Immunity, Vol. 14, 123–133, February, 2001, Copyright ©2001 by Cell Press

ULBPs, Novel MHC Class I–Related Molecules, Bind to CMV Glycoprotein UL16 and Stimulate NK Cytotoxicity through the NKG2D Receptor

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Summary

The human cytomegalovirus glycoprotein, UL16, binds to two members of a novel family of molecules, the ULBPs, and to the MHC class I homolog, MICB. The ULBPs are GPI-linked glycoproteins belonging to the extended MHC class I family but are only distantly related to MICB. The ULBP and MICB molecules are ligands for the activating receptor, NKG2D/DAP10, and this interaction is blocked by a soluble form of UL16. The ULBPs stimulate cytokine and chemokine production from NK cells, and expression of ULBPs in NK cell–resistant target cells confers susceptibility to NK cell cytotoxicity. Masking of NK cell recognition of ULBP or MIC antigens by UL16 provides a potential mechanism by which human cytomegalovirus–infected cells might evade attack by the immune system.

Introduction

Human cytomegalovirus (HCMV) is a β herpesvirus that is commonly acquired during childhood, leading to mild or inapparent symptoms. The virus persists for the lifetime of the host in a latent state with asymptomatic episodes of viral shedding. However, in immunocompromised individuals, such as those with AIDS or bone marrow transplant recipients, the virus is reactivated and replicates in an uncontrolled manner, leading to considerable morbidity and mortality (Britt and Alford, 1996). These observations show that HCMV has learned to exist in equilibrium with the immune system of the normal host and has evolved mechanisms by which to counteract or evade immune surveillance. The genome of HCMV is large and encodes a number of glycoproteins that are nonessential for virus replication in cell culture. Candidate functions for such molecules might be to allow the virus to infect or replicate in different cell types in vivo or as immunomodulators. Recent work has shown that several of these glycoproteins function through diverse mechanisms to downregulate MHC class I expression on infected cells, providing an escape mechanism from T cell surveillance (Yewdell and Bennink, 1999; Tortorella et al., 2000). However, the functions of most of the nonessential viral glycoproteins remain unknown.

NK cells are also known to play an important role in controlling CMV infection. Patients with defective NK cell function have problems with herpesvirus infections (Biron et al., 1989), and much evidence supports a role for NK cells in limiting murine cytomegalovirus (MCMV) replication (Biron et al., 1999; Farrell et al., 1999). NK cell cytotoxicity is known to be controlled by a complex interplay between inhibitory and stimulatory receptors expressed on the NK cells and their ligands on target cells (Long et al., 1997; Lanier, 1998). Important components of this control system are NK receptors for MHC class I antigens (killer inhibitory receptors [KIRs]) that inhibit NK cytotoxicity when engaged by MHC class I expressed on the target cell. These negative signals were thought to be dominant over any stimulatory signals that might be transmitted to the NK cells, so that cells expressing class I MHC antigens would be protected from NK cytotoxicity—the "missing self" hypothesis (Ljunggren and Karre, 1990). However, it was recently shown that the nonclassical MHC class I antigens, MICA and MICB, could engage a stimulatory receptor on NK cells consisting of a heterodimeric complex of NKG2D, a C-type lectin, and DAP10, a cell-surface adaptor molecule involved in signal transduction (Houchins et al., 1990; Bauer et al., 1999; Wu et al., 1999). The activation signal resulting from engagement of NKG2D/DAP10 was shown to override an inhibitory signal from MHC class I binding to KIRs, leading to target lysis (Bauer et al., 1999).

While CMV downmodulation of host classical MHC class I antigens might help to protect cells against T cell surveillance, it would theoretically leave them vulnerable to NK-mediated lysis, as the engagement of the NK KIRs would be diminished. Two mechanisms can be envisaged by which CMV could counteract this threat: the virus might encode its own MHC class I–like molecule to engage the KIRs, or it might inhibit the function of an activating molecule like MICA/B. It was proposed several years ago that the HCMV-encoded MHC class I homolog, UL18, might provide a decoy function to engage KIRs (Fahnestock et al., 1995). Expression cloning of the receptor for UL18 revealed a novel inhibitory receptor that also bound cellular MHC class I (leukocyte immunoglobulin-like receptor-1 [LIR-1]) (Cosman et al., 1997). However, LIR-1 is only expressed on some subsets of NK cells and is more widely expressed on myeloid and B cells (Cosman et al., 1997). Functional experiments designed to support the role of UL18 as an inhibitor of NK cell cytotoxicity have provided contradictory results (Reyburn et al., 1997; Leong et al., 1998), and the actual function of UL18 remains controversial (reviewed in Cosman et al., 1999). In this paper, we show that another HCMV-encoded glycoprotein, UL16, binds to MICB and to two members of a novel family of MHC class I–related molecules, the ULBPs. The ULBPs and the MICs share the property of binding to the NKG2D/DAP10 receptor and provide a positive signal for NK cell activation and cytotoxicity that can override the

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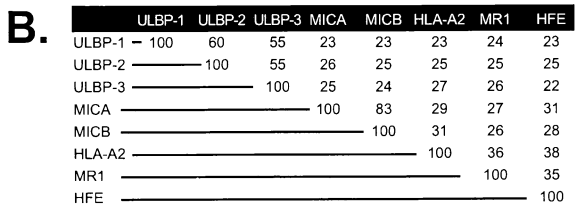
negative signal mediated by MHC class I/KIR engagement. Recombinant soluble forms of the ULBPs can bind to NK cells and induce cytokine and chemokine production. Binding and function of these two ULBPs is blocked by a soluble form of UL16. These findings suggest another possible mechanism: inhibition of an NK cell activation signal, by which a CMV-encoded protein might inhibit a cytolytic response of the innate immune system.

Results

Isolation of UL16 Binding Proteins

The protein encoded by the HCMV ORF, UL16, was chosen as a candidate immunomodulator because it is predicted to be a type 1 membrane glycoprotein and is known to be expressed by CMV-infected cells but is undetectable on virions. It is also known to be nonessential for CMV replication in tissue culture (Kaye et al., 1992). In order to identify cellular proteins with which UL16 might interact, a fusion protein was constructed in which the extracellular domain of UL16 was joined to the Fc region of human IgG1 (UL16-Fc). This protein was purified from supernatants of transiently transfected cells and used in flow cytometry experiments with various cell lines. UL16-Fc was found to bind specifically to HSB2, a human T cell line (data not shown). In order to identify the UL16 counterstructure on these cells, an HSB2 cDNA library in a mammalian expression vector was transfected into CV-1/EBNA cells and screened for UL16-Fc binding. A single cDNA was identified that conferred UL16-Fc binding on transfected cells. The protein sequence encoded by this cDNA matched that of MICB, also known as PERB11, originally identified during genomic analysis of the major histocompatibility complex (MHC) (Bahram et al., 1994; Leelayuwat et al., 1994). MICB and the closely related MICA protein are nonclassical MHC class I antigens that are highly polymorphic. They have the same basic structure as classical MHC class I antigens, namely α1, α2, and α3 domains in the extracellular region, a transmembrane domain, and a short cytoplasmic domain, but, unlike classical MHC class I molecules, they do not associate with β_2-microglobulin (β_2m) or require β_2m to be expressed on the cell surface (Groh et al., 1996).

In a similar series of experiments, UL16-Fc was also found to bind to Namalwa, a human Burkitt's lymphoma line (data not shown). Expression cloning from a Namalwa cDNA library resulted in the isolation of a second UL16 binding protein. This molecule, ULBP1 (UL16 binding protein), is a previously unidentified protein, distantly related to various members of the extended MHC class I family. Its sequence predicts α1 and α2 domains but no α3 domain. Instead of a transmembrane domain, the sequence predicts a glycosylphosphatidylinositol (GPI) linkage to the membrane (Figure 1A). Using the sequence of ULBP1, two similar sequences were identified in public EST databases. Resequencing of these clones showed that they encoded two ULBP1-related proteins that we call ULBP2 and ULBP3. The predicted domain structure of these proteins is similar to ULBP1 (Figure 1A). The amino acid sequences of the three ULBPs are 55%–60% identical; however, the ULBPs show only low



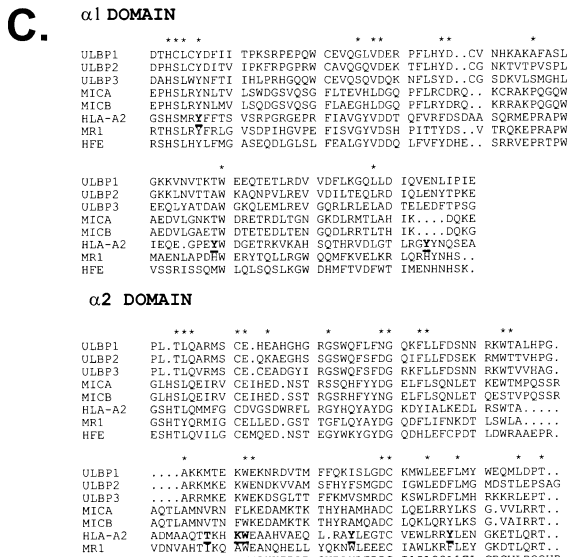


Figure 1. Amino Acid Sequence Comparisons of the ULBPs with Other Extended MHC Class I Family Members

(A) Amino acid sequence alignment of the ULBPs. Domains of the proteins are shown based on exon structures predicted from genomic sequence (data not shown). Potential N-linked glycosylation sites are indicated in bold. Sequences of the ULBPs have been deposited with GenBank (accession numbers AF304377-AF304379).

