Primary NK Cells Activate Multiple Signaling Pathways in I-Related Proteins, Bind to NKG2D and UL16-Binding Proteins, Novel MHC Class I-Related Proteins, Bind to NKG2D and Activate Multiple Signaling Pathways in Primary NK Cells

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UL16-Binding Proteins, Novel MHC Class I-Related Proteins, Bind to NKG2D and Activate Multiple Signaling Pathways in Primary NK Cells

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The UL16-binding proteins (ULBPs) are a novel family of MHC class I-related molecules that were identified as targets of the human CMV glycoprotein, UL16. We have previously shown that ULBP expression renders a relatively resistant target cell sensitive to NK cytotoxicity, presumably by engaging NKG2D, an activating receptor expressed by NK and other immune effector cells. In this study we show that NKG2D is the ULBP counterstructure on primary NK cells and that its expression is up-regulated by IL-15 stimulation. Soluble forms of ULBPs induce marked protein tyrosine phosphorylation, and activation of the Janus kinase 2, STAT5, extracellular signal-regulated kinase, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (PI 3-kinase)/Akt signal transduction pathways. ULBP-induced activation of Akt and extracellular signal-regulated kinase and ULBP-induced IFN-γ production are blocked by inhibitors of PI 3-kinase, consistent with the known binding of PI 3-kinase to DAP10, the membrane-bound signal-transducing subunit of the NKG2D receptor. While all three ULBPs activate the same signaling pathways, ULBP3 was found to bind weakly and to induce the weakest signal. In summary, we have shown that NKG2D is the ULBP counterstructure on primary NK cells and for the first time have identified signaling pathways that are activated by NKG2D ligands. These results increase our understanding of the mechanisms by which NKG2D activates immune effector cells and may have implications for immune surveillance against pathogens and tumors. The Journal of Immunology, 2002, 168: 671–679.

The UL16-binding proteins (ULBPs) are a novel family of MHC class I-related, cell surface proteins. ULBP1 was identified based on its ability to bind to the human CMV (HCMV) glycoprotein, UL16 (1). ULBP2 and ULBP3 were subsequently discovered as expressed sequence tags with high homology to ULBP1 (1). Unlike traditional MHC class I proteins, ULBPs are GPI-linked, lack an α1 domain, and do not associate with β2-microglobulin (1). UL16 also binds to a member of another family of nonclassical MHC class I proteins, MICB (2). MICB and the closely related MICA protein share some similar properties with the ULBPs, as discussed below.

NK cells are a key component of the innate immune system that recognize and lyse virally infected and neoplastic cells. Recent studies indicate that ULBPs are important activators of NK cells. Soluble recombinant forms of ULBPs bind to human NK cells and stimulate NK cytotoxicity against tumor targets (3). Soluble ULBPs also induce production of the cytokines IFN-γ, GM-CSF, TNF-α, and TNF-β, and the chemokines macrophage-inflammatory protein (MIP)-1α, MIP-1β, and I-309. In all cases, costimulation with IL-12 has a superadditive effect on the production of these factors (1, 3).

NK cells recognize cellular targets through the use of receptors that are specific for MHC class I molecules on the target cell. These NK cell receptors belong to the killer cell Ig-like receptor, Ly49, and NKG2 families and, depending on their structure, deliver either activating or inhibitory signals to the NK cell (4). Until recently, it was thought that signals generated by inhibitory NK cell receptors were dominant over those generated by any activating receptor, so that cells with down-regulated MHC class I levels would be killed, the missing-self hypothesis (5). However, expression of ULBPs or MHC class I-related chain molecules (MICs) on NK cell-resistant, MHC class I-expressing, target cells confers susceptibility to NK cell killing (1, 6). Thus, ULBPs and MICs can transduce an activating signal to NK cells that can override a negative signal generated by engagement of inhibitory receptors for MHC class I. Whether the signal delivered by ULBPs and MICs is sufficient to cause activation of NK cells in vivo is likely to depend on the relative numbers of NK cell-activating and inhibitory receptors expressed and on the avidity of receptor engagement.

