specific for these molecules were included in the cytolytic assay (Fig. 2). In the presence of either mAb, 6 day-infected fibroblasts were not lysed. Inhibition did not occur significantly before day 6 (see *Discussion*). Moreover, an irrelevant isotype-matched mAb (anti-CD44) had no effect on cytotoxicity.

The level of hCMV-infected target cell lysis is neither related with surface expression of CD85j detected using the HP-F1 mAb, nor with the cytotoxic function of the clones determined in a redirected killing assay in the presence of anti-CD3 mAb (Table I). Clones AK5 and CO1 that lyse <20% hCMV-infected targets exhibited a surface expression of CD85j and a redirected cytotoxicity comparable to that of clones lysing >20% hCMV-infected cells (Table I).

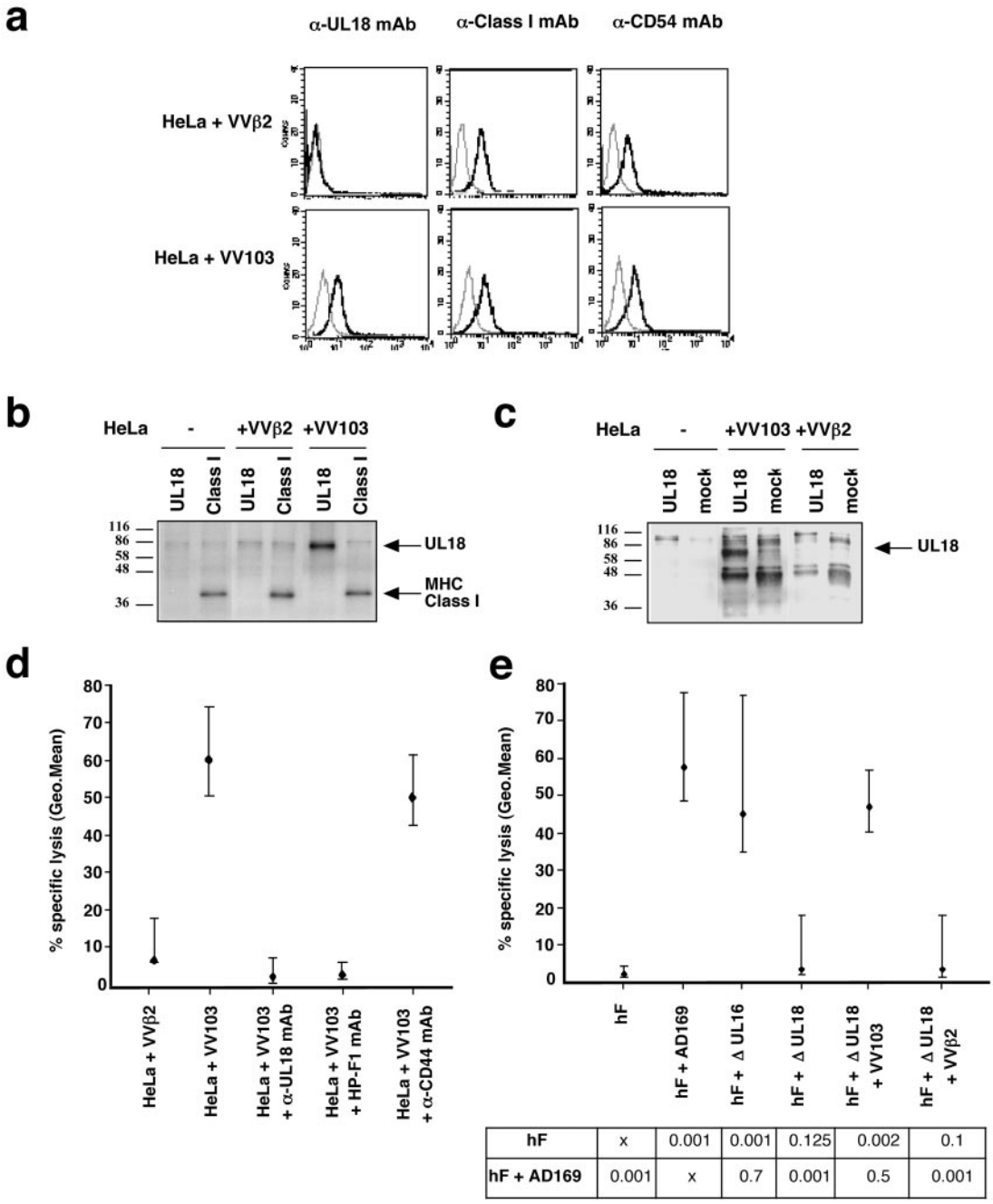
Table I. *Lysis of hCMV-infected fibroblasts by cytotoxic T cell clones is neither related to surface expression of CD85j nor to their cytotoxic activity*