(B) Percent amino acid identities between the ULBPs and selected members of the extended MHC class I family.

(C) Amino acid sequence alignment of the α1 and α2 domains of the ULBPs and selected members of the extended MHC class I family. Asterisks mark those residues conserved in six or more of the eight sequences. Bold and underlined amino acids in the HLA-A2 sequence represent some key residues for peptide binding, as discussed in the text.

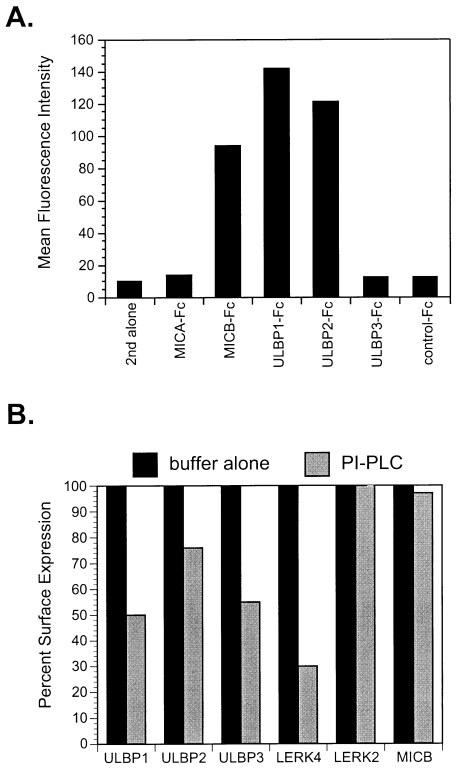


Figure 2. ULBP and MIC Binding to UL16 and GPI-Linked, Cell-Surface Expression of ULBPs

(A) 293 cells transfected with UL16 were stained with MICA-Fc, MICB-Fc, ULBP1-Fc, ULBP2-Fc, ULBP3-Fc, or a control-Fc (10 μg/ml), followed by PE-conjugated F(ab′)2 goat anti-human IgG (Fc specific). Mean fluorescence intensity values shown are representative of three independent experiments. Mean fluorescence intensity values for binding of the above Fc proteins, in the order shown in the figure, to 293 cells transfected with vector alone are as follows: 4, 7, 4, 5, 4, 4, and 7.

(B) CV-1 cells transfected with cDNAs encoding the full-length ULBP1, ULBP2, ULBP3, LERK4 (positive control), LERK2, and MICB (negative controls) were mock treated (black bars) or treated with PI-PLC (gray bars). Subsequently, cells were stained with specific monoclonal antibodies, followed by PE-conjugated sheep anti-mouse IgG. Mean fluorescence intensity values were calculated after subtraction of background staining values obtained with the second step reagent alone.

homology (23%–26% identity) to the MIC proteins, or indeed to other members of the MHC class I family (Figure 1B). Alignment of the sequences of the ULBPs and the MICs with those of representative classical and nonclassical MHC class I molecules (Figure 1C) shows the presence of ~20% highly conserved residues throughout the α1 and α2 domains, which clearly identify the ULBPs as novel members of the extended MHC class I family.

UL16 Binding of MIC and ULBP Molecules

In order to compare the binding of all the MIC and ULBP proteins to UL16, a series of fusion proteins was constructed in which the extracellular domains of the ULBPs and MICs were joined to the Fc region of human IgG1. These proteins were expressed, purified, and tested for binding to full-length UL16 expressed on the cell surface of transfected CV-1/EBNA cells (Figure 2A). Somewhat surprisingly, while the MICB, ULBP1, and ULBP2 Fc fusion proteins bound strongly to UL16, the closely related MICA and ULBP3 fusions showed no detectable binding above the negative controls. In order to determine if this result might be an artifact of expressing the MICs and ULBPs in soluble form, binding of UL16-Fc was tested to cells transfected with full-length ULBPs and MICs. The results (data not shown) were completely consistent in that UL16-Fc bound to ULBP1, ULBP2, and MICB, but not to ULBP3 or MICA.

The ULBP Molecules Are GPI-Linked Proteins

The sequence of the ULBP proteins predicts that they would be expressed on the cell surface and associated to the membrane by a GPI linkage. In order to test this, cells expressing the transiently transfected ULBP molecules were treated with phophatidylinositol-specific phospholipase C (PI-PLC), and cell-surface expression of the proteins was assessed using monoclonal antibodies specific for each of the molecules. Compared to cells undergoing a mock enzyme treatment, expression levels of ULBP1, ULBP2, and ULBP3 were significantly reduced by PI-PLC treatment, as was expression of LERK4 (ephrin-A4), a molecule known to be GPI linked (Figure 2B) (Kozlosky et al., 1995). Expression of two type 1 transmembrane proteins, LERK2 (ephrin-B1) and MICB, was not altered by PI-PLC treatment, demonstrating that the ULBPs are indeed GPI-linked, cell-surface molecules.

Expression of the ULBPs in Cells and Tissues

In order to rapidly survey the expression of the ULBPs in different cell types, a set of primers was designed that would detect all three ULBP transcripts in RT-PCR experiments. As shown in Figure 3A, expression of ULBP transcripts was detected in a variety of cells and tissues. Amplification products of the expected size were not detected when genomic DNA was used as the template (data not shown), demonstrating that the experiment was measuring ULBP RNA only. ULBP messages were detected in T, B, and erythroleukemia cell lines and in a wide range of tissues including heart, brain, lung, liver, testis, lymph node, thymus, tonsil, and bone marrow. ULBP transcripts were also abundant in fetal heart, brain, lung, and liver. Since MICA and MICB are expressed on various epithelial tumor cells and have been postulated to play a role in immune responses against tumors (Groh et al., 1999), we asked whether ULBP expression was upregulated in various tumor tissues. Although we did not perform a quantitative RT-PCR analysis, the data in Figure 3A show that, compared to matched normal tissues, ULBP messages are upregulated in colon and stomach tumors but downregulated in a kidney tumor. Further studies are required to determine whether ULBPs may be involved in tumor recognition.

RT-PCR products from various cell lines were digested with restriction enzymes selected to distinguish ULBP1 and ULBP2 transcripts. We found that ULBP1 and ULBP2 transcripts are coexpressed in all of the cell lines tested, with the exception of the Namalwa Burkitt's lymphoma line in which little or no ULBP2 message was detected (Figure 3B). In order to determine if cell-surface