MICA expression is up-regulated in certain epithelial tumors, in HCMV-infected cells, and in response to stress (2, 7). In contrast to the MICs, ULBP messages are expressed by a wide range of cells, tissues, and tumors, and ULBP proteins are expressed on various cell lines (1). Thus, several types of cells may potentially deliver ULBP-mediated signals to NK cells and be targets of ULBP-mediated killing.

Although the amino acid sequences of the ULBPs and MICs are only distantly related, both families of proteins deliver an activating signal to NK cells apparently by binding to NKG2D/DAP10 heterocomplexes. NKG2D is a homodimeric, C-type lectin that is expressed not only on NK cells, but also on T cells and activated macrophages (6, 8). In T cells, NKG2D has been shown to act as...
a costimulatory receptor, in a similar manner as CD28 (9). The cytoplasmic domain of NKG2D is short, and signaling appears to be mediated through its association with the DAP10 membrane adapter protein (10). DAP10 can bind the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and the adapter protein Grb2 (10, 11). However, despite the likely importance of NKG2D in the activation of innate and adaptive immune responses to pathogens and tumors, nothing else is known about how NKG2D transmits its signal.

We have previously shown that ULBPs bind to recombinantly expressed NKG2D/DAP10 heterodimers and that an anti-NKG2D antiseraum partially blocks ULBP binding to NKL cells (1). In this report we provide proof that NKG2D is indeed the ULBP receptor expressed on primary human NK cells. We also identify signal transduction pathways that are activated by ULBPs in NK cells. These results provide a basis for comparison with CD28 signaling pathways and give insight into the mechanisms by which NKG2D ligands can provide a powerful stimulatory signal to activate NK cell effector functions.

Materials and Methods

Fusion proteins, Abs, and cytokines

Soluble ULBP-leucine zipper (LZ), MIC-LZ, UL16-LZ, ULBP-Fc, MIC-Fc, human NKG2D-Fc, and p7.5-Fc fusion proteins were prepared as previously described (1). Fab'2 forms of the M15 anti-LZ mAb were prepared by digestion with Ficin (Sigma, Milwaukee, WI) and then purified using a protein A-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ). The 4G10 anti-phosphotyrosine mAb, the p85 anti-PI 3-kinase mAb, and the 4G10 anti-phosphotyrosine mAb, the p85 anti-PI 3-kinase mAb used for immunoblotting, and rabbit Abs used to immunoprecipitate Janus kinase (JAK)2 and PI 3-kinase were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit Abs used to immunoblot phosphorylated JAK2 (Ty1007/1008) were obtained from BioSource International (Camarillo, CA). Rabbit anti-phospho-STAT5 (Ty694), phospho-mitogen-activated protein (MAP) kinase kinase 1/2 (phospho-MEK1/2, Ser277/286), phospho-extracellular signal-regulated kinase (phospho-ERK; Thr202/Tyr204), and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-ERK1 (sc-94) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-STAT5, anti-Akt, and anti-p70 S6 kinase were obtained from Cell Signaling Technology.

mAbs M585 and M580 to human NKG2D were generated by injecting BALB/c mice at 0, 2, and 6 wk with 10^9 H9262 cells. The mAbs were from Genzyme (Cambridge, MA), whereas rhuIL-15 was obtained from ImmunoResearch Laboratories (Beverly, MA). Rabbit anti-ERK1 (sc-94) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-STAT5, anti-Akt, and anti-p70 S6 kinase were obtained from Cell Signaling Technology.

Purification of NK cells

Primary human NK cells were obtained as previously described (13). Briefly, peripheral blood was layered over isolymph and centrifuged. PBMC from the resulting interphase were depleted of monocytes by monocyte-negative selection with RosetteSep (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol.