$CD3^+CD8^+$ Clones	$HP\text{-}F1^{SURF+}$ %[a]	hF[b]	hF + hCMV	hF + hCMV + αUL18	P815[b]	P815 + αCD3
BE1	78.4	1[c]	82	1	1	60
RP1.19	11.9	6	100	1	13	88
AK2	44	1	82	18	9	96
CEG.12	9.9	15	45	26	12	88
RP.32	15.4	8	47	27	5	90
AK5	35.2	1	1	1	1	88
CO1	31.4	1	1	1	1	94

[a] Percent of surface $HP\text{-}F1^+$ T cells determined by indirect immunofluorescence and FACS analysis.
[b] Target cells were hF or the murine mastocytoma cell line P815.
[c] Cytotoxic activity of the clones is expressed as percent of specific lysis. The E:T ratio was 10:1.

$CD8^+$ T lymphocytes lyse target cells expressing surface UL18

To further define a role of UL18 for the susceptibility to lysis of hCMV-infected cells, vaccinia viruses containing UL18 (*VV103*) or β_2-microblobulin (*VVβ2*) genes, respectively, were used to infect HeLa cells. Expression of UL18 in *VV103*-infected cells was assessed by surface staining (Fig. 3*a*) and by immunoprecipitation with the anti-UL18 mAb 10C7 of either metabolically labeled (Fig. 3*b*) or biotin surface-labeled cells (Fig. 3*c*). As expected, cells infected with *VVβ2* failed to express UL18. Vaccinia-infected HeLa cells were also tested in a cytolytic assay using 20 $CD8^+$ T cell clones randomly selected among the 41 clones that were strongly cytotoxic for hCMV-infected fibroblasts. All clones lysed



hF	x	0.001	0.001	0.125	0.002	0.1
hF + AD169	0.001	x	0.7	0.001	0.5	0.001

FIGURE 3. Surface expression of UL18 on HeLa cells infected with vaccinia virus vectors and UL18-mediated lysis by $CD8^+$ T lymphocytes. *a*, Vaccinia virus containing UL18 (*VV103*) or β_2-microglubulin (*VVβ2*) genes, respectively, were used to infect HeLa cells. The presence of UL18, MHC class I, and CD54 in vaccinia-infected cells was assessed by surface immunofluorescence staining. *b*, Immunoprecipitation with anti-UL18 mAb 10C7 or with anti-MHC class I mAb of metabolically labeled HeLa cells. Immunoprecipitated proteins were subjected to SDS-PAGE electrophoresis in 8% acrylamide gels under reducing conditions. Arrows indicate bands of 67 and 45 kDa, corresponding to UL18 and MHC class I respectively. Molecular mass markers are shown on the *left*. *c*, Immunoprecipitation with anti-UL18 mAb of biotin surface-labeled HeLa cells. Arrow indicates a band of 67 kDa corresponding to UL18. *d*, Cytolytic assay of vaccinia-infected HeLa cells by 20 $CD8^+$ T cell clones used as effector cells at an E:T ratio of 10:1. Cytotoxic clones were randomly selected among the 41 clones that lysed >20% hCMV-infected hF. Data are expressed as geometric means from individual clones (◆) and dashes indicate the upper and lower 95% confidence interval. *e*, Cytolytic assay using hF as targets. hF were infected with AD169, and with the virus deleted for the UL18 gene (Δ*UL18*) or for another hCMV gene (Δ*UL16*). hF infected with Δ*UL18* were also coinfected with *VV103* or with *VVβ2*, respectively. Effector cells are the same as in *d*. Wilcoxon test has been used for statistical analysis.

significantly HeLa cells expressing UL18, but not *VVβ2*-infected cells, and lysis was inhibited by anti-UL18 or anti-CD85j mAb (Fig. 3*d*).