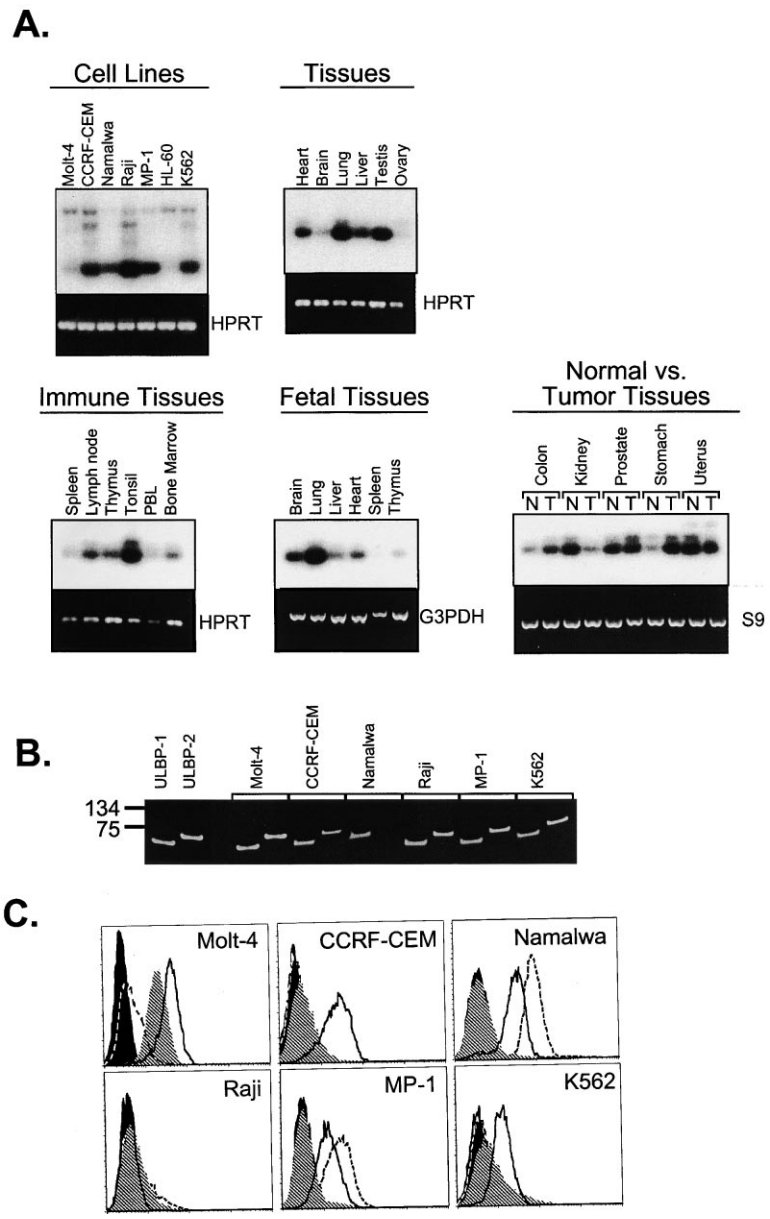


Figure 3. ULBPs Are Expressed in a Variety of Cells and Tissues

(A) Various cell lines, tissues, fetal tissues, and matched normal versus tumor tissues were analyzed for ULBP message expression by RT-PCR followed by Southern blotting with a ^{32}P-labeled ULBP specific cDNA probe (upper panels). The RT-PCR primers were degenerate for the amplification of a 322–328 bp region in ULBP1, ULBP2, and ULBP3 (corresponding to the major hybridizing band in each lane). The integrity of the cDNAs was evaluated by PCR by using HPRT, G3PDH, or S9 ribosomal protein primers as indicated to generate PCR products of the expected size (lower panels). In several tissues, most notably lung and tonsil, an additional ULBP-related band of slightly larger size was detected. This may represent a ULBP splice variant or a related transcript. The results are representative of two independent experiments.

(B) RT-PCR products from selected cell lines were digested with BamHI or BglII restriction enzymes in order to distinguish ULBP1 and ULBP2 transcripts, respectively. Positive control digests of PCR products amplified from ULBP1 or ULBP2 containing plasmids are shown on the left. The data are representative of three independent experiments. The quantities of the PCR products were normalized before digestion.

(C) ULBP cell-surface expression on various cell lines (Molt-4, CCRF-CEM, Namalwa, Raji, MP-1, and K562) was analyzed by flow cytometry. The cells were stained with monoclonal antibodies specific for ULBP1 (black line), ULBP2 (solid gray), or ULBP3 (solid stipple), or with an anti-I-309 IgG1 isotype control antibody (solid black) followed by a biotinylated goat anti-mouse IgG F(ab′)2 s step and then streptavidin-PE. The results are representative of three independent experiments.

expression of the molecules correlated with mRNA expression, selected cell lines were analyzed by flow cytometry using monoclonal antibodies specific for the ULBP proteins. Although in general ULBP protein expression correlated with ULBP message expression, some obvious discrepancies were found. For example, in Raji cells ULBP message expression was relatively high (Figure 3A), whereas ULBP protein expression was relatively low (Figure 3C). Thus, ULBP mRNA expression may not always predict protein expression. ULBP3 expression was highest on two of the three B-lineage cell lines tested. As of yet, we cannot rule out the possibilities that our monoclonal antibodies might not detect certain ULBP allelic variants or might cross-react with related proteins.

Binding of Soluble ULBPs to NK Cells

The ULBP and MIC Fc fusion proteins were tested for their binding to various primary cells and cell lines. Reproducible binding was seen to primary NK cells (Kubin et al., submitted). As shown in Figure 4A, all three ULBP and both MIC proteins bound specifically to NKL cells, a cell line derived from a natural killer cell leukemia (Robertson et al., 1996), compared to the binding of a control Fc fusion protein. To confirm the specificity of binding of the Fc fusion proteins, differently tagged soluble ULBPs and MICs were constructed in which their extracellular domains were fused at their C termini to a modified leucine zipper motif with a hexahistidine tag to facilitate purification. This modified leucine zipper has been shown to form a trimeric structure and to cause the formation of trimeric or higher order multimers when fused to heterologous proteins (Harbury et al., 1993; Morris et al., 1999). In each case, the leucine zipper (LZ) form of a given ULBP or MIC protein was able to compete the binding of the equivalent Fc fusion protein to cells (data not shown).

A leucine zipper-tagged form of the extracellular do-

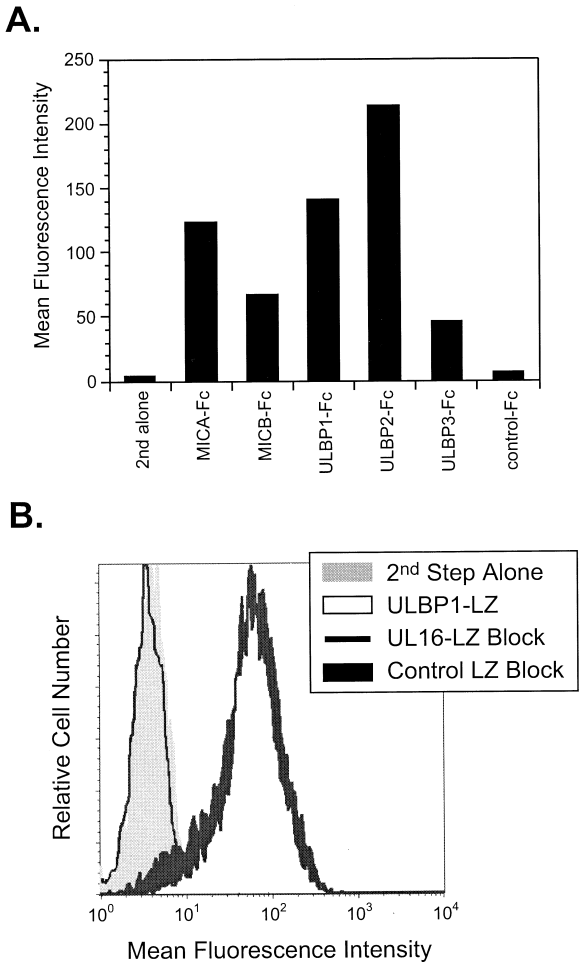


Figure 4. ULBP and MIC Binding to NKL Cells Is Blocked by UL16-LZ

(A) NKL cells were bound with ULBP-Fcs, MIC-Fcs, or a control-Fc (10 μg/ml), followed by PE-conjugated goat anti-human IgG. Mean fluorescence intensity values shown are representative of five independent experiments.

(B) ULBP1-Fc (10 μg/ml) was preincubated in the presence or absence of UL16-LZ or a CD40L-LZ control protein (250 μg/ml) and then added to NKL cells. Binding was detected using a PE-conjugated F(ab′)2 fragment goat anti-human IgG, Fc-specific reagent. Histograms displaying the fluorescence of live cells are presented. The results shown are representative of five independent experiments.