Flow cytometry

To determine whether soluble NKG2D-Fc blocks ULBP binding to primary NK cells, 1 μg/ml ULBP-LZ fusion protein or MIC-LZ protein was mixed with either 50 μg/ml NKG2D-Fc or p7.5-Fc control protein for 30 min at room temperature. Cells (1 × 10^6) were then resuspended in the NKG2D-Fc/ULBP-LZ mixture and incubated for 45 min on ice. Specific binding of LZ proteins was detected with a combination of biotin-conjugated M15 anti-LZ Ab (ImmuneX, Miltenyi Biotec), followed by PE-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA). To determine whether NKG2D-specific mAbs block ULBP binding, NK cells were treated for 45 min on ice with 4 μg/ml M585 or M580 anti-human NKG2D mAb (ImmunoResearch Laboratories) or M90 anti-NKG2D control (anti-CD40L) control mAb (ImmuneX), followed by 10 μg/ml ULBP-Fc or MIC-Fc protein. Binding of Fc proteins was detected with PE-conjugated, goat anti-murine Fcγ-specific Ab (Jackson ImmunoResearch Laboratories). To determine the effect of cytokine stimulation on NKG2D expression, NK cells were cultured for 20 h with IL-4 at 10 ng/ml, IL-10 at 10 ng/ml, IL-12 at 1 ng/ml, IFN-α at 1000 U/ml, or IL-15 at 50 ng/ml. Cells were washed and stained with M585 anti-NKG2D mAb, followed by detection with PE-conjugated, goat anti-mouse IgG (Sigma). After staining, cells were analyzed for ULBP-LZ, MIC-LZ, ULBP-Fc, and MIC-Fc binding or for NKG2D expression using a FACScan (BD Biosciences, Mountain View, CA).

Cell stimulation and preparation of cell lysates

Primary human NK cells were cultured overnight with RPMI 1640 containing 50 ng/ml rhuIL-15, 1% heat-inactivated FBS, and 2 mM l-glutamine. The cells were serum-starved for an additional 4 h in RPMI 1640 supplemented with 2 mM l-glutamine, then resuspended in RPMI 1640 to 3 × 10^6/ml. Where indicated, cells were pretreated for 30 min with 1 μM of the PI 3-kinase inhibitor wortmannin (Upstate Biotechnology), 50 μM of the PI 3-kinase inhibitor Ly294002 (Upstate Biotechnology), 5 μM of the MEK1 inhibitor PD98059 (New England Biolabs, Beverly, MA), or DMSO vehicle alone. For the NKG2D-Fc blocking experiments, cells were pretreated for 30 min with various concentrations of NKG2D-Fc or with p7.5-Fc control protein.

Cells were stimulated with 1 μg/ml ULBP-LZ fusion protein in the presence of 1 μg/ml M15 Fab'2 (anti-LZ) cross-linking Ab (ImmuneX). UL16-LZ and CD40-LZ were used as negative control LZ proteins. Reactions were stopped by adding cold PBS containing 1 mM Na_3 VO_4 and then centrifuging the cells for 3 min in the cold. The cells were solubilized in 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 100 mM glycerol, 137 mM NaCl, 2 mM EDTA, 1 mM Na_3 VO_4, 1 mM Na_2 MoO_4, 20 μM leupeptin, 20 μM aprotinin, and 1 mM PMSF. After 10 min on ice, detergent-insoluble material was removed by centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Immunoprecipitations

Cell lysates (0.4–0.75 mg of protein) were preincubated with mixing 15 μg of protein A-Sepharose for 1 h at 4°C. JAK2 or PI 3-kinase was immunoprecipitated by incubating the precleared lysates for 3 h at 4°C with anti-JAK2 or anti-p85 Ab, respectively. Immune complexes were collected by mixing the lysate with 15 μl of protein A-Sepharose for 1 h at 4°C. The beads were washed four times with lysis buffer before eluting bound protein with SDS-PAGE sample buffer.