Infection with *VV103* did not induce over-expression of CD54 compared with that of *VVβ2*- (Fig. 3*a*) or of noninfected HeLa cells. Thus, although it has been suggested that killing of hCMV-infected fibroblasts by NK cells is, at least in part, related to increased expression of CD54 (17), lysis in our model is attributable exclusively to UL18. Additional evidence for the ability of UL18 to induce T cell-mediated lysis of hCMV-infected cells has been provided by the use of a virus deleted for the UL18 gene (Δ*UL18*), or for another hCMV gene (Δ*UL16*). Fibroblasts infected with Δ*UL16* or AD169 virus were susceptible to lysis whereas cells infected with Δ*UL18* were resistant. Coinfection of fibroblasts with Δ*UL18* and vaccinia virus *VV103* containing the UL18 gene re-established the susceptibility of target cells to lysis (Fig. 3*e*).

Resting and activated PBMC-derived CD8$^+$ T lymphocytes lyse UL18$^+$ HeLa cells

To assess the role of resting CTL for the control of hCMV infection, CD8$^+$ T lymphocytes were purified from peripheral blood cells by subtraction of CD4$^+$, CD16$^+$, and CD19$^+$ lymphocytes. The remaining cells were >95% CD3$^+$CD8$^+$ and were used as effector cells in cytolytic assays where the targets were uninfected HeLa cells or cells infected with vaccinia viruses *VV103* or *VVβ2* containing the UL18 or β_2-microglobulin genes, respectively. In this assay, resting CD8$^+$ lymphocytes lysed *VV103*-infected HeLa cells but failed to kill uninfected or *VVβ2*-infected targets. The level of lysis was increased when CD8$^+$ T lymphocytes were cultured for 48 h with rhIL-2 or activated by immobilized anti-CD3 mAb (Table II). Clones RP1.19 and BE1 were used in these experiments as positive controls.

Table II. *PBMC-derived CD8$^+$ T cells, both resting or activated, lyse UL18$^+$ target cells*

		Target Cells[a] (% specific lysis)		
Effector T Cells	E:T Ratio	HeLa	HeLa + *VVβ2*	HeLa + *VV103*
CD8$^+$ resting	100:1	ND	4.2	29.9
	50:1	1.2	1.0	20.4
	20:1	1.3	2.9	22.7
	10:1	1.0	1.8	17.8
	5:1	1.0	1.0	11.6
CD8$^+$ + rhIL2	100:1	2.1	1.0	30.9
	50:1	1.7	1.0	24.6
	20:1	1.0	1.7	25.2
	10:1	1.0	1.0	13.0
	5:1	1.0	1.0	15.1
CD8$^+$ + OKT3	100:1	1.2	1.0	49.9
	50:1	1.4	1.0	34.7
	20:1	1.0	1.0	28.1
	10:1	1.0	1.0	25.8
	5:1	1.0	1.0	22.9
RP1.19	100:1	22.2	25.4	57.7
	50:1	12.4	9.5	41.4
	20:1	11.2	12.4	35.5
	10:1	5.0	6.0	33.2
	5:1	4.0	2.0	23.0
BE1	100:1	10.6	11.7	95.5
	50:1	3.3	4.7	70.3
	20:1	4.2	5.2	62.8
	10:1	1.0	1.0	52.6
	5:1	1.0	1.0	42.0

[a] Target cells were HeLa cells, noninfected or infected with vaccinia viruses carrying the β2 microglobulin gene (*VVβ2*) or the UL18 gene (*VV103*).