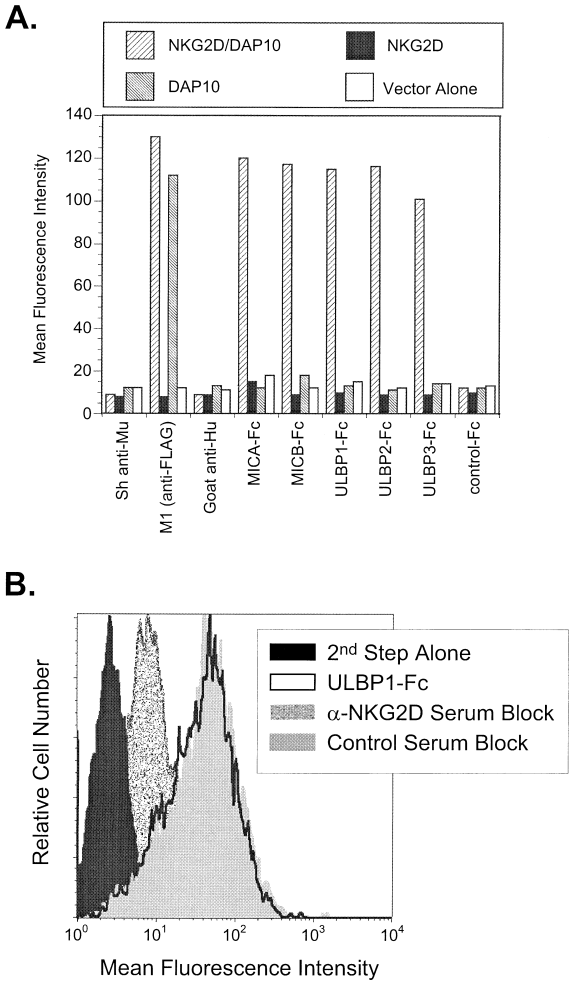


Figure 5. ULBP1, ULBP2, ULBP3, MICA, and MICB Are Ligands for NKG2D/DAP10

(A) 293 cells were transfected with cDNAs encoding NGK2D, DAP10, NKG2D + DAP10, or vector alone. Transfected cells were stained with MICA-Fc, MICB-Fc, ULBP1-Fc, ULBP2-Fc, ULBP3-Fc, or control-Fc (10 μg/ml) followed by PE-conjugated F(ab′)2 goat anti-human IgG (Fc specific) and with the M1 mAb (anti-FLAG) (10 μg/ml) followed by PE-conjugated sheep anti-mouse IgG. The results shown are representative of three independent experiments.

(B) NKL cells were preincubated in the presence of 10 μl of polyclonal murine anti-NKG2D antiserum or 10 μl of a control antiserum in a total volume of 100 μl. Cells were then incubated with ULBP1-Fc (10 μg/ml) followed by PE-conjugated F(ab′)2 goat anti-human IgG (Fc specific). Histograms displaying the fluorescence of live cells are presented. The results shown are representative of two independent experiments.

main of UL16 was also constructed. Figure 4B shows that a 10-fold excess of UL16-LZ completely competed binding of ULBP1-Fc to NKL cells, demonstrating that UL16 binds to the same (or overlapping) epitopes that ULBP1 uses to bind to its cell-surface receptor and that UL16 is a potential inhibitor of the biological activity of ULBP1. UL16-LZ was also able to block binding of ULBP2-Fc and MICB-Fc, but not ULBP3-Fc or MICA-Fc, to NKL cells (data not shown), as expected from the results shown in Figure 2A.

Binding of ULBPs to Recombinantly Expressed NKG2D/DAP10

Because the cell-surface receptors for MICA and MICB were recently shown to consist of NKG2D/DAP10 heterodimers, we tested the binding of the ULBP-Fc fusion proteins to 293 cells transiently transfected with NKG2D and DAP10 expression plasmids. The DAP10 protein was modified by the addition of an N-terminal Flag tag to allow cell-surface expression to be detected. As shown in Figure 5A, transfection of DAP10 alone or together with NKG2D allowed detection of the Flag epitope on the cell surface. In contrast, no binding of the ULBP or MIC-Fc proteins was seen to cells transfected with DAP10 alone or NKG2D alone, showing that NKG2D requires DAP10 for cell-surface expression. All the ULBP

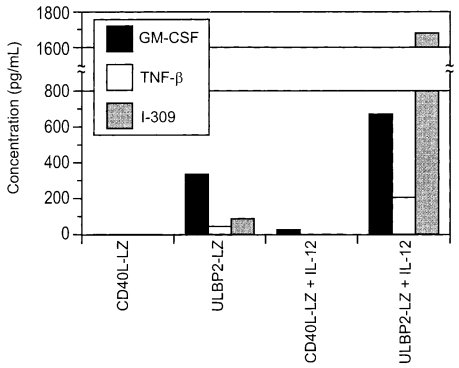


Figure 6. ULBPs Induce Cytokine and Chemokine Production by NK Cells

Purified NK cells were stimulated with IL-15 (50 ng/ml) for 20 hr, washed, and stimulated for an additional 24 hr with ULBP2-LZ (1 μg/ml) or a CD40L-LZ control protein (1 μg/ml) in the presence or absence of IL-12 (1 ng/ml). Supernatants were analyzed for the presence of GM-CSF, TNF-β, and I-309 proteins as described in Experimental Procedures. Results shown are representative of three experiments performed on cells from different donors.

and MIC-Fc fusion proteins bound specifically to cells transfected with both NKG2D and DAP10, compared to background binding seen with a control Fc fusion protein. This result suggests that at least a component of the ULBP and MIC receptors detected on NK cells is probably NKG2D/DAP10, as this receptor is known to be expressed by all NK cells (Bauer et al., 1999).

An antiserum was raised against a recombinant NKG2D-Fc fusion protein and tested for its ability to block binding of the ULBP and MIC-Fc fusion proteins to NKL cells. The results (Figure 5B) show that the anti-NKG2D antiserum effectively blocked binding of the ULBP1-Fc protein to NKL cells. An antiserum raised against an irrelevant Fc fusion protein had no effect. The anti-NKG2D antiserum also blocked binding of all other ULBP and MIC Fc fusion proteins to NKL cells (data not shown). This result confirms that NKG2D is an essential component of the MIC and ULBP receptors on NK cells but does not rule out the existence of additional receptor subunits.

Biological Activity of ULBPs on NK Cells

The presence of receptors for the ULBPs on NK cells prompted an examination of the biological consequences of binding to these cells. In order to avoid any possible interactions with Fc receptors on the target cells, the ULBP-LZ fusion proteins were used in these experiments. The NK cells were stimulated overnight with IL-15 to maximize binding of the ULBPs (Kubin et al., submitted) and then stimulated with the ULBP2-LZ protein, either alone or with the addition of IL-12, a known stimulus for NK cell cytokine production. Supernatants from the stimulated NK cells were analyzed for cytokine and chemokine production. As shown in Figure 6, ULBP2-LZ stimulated production of GM-CSF, TNF-β (lymphotoxin-α), and the CC chemokine I-309 (a ligand for CCR8), whereas IL-12 alone had little effect. The combination of IL-12 and ULBP2-LZ had a superadditive effect on GM-CSF and TNF-β production and a strong synergistic effect on I-309 production. Similar results were seen with ULBP1-LZ and ULBP3-LZ (data not shown).

Activation of NK Cytotoxicity by the ULBPs

It was previously shown that MICA could overcome an MHC class I–mediated inhibitory signal and trigger NK cytotoxicity when expressed on a target cell (Bauer et al., 1999). In order to test if the ULBP molecules would function similarly, Daudi cells, which do not express cell-surface MHC class I due to a mutation in β_2m, were transduced with a retroviral vector encoding wild-type β_2m in order to restore MHC class I expression. The MHC class I–expressing Daudi cells were then further transduced with retroviral vectors encoding full-length ULBPs or MICA. Cell populations expressing the transduced cell-surface molecules were obtained as described in Experimental Procedures. As shown in Figure 7A, MHC class I–negative Daudi cells were efficiently killed, whereas β_2m-mediated reexpression of cell-surface MHC class I afforded protection against NK cytotoxicity. This protection was substantially reversed by Fab fragments of an antibody directed against MHC class I molecules (Figure 7B). Introduction of any of the ULBP or MIC molecules into the MHC class I–expressing Daudi cells overcame the MHC class I–induced protection and allowed efficient killing by the effector cells (Figure 7A). The specificity of this stimulation of cytotoxicity was demonstrated by using a Fab fragment of a monoclonal antibody against ULBP1 to restore protection against killing (Figure 7C). These experiments demonstrate that the ULBPs and MICs function to transduce a dominant stimulatory signal to NK cells, overcoming an inhibitory signal generated by MHC class I engagement of KIRs.