Immunoblotting

Proteins (20 μg of total cell lysate or immunoprecipitates) were separated on 1-mm-thick, 4–20% Tris-glycine gels and then transferred to nitrocellulose. Equal protein loading in each of the lanes was confirmed by staining the filters with Ponceau S (Sigma). The filters were blocked with 5% nonfat dry milk powder in TBS (10 mM Tris-HCl (pH 8) and 150 mM NaCl). Primary Abs were diluted 1/1000 in TBS containing 3 mg/ml BSA (Sigma Immunochemicals, Aurora, OH) and 0.02% sodium azide and incubated with the filters overnight at 4°C. After washing with TBS/0.1% Tween 20 (TBST), the filters were incubated 1 h with HRP-conjugated goat anti-rabbit IgG (1/2000 in TBST) or HRP-conjugated sheep anti-mouse IgG (1/100,000). Filters were washed extensively with TBST, and immunoreactive bands were visualized by ECL (Amersham Pharmacia Biotech).

PI 3-kinase enzyme assays

Lysates (300 μg) from NK or 293 cells were immunoprecipitated as described above, and kinase assays were performed as described previously (14).

Chemokine and cytokine assays

Primary human NK cells were cultured in the presence of rhuIL-15 (50 ng/ml) for 20 h and washed. The cells were then stimulated with ULBP-LZ fusion proteins, IL-12 (1 ng/ml), or the combination of ULBP-LZ and IL-12 in the presence of 0.2 μM wortmannin (Upstate Biotechnology), 50 μM Ly294002 (Upstate Biotechnology), 1 μM PD98059 (New England Biolabs), or DMSO vehicle alone. GM-CSF, MIP-1α, and IFN-γ levels in tissue culture supernatants were determined by ELISA. GM-CSF levels were determined using the M5 and P5 paired Abs (ImmuneX), whereas MIP-1α and IFN-γ levels were determined using paired Abs from R&D
Results

NKG2D-Fc and anti-NKG2D mAbs block ULBP binding to primary NK cells

We have shown earlier that ULBPs bind to recombinantly expressed NKG2D/DAP10 heterocomplexes and that an antisera raised against recombinant NKG2D-Fc fusion protein partially blocks binding of ULBPs to NK cells (1). In addition, another group has shown that anti-NKG2D mAbs block MIC binding to NK cells (6). To determine whether NKG2D/DAP10 is the ULBP receptor on primary human NK cells, we tested the effects of soluble NKG2D-Fc fusion proteins and mAbs against NKG2D on the binding of soluble forms of the ULBPs to these cells. We found that NKG2D-Fc (Fig. 1A) and anti-NKG2D mAb M580 (Fig. 1B) completely block binding of ULBP1, ULBP2, ULBP3, and the MICs to primary NK cells. In contrast, a control Fc fusion protein (Fig. 1A) and an isotype-matched control mAb (Fig. 1B) did not block ULBP or MIC binding. These results demonstrate that NKG2D is the major binding component of the ULBP counterstructure on primary NK cells. However, there appear to be differences in the NKG2D epitopes used for binding individual NKG2D ligands. For example, we found that anti-NKG2D mAb M580 partially blocks binding of ULBP2 (Fig. 1C) and MICA (M. Kubin, unpublished observation) to NK cells. In contrast, M580 consistently causes a slight increase in binding of ULBP1, ULBP3 (Fig. 1C), and MICB (M. Kubin, unpublished observation) to NK cells, perhaps by causing a conformational change in NKG2D that favors binding of these ligands.

We have previously shown that overnight culture in IL-15 greatly enhances the ability of NK cells to bind soluble ULBPs (3). In this study we found that IL-15 treatment markedly up-regulates NKG2D expression and that this correlates with the ability of ULBPs to bind to primary NK cells (Fig. 1D). NKG2D expression and ULBP binding are also up-regulated in response to IL-10, IFN-α, and IL-12, but weakly or not at all in response to IL-4 treatment (Fig. 1D).