UL18Fc and the anti-CD85j mAb HP-F1 recognize the same surface receptor on CD8$^+$ T lymphocytes

As CD85j is the only human receptor known to recognize UL18, the HP-F1 mAb was included in all cytotoxicity assays. As mentioned above, inhibition of UL18-expressing cell lysis by the HP-F1 mAb indicates an involvement of this receptor. To assess whether UL18 could recognize molecules other than CD85j on T cells, a soluble recombinant UL18Fc protein was produced and used to precipitate receptors from human T lymphocytes. HP-F1 and UL18Fc immunoprecipitates from biotin surface-labeled T cell clones and CD85j-transfected Jurkat cells were compared. As shown in Fig. 4*a*, the two reagents yielded an identical pattern. Moreover, sequential immunoprecipitation performed on clone BE1 demonstrated that no HP-F1 reactive molecules were left after UL18Fc immunoprecipitation and vice versa (Fig. 4*c*). These data suggest that UL18Fc and the HP-F1 mAb challenge the same molecular species on the surface of T cells.

Next, we addressed the issue of the multiple bands forming the HP-F1/UL18Fc immunoprecipitation pattern. Fig. 4 and previous data (21) show that the HP-F1 Ag displays a wide M_r, between 120 and 95 kDa. Such pattern can be dissected in up to three discrete bands when proteins are immunoprecipitated from cells expressing high surface levels of Ag, as in the CD85j Jurkat transfectants (J-ILT2), and separated on an 8% polyacrylamide gel (Fig. 4*a*). Deglycosilation with *N*-glycosidase F, although partial, resulted in an enrichment of the middle band, which can therefore be identified as a glycosilation variant (data not shown). Moreover, and in

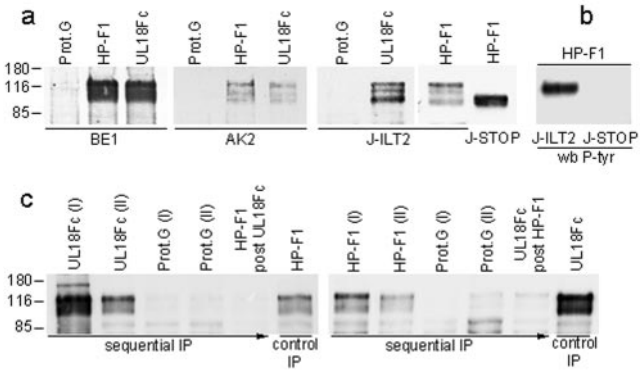


FIGURE 4. UL18Fc and the anti-CD85j mAb HP-F1 recognize the same receptor on the surface of CD8$^+$ T lymphocytes. *a*, Two T cell clones, BE1 and AK2, and J-ILT2 or J-STOP transfected Jurkat cells were surface labeled with biotin and immunoprecipitated with HP-F1 mAb or with UL18Fc. Immunoprecipitates were subjected to SDS-PAGE electrophoresis on 8% polyacrylamide gels under reducing conditions. Biotin was revealed by Western blotting, streptavidin-HRP probing and enhanced chemiluminescence visualization, using standard procedures. Proteins were precipitated from equal amounts of cells. Molecular mass weights are shown on the *left*. HP-F1 mAb and UL18Fc show an identical pattern of immunoprecipitated proteins, characterized by a wide M_r, spanning from 120 to 95 kDa, and dissected in up to three discrete bands. *b*, J-ILT2 and J-STOP transfected Jurkat cells were treated with pervanadate, subjected to immunoprecipitation with the HP-F1 mAb, and immunoblotted with an anti-phosphotyrosine Ab. A ~120-kDa band is detected in immunoprecipitates from J-ILT2 cells, whereas no signal is present in the HP-F1 Ag precipitated from J-ILT2-STOP cells, lacking the ITIMs-bearing intracellular region. *c*, Biotin surface-labeled BE1 T cells were subjected to sequential immunoprecipitation with the HP-F1 mAb and with UL18Fc. The HP-F1 mAb is unable to precipitate any protein from lysates precleared with UL18Fc (*left panel*), and vice versa (*right panel*). In the same experiment, control HP-F1 mAb and UL18Fc immunoprecipitates from nonprecleared lysates are shown in comparison (control IP).

accordance with previous data (21), only the upper species but not the 95-kDa band was phosphorylated on tyrosines following pervanadate treatment of the CD85j-transfected Jurkat cells (Fig. 4*b*), indicating the presence of the ITIMs-bearing intracellular region only in the former.