Discussion

The results presented here represent another example of the use of viral proteins as probes to identify novel cellular antigens and to investigate the complexities of virus-host interactions. The discovery of the ULBP molecules followed a similar path to that previously taken to identify LIR-1, the receptor for the HCMV-encoded MHC class I homolog, UL18 (Cosman et al., 1997), and VESPR, the receptor for the poxvirus-encoded semaphorin homolog, A39R (Comeau et al., 1998). Unlike many virus-encoded immunomodulatory molecules, UL16 has no detectable homology to any cellular protein, and its binding to MICB and the ULBPs could not have been predicted. Similarly, the poxvirus-encoded soluble chemokine binding protein, p35, shows no homology to cellular chemokine receptors (Smith et al., 1997), suggesting that viruses can evolve proteins with novel functions by mechanisms other than acquiring cellular genes with similar properties.

It is interesting that UL16 only binds to ULBP1, ULBP2, and MICB, but not to the closely related molecules, ULBP3 and MICA. This finding might suggest that there are distinct biological roles for the different ULBPs and MICs. We have tested two alleles of MICB but only one allele of MICA for UL16 binding, so it is possible that other MIC alleles might show different properties. Another possibility is that there might be strain to strain variation in UL16 sequences resulting in changes in the

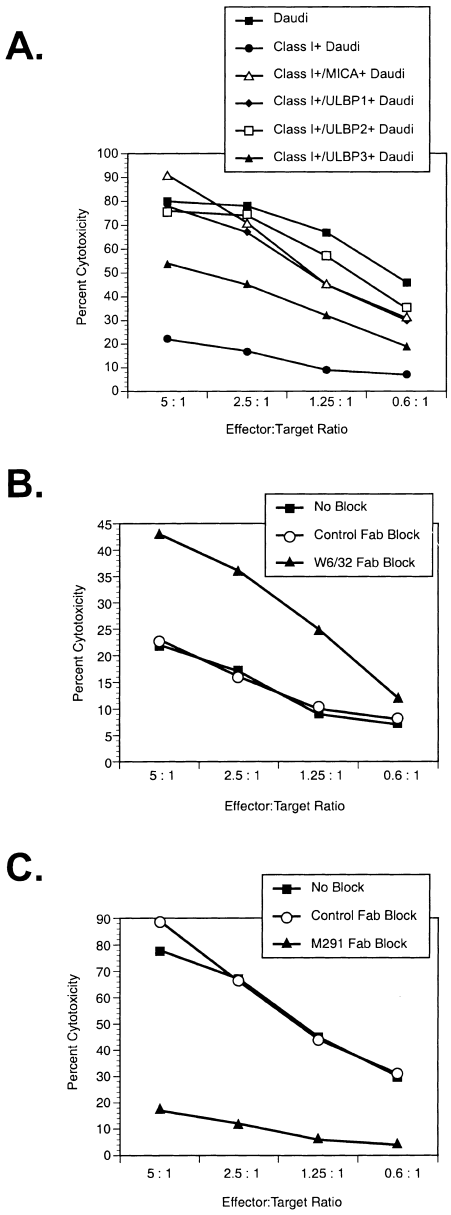


Figure 7. NK Cell Killing of MHC Class I$^+$ Daudi Target Cells Is Restored by Transduction of MICA, ULBP1, ULBP2, or ULBP3

Daudi cells were transduced with amphotropic retroviruses encoding β_2m. The MHC class I$^+$, β_2m transduced cells were further transduced with retroviruses encoding MICA, ULBP1, ULBP2, or ULBP3. Transduced cells were enriched by panning on magnetic beads coated with monoclonal antibodies specific for each molecule and further enriched by fluorescence activated cell sorting for uniform expression of the transduced proteins. When stained with specific monoclonal antibodies, the following MFIs were measured on the target cells: Class I$^+$ Daudi – MHC class I = 3500; Class I$^+$/MICA$^+$ Daudi – MHC class I = 2300 and MICA = 2100; Class I$^+$/ULBP1$^+$ Daudi – MHC class I = 3700 and ULBP1 = 120; Class I$^+$/ULBP2$^+$ Daudi – MHC class I = 3000 and ULBP2 = 150; Class I$^+$/ULBP3$^+$ Daudi – MHC class I = 3500 and ULBP3 = 200. The MFIs for non-transduced Daudi cells were: MHC class I = 15, MICA = 8, ULBP1 = 22, ULBP2 = 14, ULBP3 = 6. 3 hr ^{51}Cr-release assays were performed using IL-15 activated NK cells from human donors at the indicated effector to target ratios.

(A) The following cell lines were used as targets: Daudi, Class I$^+$ Daudi, Class I$^+$/MICA$^+$ Daudi, Class I$^+$/ULBP1$^+$ Daudi, Class I$^+$/ULBP2$^+$ Daudi, and Class I$^+$/ULBP3$^+$ Daudi.

(B) Class I$^+$ Daudi targets were preincubated with the W6/32 (anti-MHC class I) Fab, a control Fab (50 μg/ 0.5 $\times$ 10^6 targets), or in the absence of Fab, before addition to effector cells.

(C) Class I$^+$/ULBP1$^+$ Daudi targets were preincubated with the M291 (anti-ULBP1) Fab, a control Fab (50 μg/0.5 $\times$ 10^6 targets), or in the absence of Fab, before addition to effector cells.

Individual data points are calculated from the averages of triplicate samples. The results shown are from a single donor but are representative of seven independent experiments done on a total of four separate donors (A) or three independent experiments done on a total of two separate donors (B) and (C).

spectrum of ULBPs and MICs that are bound. In this respect, the MIC genes show extensive polymorphism (Bahram et al., 1994; Leelayuwat et al., 1994; Fodil et al., 1996; Ando et al., 1997) that might be driven by interaction with a rapidly evolving microbial antigen. Polymorphism in the ULBP genes has not yet been examined.

Relationship of the ULBPs to the Extended MHC Class I Superfamily

The ULBPs are unusual members of the extended MHC class I superfamily in several respects. They do not contain the α3 domain that associates with β_2m in many of the classical and nonclassical MHC class I molecules. Unlike most members of the family, they lack a transmembrane domain and use a GPI linkage to the cell surface. The ULBP genes are not located in the MHC but instead map to chromosome 6q25 (data not shown). However, the sequence alignments (Figure 1C) clearly show that the ULBPs belong to the MHC class I family. The classical MHC class I molecules, and some other family members, bind peptides in a cleft formed by the structure of the α1 and α2 domains. Important contacts for hydrogen bonding to the N termini of the bound peptides are the conserved tyrosine residues at positions 7, 59, 159, and 171 of the classical MHC class I molecules (Matsumura et al., 1992). All but the equivalent of tyrosine 7 are absent from the ULBP sequences (Figure 1C). At the binding site for the C termini of displayed peptides are the conserved residues, Y84, T143, K146, and W147, that also form hydrogen bonds and a salt bridge with the peptide (Matsumura et al., 1992). Only the equivalents of K146 and W147 are found in the ULBP sequences. Taken together, the absence of so many conserved amino acids that are known to be important for peptide binding by classical MHC class I molecules suggests that the ULBPs are unlikely to present peptides.

Relationship of the ULBPs to Other NKG2D Ligands

Although the ULBPs and the MIC proteins bind to the NKG2D/DAP10 complex, the ULBP sequences are no more closely related to the MIC sequences than to any other members of the extended MHC class I family (Figure 1B). During the preparation of this manuscript, a number of mouse ligands for NKG2D/DAP10, the RAE-1 family and H60 proteins, were identified (Cerwenka et al., 2000; Diefenbach et al., 2000). Interestingly, the overall structure of these proteins resembles the ULBPs rather than the MICs in that they contain only α1 and α2 domains. The RAE-1 proteins are GPI linked whereas H60 appears to be a transmembrane protein. However, the

RAE-1 and H60 sequences show only 22%–24% amino acid identity to the ULBP sequences, suggesting that the NKG2D/DAP10 complex is capable of binding a number of ligands of diverse sequence. There are very few residues conserved between the proteins capable of binding NKG2D/DAP10 that are not also conserved in the extended MHC class I family (data not shown). More extensive structural analysis will be required to determine if all the NKG2D/DAP10 ligands bind in the same way. Given the low sequence identities between the RAE-1 and H60 proteins and the ULBPs, it is difficult to assert that these are the true species homologs of the ULBPs. Evidence has been presented that expression of the RAE-1s and H60 is higher in transformed cell lines than in normal tissues (Cerwenka et al., 2000; Diefenbach et al., 2000). However, direct comparison of ULBP mRNA levels in several matched normal and tumor tissue samples shows no consistent difference in expression (Figure 3A). H60 expression was induced upon activation of mouse splenocytes (Diefenbach et al., 2000), but we have been unable to detect ULBP surface expression on activated human peripheral blood mononuclear cells (data not shown). It therefore remains to be determined if true mouse homologs of the ULBPs exist. There is precedent for a lack of conservation of immune recognition molecules between mouse and human; despite much effort, human homologs of the mouse Ly49 family and mouse homologs of the human killer cell immunoglobulin-like receptors (KIRs) have yet to be identified (Lopez-Botet et al., 2000).