ULBPs stimulate marked protein tyrosine phosphorylation

Protein tyrosine phosphorylation is an important mechanism used by many receptors to transmit signals into cells. To determine whether ULBPs induce activation of protein tyrosine kinases, we performed anti-phosphotyrosine immunoblots on lysates from ULBP-LZ-treated NK cells. Both ULBP1 and ULBP2 induced marked protein tyrosine phosphorylation within 1 min (Fig. 2A). The most prominent tyrosine-phosphorylated proteins present in response to ULBP treatment had apparent molecular masses of approximately 40, 45, 100, and 120 kDa (Fig. 2A). UL16-LZ control protein, which shows no detectable binding to NK cells, had little or no effect on protein tyrosine phosphorylation (Fig. 2A). We have shown that ULBPs synergize strongly with IL-12 in inducing IFN-γ production from NK cells (3). In this study we found that IL-12 has no obvious effect on the pattern of ULBP-induced protein tyrosine phosphorylation (Fig. 2A). Together, these data suggest that protein tyrosine kinase activation may be a signaling mechanism used by ULBPs to transmit their effects on NK cells.

ULBPs induce phosphorylation of JAK2 and STAT5

After determining that ULBPs cause marked protein tyrosine phosphorylation, we set out to identify ULBP-activated tyrosine kinase(s). We focused on the JAKs and their downstream effectors, the STAT transcription factors, because JAKs and STATs are activated by tyrosine phosphorylation, and major phosphotyrosine proteins identified in Fig. 2A have similar sizes, ~110-130 kDa, as JAK and STAT proteins. We assessed JAK activation by stimulating NK cells with ULBP2-LZ, followed by immunoprecipitation of the JAKs from cell lysates and assessment of their activation states by immunoblotting with Abs specific for the tyrosine-phosphorylated, active forms of these kinases. We found that ULBP2-LZ induces phosphorylation of JAK2 (Fig. 2B) but has no detectable effect on phosphorylation of JAK1, JAK3, and Tyk2 (data not shown).

To determine whether ULBPs activate the main downstream effectors of the JAKs, the STATs, NK cells were stimulated with ULBPs for 5 min, and then cell lysates were immunoblotted with phosphospecific STAT1, STAT3, STAT5, or STAT6 Abs. Alternatively, STAT4 was immunoprecipitated and then immunoblotted with 4G10 anti-phosphotyrosine mAb. We found that STAT5 is strongly phosphorylated in response to ULBP1 and ULBP2 (Fig. 2C) and weakly phosphorylated in response to ULBP3 (data not shown). In contrast to STAT5, we found that ULBP1 and ULBP2 weakly phosphorylate STAT1 but have no apparent effect on phosphorylation of STAT3, STAT4, and STAT6 (C. L. Sutherland, unpublished observations). NKG2D-Fc completely blocks ULBP-induced STAT5 phosphorylation (Fig. 2D), consistent with the idea that STAT5 is activated in response to ULBP engagement of NKG2D.

We have shown that ULBPs synergize strongly with IL-12 in inducing IFN-γ production from NK cells (3). To analyze the mechanism underlying the synergistic effect of ULBPs and IL-12 on NK cells, we tested the effect of ULBP and IL-12 cotreatment on STAT activation. We were particularly interested in determining whether ULBPs synergized with IL-12 for STAT4 activation, because STAT4 is required for IL-12-induced IFN-γ production (15, 16). While IL-12 caused a small increase in ULBP-induced STAT5 phosphorylation (Fig. 2C), it had no detectable effect on the ability of ULBPs to induce phosphorylation of STAT1, STAT3, STAT4, or STAT6 (C. L. Sutherland, unpublished observations).

ULBPs induce activation of the ERK MAP kinase pathway

After finding that ULBPs induce activation of JAK2 and STAT5 in NK cells, we tested whether ULBPs activate the ERK, c-Jun N-terminal kinase (JNK), and p38 MEKs. We were interested in testing whether ULBPs activate MEKs, because these serine/threonine kinases play key roles in transducing extracellular signals from the cytosol to the nucleus. In particular, ERK2 has recently been shown to play a critical role in driving NK cell lysis of tumor targets (17). After stimulating cells with ULBP-LZ proteins, MEK activity was assessed by immunoblotting cell lysates with Abs specific for the phosphorylated, activated forms of ERK, JNK, and p38. We found that ULBP1-LZ, ULBP2-LZ, and the MICs induce marked phosphorylation of p42 ERK2 and modest phosphorylation of p44 ERK1 within 1 min (Fig. 3, upper panels). In contrast, ULBP3-LZ only weakly phosphorylates the ERKs, and a UL16 control LZ protein has no effect (Fig. 3, upper panels). Consistent with the finding that ULBPs activate ERK1 and ERK2, ULBPs induce phosphorylation of kinases upstream of ERK1 and ERK2, MEK1/2 (Fig. 3, lower panel).