The identity of the 120-kDa and of the 95-kDa molecular species was then investigated by MALDI-TOF peptide mass mapping. Both bands identified the ILT2 protein, with 7 peptides covering 13% of the sequence with a mass accuracy within 40 ppm for the upper band and 15 peptides covering 29% of the sequence with a mass accuracy within 40 ppm for the lower band. Therefore, the 95-kDa band is the ILT2 protein, possibly lacking part or all of the ITIMs-bearing region. In addition, we analyzed the HP-F1 reactive molecule expressed by Jurkat cells transfected with a CD85j mutant without ITIMs (J-STOP) as control. It yielded a 100-kDa protein (Fig. 4*a*), not phosphorylated on tyrosines following pervanadate treatment of the cells (Fig. 4*b*).

Discussion

We show that UL18 is expressed in tissues from patients infected in vivo by wild-type hCMV strains. It is of note that all samples obtained from three patients were positive for the viral protein. In addition, the two hCMV genomes that have been fully sequenced and deposited in GenBank, i.e., the AD169 and the Towne strains, contain the UL18 gene. This suggests that UL18 plays a relevant role in virus biology. As the UL18 gene seems to be conserved, it should confer an advantage to virus spreading in vivo. Host death obviously impairs virus spreading. To ensure the highest efficiency of its dissemination, the virus must therefore reach a steady state with the host. In developed countries, latent hCMV infection affects ~60% of the individuals (5). Consequently, in addition to mechanisms that allow evasion from immune recognition, hCMV must have developed a counterbalance strategy that provides control of the infection aimed at preventing host death.

A relevant point in our work is the demonstration that UL18 expression leads to lysis of infected cells and may thus be responsible for the control of infection in vivo. This would explain why wild-type viruses express UL18, although this protein is not directly involved in viral replication and infectivity in vitro (27). We suggest that the control of infection occurs via interactions between CD85j/LIR-1/ILT2 on cytolytic T cells and UL18 expressed by target cells that leads to lysis of hCMV-infected cells. Because CD85j and UL18 are monomorphic proteins, lysis does not require involvement of TCR and MHC-restriction. The large majority of cytolytic T cell clones display this unrestricted function. Resting and activated $CD8^+$ T lymphocytes derived from peripheral blood are also able to lyse hCMV-infected cells. Altogether, the data suggest that, in vivo, the number of T lymphocytes is the major control mechanism for the onset of productive infection.

It is of note that the immune system of the patients studied for the in vivo expression of UL18 was strongly impaired. Two adults had undergone bone marrow transplantation less than a month earlier and were severely immunocompromised. Accordingly, the 25-wk-old fetus had an immune system not yet fully developed and thus unable to control hCMV infection. To reinforce this contention, immunohistochemical analyses of autopsy or biopsy specimens from these patients for the expression of CD3 failed to detect infiltrating T lymphocytes (data not shown). A recent report on allogeneic peripheral blood stem cell transplant patients, with or without hCMV viremia or disease, further supports the relevance of T cell numbers. The study shows that viremic patients who had progressed to hCMV disease displayed, on day 60 after transplantation, $CD8^+$ T cell counts significantly lower than those of non-viremic patients or of viremic patients who had not progressed to disease (35).

Activation via CD3/TCR and addition of rhIL-2 enhanced the ability of T cells to kill infected cells. Therefore, a role could be also envisaged for $CD4^+$ T lymphocytes, as they could help $CD8^+$ T cells by producing IL-2. Our preliminary results suggest that $CD4^+$ T cells are activated via CD85j/UL18 and that this interaction leads to IL-2 production.

A novel mechanism of recognition and lysis of hCMV-infected cells by cytolytic T lymphocytes, mediated by two monomorphic receptors, is demonstrated in the present study. Usually, T cells recognize antigenic peptides associated with MHC molecules through clonally distributed receptors. Ag experience results in expansion of specific T cells and generation of immunological memory, a distinct feature of adaptive immunity. Our data indicate that an invariant receptor expressed on $CD8^+$ T cells is capable of specific recognition of a viral component, and provide a novel example that extends the characteristics of innate immunity to cells that are mainly involved in adaptive immunity. One mechanism mediated by monomorphic proteins expressed by T lymphocytes and target cells, respectively, has been described previously (36). In hCMV-infected cells, the NKG2D-activating receptor present on most $CD8\alpha\beta$ T cells recognizes the stress-induced class I-like molecule MIC; this provides a costimulus that overcomes viral interference with MHC class I Ag presentation (36).