Possible Functions of ULBPs in Immune Responses to Viruses and Tumors

Binding of ULBP proteins to NK cells triggers production of multiple cytokines and chemokines. These include LTα/TNFβ, GM-CSF, and I-309 (this paper) and TNFα, IFNγ, and MIP-1β (Kubin et al., submitted). In all cases, IL-12 enhances production of these factors, although the magnitude of this enhancement varies. In vivo, these cytokines and chemokines are likely to contribute to the recruitment and activation of NK cells, macrophages, and other cellular components of the innate and adaptive immune response. TNFα and IFNγ are known to be important antiviral cytokines, acting directly and indirectly against virus-infected cells (Biron et al., 1999). Expression of ULBP proteins in cells that are relatively resistant to NK cell cytotoxicity (MHC class I–expressing Daudi cells) generates potent activation of killing. Together, these data argue for an important role of the ULBPs, like the MICs, in activation of innate immune responses. It remains to be determined how ULBP expression and/or activation is controlled under physiological and pathological circumstances.

NKG2D has been shown to be expressed by $\gamma\delta$ T cells, CD8$^+$ T cells, and activated macrophages, as well as NK cells (Yabe et al., 1993; Bauer et al., 1999; Diefenbach et al., 2000). In addition to their roles in innate and adaptive immune responses to infections, these cells are also believed to mediate antitumor activities. Do the NKG2D ligands play a role in the recognition of tumor cells by the immune system? The MIC proteins have been shown to be more highly expressed by tumor cells than by normal cells (Groh et al., 1999), and the mouse NKG2D ligands have been suggested to be more prevalent on transformed cells (Cerwenka et al., 2000; Diefenbach et al., 2000). Although the data presented here show no consistent increase of ULBP mRNA levels in tumor cells compared to normal tissue, this issue requires further investigation at the level of ULBP protein expression. It is also possible that the ULBPs may act in concert with other target cell antigens to activate immune effector cells.

Possible Mechanism of Immune Evasion by UL16

We have presented evidence that the interaction between ULBPs and their receptor on NK cells can be blocked by a soluble form of UL16. Data presented elsewhere (Kubin et al., submitted) also demonstrate blocking of ULBP-mediated biological activities by soluble forms of UL16. Extrapolation from these results to what might occur in the context of a CMV infection leads to the hypothesis that UL16 could mask the recognition of ULBPs or MICB by NKG2D-expressing cells and thus prevent the activation of these cells to kill virus-infected targets and to secrete increased amounts of cytokines and chemokines. UL16 expression on the surface of the infected cell might lead to the formation of a complex with ULBPs or MICB; alternatively, UL16 binding might retain ULBPs or MICB within the infected cell. Whether infection by HCMV or other pathogens might induce expression of NKG2D ligands is also of interest, as it would provide a potential mechanism by which NK cells could recognize and kill infected cells even in the absence of MHC class I antigen downregulation. Further investigation of these issues may provide additional insight into how HCMV can maintain a lifelong relationship with its host and add to our knowledge of the controls that exist to enable cells of the innate immune system to distinguish self from infected, "stressed," or transformed self.

Experimental Procedures

Cell Lines and Purification of Cells

Daudi, Raji, and Namalwa, Burkitt's lymphoma cell lines (ATCC CRL-213, ATCC CCL 86, and ATCC CRL 1432, respectively), Molt-4, a T cell leukemia (ATCC CRL 1582), CCRF-CEM, a T lymphoblastoid cell line (ATCC CCL 119), MP-1, a B lymphoblastoid cell line, and K562, an erythroleukemia cell line (ATCC CCL 243), were all grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS). The human NK cell line NKL was a generous gift from Dr. Michael J. Robertson and was cultured as described (Robertson et al., 1996). Short-term cultured primary human NK cells were obtained as described (Perussia et al., 1987) and purified by magnetic negative selection using a MACS column (Miltenyi Biotec, Auburn, CA) as described (Cosman et al., 1997). Primary NK cells were cultured overnight in the presence of 50 ng/ml rhuIL-15 (Immunex). 293EBNA (Invitrogen) and CV-1/EBNA were grown and transfected as previously described (McMahan et al., 1991).

Construction and Expression of Fc Fusion Protein Constructs

All Fc fusion proteins contain the extracellular domain of the protein of interest fused to the amino terminus of the hinge region of a modified human IgG1 Fc region (Baum et al., 1994) and were then subcloned into the mammalian expression vector pDC409 (Giri et al., 1994) or pDC412 (the pDC412 vector is identical to pDC409 except that the NotI and BglII cloning sites have been transposed).

The UL16-Fc fusion protein contains the extracellular domain of UL16 fused to the Fc region after amino acid 183. The MICA-Fc fusion protein contains the extracellular domain of MICA fused to

the Fc region after amino acid 297. The MICA allele used corresponded to GenBank accession number AAD52060 with the single exception of a conservative amino acid substitution at position 256 of Val to Ile. The MICB-Fc fusion protein contains the extracellular domain of MICB fused to the Fc region after amino acid 297. The MICB allele used corresponded to GenBank accession number AAB71643. Another MICB allele corresponding to accession number AF021222 also bound to UL16. The ULBP1-Fc fusion protein contains the extracellular region of ULBP1 fused to the Fc region after amino acid 220. The ULBP2-Fc fusion protein contains the extracellular region of ULBP2 fused to the Fc region after amino acid 222. The ULBP3-Fc fusion protein contains the Ig κ leader sequence fused to the ULBP3 extracellular region at amino acid 23, and the ULBP3 extracellular region fused to the Fc region at amino acid 220. The NKG2D-Fc fusion protein contains the extracellular region of NKG2D fused to the Fc region at amino acid 74. In this construct, the NKG2D extracellular region is placed in frame with the COOH terminus of the Fc region to position it in the proper orientation for a type II fusion protein. The sequences of the oligonucleotide primers used to amplify the desired sequences by PCR are available upon request.

Plasmids encoding the Fc fusion proteins were transfected into CV-1/EBNA (ATCC CRL-10478) cells, and the fusion proteins were purified from culture supernatants by chromatography on a protein A-Poros column (PerSeptive Biosystems) as described (Fanslow et al., 1992).

A control Fc fusion protein, p7.5-Fc, has been previously described (Yao et al., 1995).

Construction and Expression of LZ Protein Constructs

All LZ fusion proteins contain the extracellular domain of the protein of interest fused to a trimerization domain based upon a leucine zipper motif (O'Shea et al., 1989) modified to form a trimer (Harbury et al., 1993) followed by six histidine residues and were then subcloned into the mammalian expression vector pDC409 or pDC412.

The UL16-LZ fusion protein contains the extracellular domain of UL16 fused to the LZ domain after amino acid 187. The MICA-LZ fusion protein contains the extracellular domain of MICA fused to the LZ domain after amino acid 297. The ULBP1-LZ fusion protein contains the extracellular region of ULBP1 fused to the LZ domain after amino acid 220. The ULBP2-LZ fusion protein contains the extracellular region of ULBP2 fused to the LZ domain after amino acid 222. The ULBP3-LZ fusion protein contains the Ig κ leader sequence fused to the ULBP3 extracellular region at amino acid 23 and the ULBP3 extracellular region fused to the LZ domain at amino acid 221. The sequences of the oligonucleotide primers used to amplify the desired sequences by PCR are available upon request.

Plasmids encoding the LZ fusion proteins were transfected into CV-1/EBNA cells, and the fusion proteins were purified from culture supernatants using immobilized metal affinity chromatography. Supernatants were passed over an Alltech column packed with Co-bound TALON Superflow resin according to the manufacturer's specifications (Clontech).

Construction of FLAG-DAP10

The DAP10 gene was amplified by PCR using a full-length DAP10 cDNA as a template (forward primer 5′-CTAGTCGAGACTAGTCA GACGACTCCAGGAGAGAGATC-3′; reverse primer 5′-CTAGTCGA GAGATCTGAAGTCAAAGGTCCAAGCTGCAG-3′). The amplification product was digested with SpeI and BglII and ligated into the pDC409 vector, containing a 5′ sequence encoding the FLAG peptide, cut with SpeI and BglII.