ULBPs-induced ERK activation in NK cells is dependent on PI 3-kinase, as the PI 3-kinase inhibitors wortmannin (Fig. 3) and Ly294002 (data not shown) completely blocked ULBP-induced phosphorylation of ERK1/2 and MEK1/2 (Fig. 3). Examples of PI 3-kinase-dependent activation of ERK have been cited previously in the literature (18, 19). In contrast to the ERKs, we did not detect ULBP-induced activation of JNK or p38 in NK cells (data not shown).
Figure 1. NKG2D-Fc and NKG2D mAbs block ULBP binding to NK cells; IL-15 up-regulates NKG2D expression. The ability of soluble NKG2D-Fc and anti-NKG2D mAb to block ULBP binding to primary NK cells was analyzed by flow cytometry. A, ULBP1-LZ or ULBP2-LZ (1.5 μg/ml) was premixed with NKG2D-Fc (5–50 μg/ml) or p7.5-Fc control protein (50 μg/ml) and then applied to cells. Binding of LZ proteins was detected with biotin-conjugated M15 F(ab')₂, followed by PE-conjugated streptavidin. Note that neither NKG2D-Fc nor p7.5-Fc shows detectable binding to NK cells (data not shown). B and C, In the top left panels, NK cells were stained with 4 μg/ml anti-NKG2D mAb M585 or M580 to assess NKG2D expression or M90 isotype control mAb. Binding of mAbs was detected with PE-conjugated, goat anti-mouse IgG. In the other panels, NK cells were pretreated with PBS alone (filled histograms), 4 μg/ml mAb M585 or M580 (hatched histograms), or M90 mAb (checked histograms) and then stained with 10 μg/ml ULBP-Fc or MIC-Fc protein. Binding of Fc proteins was detected with PE-conjugated, Fc-specific goat anti-murine IgG. D, To determine the effects of cytokines on NKG2D expression and ULBP binding, NK cells were treated with IL-4, IL-10, IL-12, IL-15, or IFN-α. M585 mAb and ULBP2-Fc binding were then analyzed as described in B. Data in A–C are representative of experiments on four different donors. The data in D show the mean and SD for the fold change in mean fluorescent intensity based on independent experiments with four different donors. The values for anti-NKG2D and control mAb binding are normalized to those for anti-NKG2D binding in the presence of medium alone; the values for ULBP2-Fc binding are normalized to those for ULBP2-Fc binding in the presence of medium alone.
ULBPs induce activation of PI 3-kinase and the antiapoptotic kinase, Akt

The receptor for MICA and MICB is a heterocomplex composed of the C-type lectin, NKG2D, and the DAP10 adapter protein (6). Studies in pervanadate-treated NKL cells (10) and in CD4-DAP10 chimera-transfected Jurkat cells (11) have shown that DAP10 can recruit the p85 subunit of PI 3-kinase. However, it is not known whether NKG2D engagement by ULBPs or MICs induces PI 3-kinase activation. To determine whether ULBP triggering induces phosphorylation and activation of PI 3-kinase, NK cells were treated with ULBP2-LZ, and then the p85 subunit of PI 3-kinase was immunoprecipitated from cell lysates. We found that both the p85 and p110 subunits of PI 3-kinase are phosphorylated within 2 min of ULBP addition, whereas CD40L-LZ control protein has no effect (Fig. 4A). Furthermore, ULBP triggering induces activation of PI 3-kinase, as assessed by in vitro lipid kinase assay (Fig. 4B).