The non-MHC-restricted T cell-mediated cytotoxic function described in this study reaches its peak on day 6 of infection. This has not been observed in several reports where hCMV-specific T cell clones have been investigated. In these studies, however, fibroblasts were analyzed on day 2 after infection (36, 37). Lysis that we show 6 days after infection is not due to a cytopathic effect of hCMV, as fibroblast viability is >85%, and it is inhibited by anti-CD85j (HP-F1) or anti-UL18 (10C7) mAb. More importantly, infection with an hCMV strain deleted for the UL18 gene does not confer to cell targets a susceptibility to lysis by $CD8^+$ T lymphocytes. Altogether, our data indicate a specific role of UL18 for the lysis of hCMV-infected fibroblasts.

Interaction between CD85j and UL18 might not be the only non-MHC-restricted mechanism involved in hCMV-infected cell lysis. Lysis inhibition by addition of HP-F1 or anti-UL18 mAb has been particularly effective on day 6 of infection and only a weak inhibition by these mAb was observed on days 4 and 5. It has been suggested that lysis of hCMV-infected fibroblasts by NK cells, in addition to a direct involvement of UL18, is at least in part due to enhanced expression of CD54 (17). Up-regulation of CD58 during infection by some hCMV strains has been considered as a possible mechanism of NK cell-mediated lysis (38). Although increased expression of CD54 and CD58 was not confirmed in another study (39), an explanation could be provided by the expression kinetics of these two molecules, that is highest between day 3 and 5 postinfection (33).

Lysis of fibroblasts 6 days after infection is due to expression of UL18 as shown by its abrogation in the presence of anti-UL18 mAb or when Δ*UL18* hCMV is used to infect target cells. Thus, in the early phase of infection, when inhibition by anti-CD85j or anti-UL18 mAb is incomplete, CD54 and CD58 could be operational in T cell-mediated lysis.

It remains to be determined how CD85j, despite its inhibitory properties, can mediate activating signals. In this context, it is of note that UL18 displays a much higher affinity for CD85j than MHC class I (40, 41); thus, it might compete with CD85j-MHC class I interactions that yields inhibitory signals. This paradox has already been described for other receptors. Indeed, the stem cell factor receptor, the erythropoietin receptor, the β-chain of the IL-3

receptor, the NKp44 receptor, and the CD66a adhesion molecule are involved in cell activation despite possessing ITIM sequences in their cytoplasmic regions (42–46). Two possible nonmutually exclusive models can be considered. The activation may depend on a truncated form of CD85j lacking the cytoplasmic tail and thus becoming unable to transduce inhibitory signals via ITIMs. Accordingly, an alternatively spliced form of CD85j, with a truncated cytoplasmic tail lacking ITIMs (ILT2c), has been described (20). It has also been described recently that, in a murine model, CMV infection is kept under control by an activation receptor of the Ly49 gene family that encodes for both activating and inhibitory NK cell receptors (8, 9). In the second model, according to the observation that CD85j is not phosphorylated by cross-linking of the receptor alone (25), it is possible that, in the absence of an activation signal able to recruit $p56^{lck}$, CD85j does not transduce an inhibitory signal. In this context, it is relevant that UL18 is recognized by CD85j independently of TCR.

In conclusion, our data suggest that the strategy used in vivo by the hCMV to ensure survival of the host and, hence, its spreading in the population, is mediated by activation signals triggered by the infected cells when their surface receptor UL18 binds to CD85j on T lymphocytes. As both receptors are monomorphic, one could envisage immunotherapeutic interventions against productive hCMV infection using infusions of allogeneic T lymphocytes from MHC class I-matched donors. To avoid graft-vs-host disease, donor lymphocytes could be genetically manipulated to express drug-inducible suicide genes (47).

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