Expression Cloning

The procedures used were those previously described (McMahan et al., 1991; Goodwin et al., 1993). In brief, plasmid DNA from pools of 2000 cDNA clones were transfected into CV-1/EBNA cells grown on one-chambered glass slides. Two or three days after transfection, the cells were incubated with 1 μg/ml of UL16-Fc protein or an unrelated Fc protein control in binding buffer followed by ^{125}I-Fc-specific mouse anti-human IgG for 30 min at room temperature. The cells were washed, fixed, and processed for autoradiography. Positive pools were subdivided until a single clone was obtained that conferred UL16-Fc binding. The HSB2 and Namalwa cDNA libraries screened were constructed in the mammalian expression vector pDC410 as previously described (Kozlosky et al., 1995).

Sequence Analysis

The amino acid sequence of ULBP1 was used to search the GenBank dbEST database using the TBLASTN program. The clones corresponding to accession numbers R25716 (ULBP2) and AI091180 (ULBP3) were purchased from Research Genetics and completely sequenced. The first five amino acids of ULBP3 sequence were predicted from GenBank high throughput genomic sequence (accession number AL138890). Sequence alignments and comparisons used the Pileup and Gap programs of the Genetics Computer Group, Inc.

Generation of Monoclonal Antibodies

BALB/C mice were immunized at 0, 2, and 6 weeks with 10 μg of the appropriate Fc protein as described (Cosman et al., 1997). Positive supernatants were subsequently confirmed by flow cytometry and immunoprecipitation using CV-1 cells transfected with cDNAs encoding full-length ULBP1, ULBP2, ULBP3, or MICA/MICB. Antibodies were chosen with specific reactivity to the protein used for immunization. Hybridomas were cloned and followed using the same assay. Monoclonal cultures were expanded and monoclonal antibodies purified by protein A affinity chromatography (BioRad protein A agarose).

Flow Cytometric Analysis

The following monoclonal antibodies were used for flow cytometric analysis: M1, IgG1 anti-FLAG; M90, anti-hCD40L used as an IgG1 isotype control; M5, IgG1 anti-FLAG used as an IgG1 isotype control; M230, IgG1 anti-UL16; M291, M293, M294, and M295, IgG1 anti-ULBP1; M292, IgG2a anti-ULPB1; M310, M311, and M312, IgG1 anti-ULBP2; M550 and M551, IgG1 anti-ULBP3; M363 and M364, IgG1 anti-MICA/MICB (generated as described above); W6/32, IgG2a pan anti-HLA-A,-B,-C (ATCC HB-95); OKT3, anti-CD3 used as an IgG2a isotype control (ATCC CRL 8001); MAB272, anti-I-309 used as an IgG1 isotype control (R&D Systems).

Cells (5×10^5) were incubated with 1 μg antibody or 1 μg Fc fusion protein for 30 min on ice. Specific binding was detected with either a PE-conjugated sheep anti-mouse IgG (Sigma), a PE-conjugated F(ab′)2 fragment goat anti-human IgG, Fcγ fragment specific (Jackson Immunoresearch), or a combination of a biotin-conjugated goat anti-mouse IgG F(ab′)2 followed by PE-conjugated streptavidin (Jackson Immunoresearch). In UL16-LZ blocking assays, 1 μg of the binding protein was preincubated with 25 μg of the UL16-LZ for 30 min on ice prior to addition to the cells. After staining, cells were analyzed on a Becton Dickinson FACScan.

RT-PCR and Southern Expression Analysis

To examine ULBP message expression, degenerate primers (forward primer: 5′-CTGCAGGYMAGGATGTCTTGTGAG-3′; reverse primer: 5′-T GAGGGTGGTGGCYRTGGC-3′) were used to amplify a 322 bp region of ULBP1 and ULBP3 and a 328 bp region of ULBP2 from various cell lines and tissues. Tissue cDNA panels and matched normal and tumor cDNA pairs were purchased from Clontech. The RT-PCR consisted of 32 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The amplified PCR products were transferred to Hybond N^+ (Amersham) and probed with a random-primed probe that was transcribed from a mixture of 322-328 bp ULBP1, ULBP2, and ULBP3 PCR products as template. To distinguish ULBP1 and ULBP2 transcripts, RT-PCR products from selected cell lines were digested with BamHI or BglII, respectively.

Phosphatidyl-Inositol-Specific Phospholipase C Treatment

To obtain evidence for GPI linkage of the ULBP proteins, cells transfected with cDNAs encoding these proteins were treated with 2 U/ml phospatidyl-inositol-specific phospholipase C (PI-PLC) (Sigma) at 37°C for 1 hr. Subsequently, cells were washed with 1 × PBS and stained on ice by indirect immunofluorescence using specific antibodies as described above.

^{51}Chromium Release Assay

The ^{51}Cr release assay was performed using 10^4 ^{51}Cr-labeled targets per well. Effectors were added at the indicated concentrations and plates were incubated for 2–3 hr at 37°C, 5% CO_2. The released radioactivity in the cell-free supernatants was measured with a gamma counter (Micromedic). Specific lysis was calculated by the standard formula: (experimental − spontaneous release)/(total − spontaneous release) × 100.

Blocking assays were carried out using Fab fragments of specific antibodies or the UL16LZ protein. The generation of Fab fragments of monoclonal antibodies was performed as previously described (Goding, 1983). ^{51}Cr-labeled targets (5×10^5) were incubated with 50 μg of the Fab fragments for 20 min at 37°C, washed with media, and plated at 10^4 cells per well. Effectors were added and assays carried out as described above.

Chemokine and Cytokine Assays

Primary human NK cells were treated with IL-15 (50 ng/ml) for 20 hr, washed, and then stimulated with the indicated fusion proteins at 1 μg/ml with or without rhuIL-12 (1 ng/ml) (R&D Systems) for 20 hr at 37°C, 5% CO_2. Cytokine and chemokine levels in cell-free supernatants were determined by ELISA. GM-CSF levels were determined using the M8 and P5 paired antibodies (Immunex), whereas I-309 and TNFβ levels were determined using paired antibodies (R&D Systems) according to manufacturer's specifications. All assays were performed in triplicate wells, and results are presented as the mean from the given culture conditions.

Retroviral Constructs and Transduction

Daudi cells were transduced with amphotropic retroviruses generated using the LZRSpBMN-Z vector (Kinsella and Nolan, 1996) in the Phoenix packaging line (kindly provided by the Nolan lab, Stanford University). Transduced cells expressing class I, MICA, and the ULBP antigens were selected using magnetic beads. Transduced cells were stained with monoclonal antibodies specific for the antigens of interest followed by PE-conjugated sheep anti-mouse IgG. Stained cells were incubated with anti-PE microbeads and passed over MACS separation columns according to manufacturer's specifications (Miltenyi Biotec). To isolate cell populations expressing homogeneous levels of the antigens of interest, these cells were further sorted by flow cytometry.

Generation of Polyclonal Murine anti-NKG2D Serum

Balb/c mice were immunized subcutaneously with 10 μg of NKG2D-Fc protein in Titermax (Cytrx) adjuvant. Four weeks after immunization, serum samples were obtained by tail bleed.

Acknowledgments

We thank the Immunex flow cytometry, DNA sequencing and hybridoma groups, especially JoDee Zappone for generation of antibodies against ULBP1, ULBP2, and MICA/MICB, Stacey Culp for generation of antibodies to ULBP3 and NKG2D, and Lori Peterson for generation of Fab fragments. We thank Ray Goodwin for cell line cDNAs, Luis Borges for the Flag-DAP10 construct, Pamela Bjorkman and Tara Chapman for helpful discussions, Melanie Spriggs for comments on the manuscript, Gary Carlton for preparation of figures, and Anne Aumell for editorial assistance.

Received September 18, 2000; revised November 14, 2000.

References

Ando, H., Mizuki, N., Ota, M., Yamazaki, M., Ohno, S., Goto, K., Miyata, Y., Wakisaka, K., Bahram, S., and Inoko, H. (1997). Allelic variants of the human MHC class I chain-related B gene (MICB). Immunogenetics *46*, 499–508.

Bahram, S., Bresnahan, M., Geraghty, D.E., and Spies, T. (1994). A second lineage of mammalian major histocompatibility complex class I genes. Proc. Natl. Acad. Sci. USA *91*, 6259–6263.

Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J.H., Lanier, L.L., and Spies, T. (1999). Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science *285*, 727–729.

Baum, P.R., Gayle, R.B., III, Ramsdell, F., Srinivasan, S., Sorensen, R.A., Watson, M.L., Seldin, M.F., Baker, E., Sutherland, G.R., Clifford, K.N., et al. (1994). Molecular characterization of murine and human OX40/OX40 ligand systems: identification of a human OX40 ligand as the HTLV-1-regulated protein gp34. EMBO J. *13*, 3992–4001.

Biron, C.A., Byron, K.S., and Sullivan, J.L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells. N. Engl. J. Med. *320*, 1731–1735.

Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., and Salazar-Mather, T.P. (1999). Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol. *17*, 189–220.

Britt, W., and Alford, C. (1996). Cytomegalovirus. In Field's Virology, B.N. Field, D.M. Knipe, and P.M. Howley, eds. (Philadelphia: Lippincott-Raven), pp. 2493–2523.

Cerwenka, A., Bakker, A.B., McClanahan, T., Wagner, J., Wu, J., Phillips, J.H., and Lanier, L.L. (2000). Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. Immunity *12*, 721–727.

Comeau, M.R., Johnson, R., DuBose, R.F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Farrah, T., Buller, R.M., Cohen, J.I., et al. (1998). A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity *8*, 473–482.

Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L., and Hsu, M.L. (1997). A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. Immunity *7*, 273–282.

Cosman, D., Fanger, N., and Borges, L. (1999). Human cytomegalovirus, MHC class I and inhibitory signalling receptors: more questions than answers. Immunol. Rev. *168*, 177–185.

Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N., and Raulet, D.H. (2000). Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. Nat. Immunol. *1*, 119–126.

Fahnestock, M.L., Johnson, J.L., Feldman, R.M., Neveu, J.M., Lane, W.S., and Bjorkman, P.J. (1995). The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides. Immunity *3*, 583–590.

Fanslow, W.C., Anderson, D.M., Grabstein, K.H., Clark, E.A., Cosman, D., and Armitage, R.J. (1992). Soluble forms of CD40 inhibit biologic responses of human B cells. J. Immunol. *149*, 655–660.

Farrell, H.E., Degli-Esposti, M.A., and Davis-Poynter, N.J. (1999). Cytomegalovirus evasion of natural killer cell responses. Immunol. Rev. *168*, 187–197.

Fodil, N., Laloux, L., Wanner, V., Pellet, P., Hauptmann, G., Mizuki, N., Inoko, H., Spies, T., Theodorou, I., and Bahram, S. (1996). Allelic repertoire of the human MHC class I MICA gene. Immunogenetics *44*, 351–357.

Giri, J.G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L.S., Cosman, D., and Anderson, D. (1994). Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. *13*, 2822–2830.

Goding, J.W. (1983). Monoclonal Antibodies: Principles and Practice, Third Edition (San Diego: Academic Press, Inc.), pp. 544.

Goodwin, R.G., Alderson, M.R., Smith, C.A., Armitage, R.J., VandenBos, T., Jerzy, R., Tough, T.W., Schoenborn, M.A., Davis-Smith, T., Hennen, K., et al. (1993). Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. Cell *73*, 447–456.

Groh, V., Bahram, S., Bauer, S., Herman, A., Beauchamp, M., and Spies, T. (1996). Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc. Natl. Acad. Sci. USA *93*, 12445–12450.

Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K.H., and Spies, T. (1999). Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc. Natl. Acad. Sci. USA *96*, 6879–6884.

Harbury, P.B., Zhang, T., Kim, P.S., and Alber, T. (1993). A switch

between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science *262*, 1401–1407.

Houchins, J.P., Yabe, T., McSherry, C., Miyokawa, N., and Bach, F.H. (1990). Isolation and characterization of NK cell or NK/T cell-specific cDNA clones. J. Mol. Cell. Immunol. *4*, 295–304.

Kaye, J., Browne, H., Stoffel, M., and Minson, T. (1992). The UL16 gene of human cytomegalovirus encodes a glycoprotein that is dispensable for growth in vitro. J. Virol. *66*, 6609–6615.

Kinsella, T.M., and Nolan, G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Hum. Gene Ther. *7*, 1405–1413.

Kozlosky, C.J., Maraskovsky, E., McGrew, J.T., VandenBos, T., Teepe, M., Lyman, S.D., Srinivasan, S., Fletcher, F.A., Gayle, R.B., 3rd, Cerretti, D.P., et al. (1995). Ligands for the receptor tyrosine kinases hek and elk: isolation of cDNAs encoding a family of proteins. Oncogene *10*, 299–306.

Lanier, L.L. (1998). NK cell receptors. Annu. Rev. Immunol. *16*, 359–393.

Leelayuwat, C., Townend, D.C., Degli-Esposti, M.A., Abraham, L.J., and Dawkins, R.L. (1994). A new polymorphic and multicopy MHC gene family related to non-mammalian class I. Immunogenetics *40*, 339–351.

Leong, C.C., Chapman, T.L., Bjorkman, P.J., Formankova, D., Mocarski, E.S., Phillips, J.H., and Lanier, L.L. (1998). 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. J. Exp. Med. *187*, 1681–1687.

Ljunggren, H.G., and Karre, K. (1990). In search of the "missing self": MHC molecules and NK cell recognition. Immunol. Today *11*, 237–244.

Long, E.O., Burshtyn, D.N., Clark, W.P., Peruzzi, M., Rajagopalan, S., Rojo, S., Wagtmann, N., and Winter, C.C. (1997). Killer cell inhibitory receptors: diversity, specificity, and function. Immunol. Rev. *155*, 135–144.

Lopez-Botet, M., Bellon, T., Llano, M., Navarro, F., Garcia, P., and de Miguel, M. (2000). Paired inhibitory and triggering NK cell receptors for HLA class I molecules. Hum. Immunol. *61*, 7–17.

Matsumura, M., Fremont, D.H., Peterson, P.A., and Wilson, I.A. (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science *257*, 927–934.

McMahan, C.J., Slack, J.L., Mosley, B., Cosman, D., Lupton, S.D., Brunton, L.L., Grubin, C.E., Wignall, J.M., Jenkins, N.A., Brannan, C.I., et al. (1991). A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J. *10*, 2821–2832.

Morris, A.E., Remmele, R.L., Jr., Klinke, R., Macduff, B.M., Fanslow, W.C., and Armitage, R.J. (1999). Incorporation of an isoleucine zipper motif enhances the biological activity of soluble CD40L (CD154). J. Biol. Chem. *274*, 418–423.

O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. Science *243*, 538–542.

Perussia, B., Ramoni, C., Anegon, I., Cuturi, M.C., Faust, J., and Trinchieri, G. (1987). Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat. Immun. Cell Growth Regul. *6*, 171–188.

Reyburn, H.T., Mandelboim, O., Vales-Gomez, M., Davis, D.M., Pazmany, L., and Strominger, J.L. (1997). The class I MHC homologue of human cytomegalovirus inhibits attack by natural killer cells. Nature *386*, 514–517.

Robertson, M.J., Cochran, K.J., Cameron, C., Le, J.M., Tantravahi, R., and Ritz, J. (1996). Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. Exp. Hematol. *24*, 406–415.

Smith, C.A., Smith, T.D., Smolak, P.J., Friend, D., Hagen, H., Gerhart, M., Park, L., Pickup, D.J., Torrance, D., Mohler, K., et al. (1997). Poxvirus genomes encode a secreted, soluble protein that preferentially inhibits beta chemokine activity yet lacks sequence homology to known chemokine receptors. Virology *236*, 316–327.

Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., and Ploegh, H.L. (2000). Viral subversion of the immune system. Annu. Rev. Immunol. *18*, 861–926.

Wu, J., Song, Y., Bakker, A.B., Bauer, S., Spies, T., Lanier, L.L., and Phillips, J.H. (1999). An activating immunoreceptor complex formed by NKG2D and DAP10. Science *285*, 730–732.

Yabe, T., McSherry, C., Bach, F.H., Fisch, P., Schall, R.P., Sondel, P.M., and Houchins, J.P. (1993). A multigene family on human chromosome 12 encodes natural killer-cell lectins. Immunogenetics *37*, 455–460.

Yao, Z., Fanslow, W.C., Seldin, M.F., Rousseau, A.M., Painter, S.L., Comeau, M.R., Cohen, J.I., and Spriggs, M.K. (1995). Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. Immunity *3*, 811–821.

Yewdell, J.W., and Bennink, J.R. (1999). Mechanisms of viral interference with MHC class I antigen processing and presentation. Annu. Rev. Cell Dev. Biol. *15*, 579–606.

GenBank Accession Numbers

Nucleic acid sequences of the ULBPs have been deposited in GenBank under accession numbers AF304377–AF304379.