To determine whether all the ULBP and MIC proteins can activate the PI 3-kinase pathway, we tested whether they activate a serine/threonine kinase downstream of PI 3-kinase, Akt. After stimulating NK cells with ULBP-LZ proteins, Akt activation was assessed by immunoblotting cell lysates with an Ab specific for the phosphorylated, activated form of Akt. We found that ULBP1, ULBP2, and the MICs induce marked phosphorylation of Akt within 1–5 min, whereas ULBP3 only weakly phosphorylates Akt (Fig. 4C). ULBP-induced activation of Akt is dependent on PI 3-kinase, because the PI 3-kinase inhibitors wortmannin and Ly294002 block Akt phosphorylation (Fig. 4C). In addition to blocking ULBP binding to NK cells (Fig. 1A), we found that NKG2D-Fc blocks ULBP-induced phosphorylation of Akt (Fig. 4D).

PI 3-kinase is required for ULBP-induced cytokine and chemokine production

We have shown that ULBPs induce NK cells to produce a variety of cytokines and chemokines. IL-12 cotreatment greatly enhances the production of these factors and has a synergistic effect on IFN-γ production (1, 3). To determine whether the PI 3-kinase and ERK pathways mediate ULBP-induced cytokine and chemokine production, we tested the effects of inhibitors of these pathways on GM-CSF, MIP-1β, and IFN-γ synthesis. We found that the PI 3-kinase inhibitor, wortmannin (0.2 μM), completely blocks ULBP-induced GM-CSF production and caused a 60–70% reduction of MIP-1β and IFN-γ production (Fig. 5). Wortmannin phosphorylated in response to ULBP treatment. B, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated.

**FIGURE 2.** ULBPs induce marked protein tyrosine phosphorylation and activate JAK2 and STAT5. A, NK cells were stimulated with 1 μg/ml ULBP-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. B, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. C, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. D, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment.

**FIGURE 2.** ULBPs induce marked protein tyrosine phosphorylation and activate JAK2 and STAT5. A, NK cells were stimulated with 1 μg/ml ULBP-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. B, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. C, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment. D, NK cells were stimulated with 1 μg/ml ULBP2-LZ or UL16-LZ control protein in the presence of 1 μg/ml M15 (anti-LZ) F(ab’)2, for 1 or 5 min. Cell lysates were normalized for protein concentration and then analyzed by immunoblotting with the 4G10 anti-phosphotyrosine mAb. Molecular mass standards in kilodaltons are indicated to the left. Arrows to the right denote major proteins that are tyrosine phosphorylated in response to ULBP treatment.
also caused a large reduction in IFN-γ synthesis induced by ULBP2 and IL-12 cotreatment (Fig. 5). While wortmannin had a dramatic effect on ULBP2-induced cytokine and chemokine induction, it did not affect NK cell proliferation in response to IL-15, suggesting that there was no nonspecific cytotoxic effect at this dose (data not shown). The PI 3-kinase inhibitor, Ly294002, had similar effects as wortmannin on ULBP2-induced cytokine production (data not shown). Compared with PI 3-kinase inhibitors, the MEK1/2 inhibitor PD98059 (1 μM) weakly inhibited production of GM-CSF, MIP-1β, and IFN-γ (Fig. 5). Although the data shown here are for ULBP2, wortmannin and PD98059 had similar effects on ULBP1- and ULBP3-induced GM-CSF, MIP-1β, and IFN-γ production (data not shown). Taken together, these results suggest that PI 3-kinase is a major mediator of ULBP-induced cytokine and chemokine production in NK cells, whereas the ERK pathway plays at most a minor role.

**Discussion**

ULBPs are a novel family of MHC class I-related molecules that bind to and activate human NK cells for cytotoxicity and cytokine production. Moreover, ULBPs deliver an activating signal to NK cells that can overcome an inhibitory signal generated by NK cell recognition of MHC class I molecules (1). In this report we show that the C-type lectin, NKG2D, is the ULBP counterstructure expressed on primary human NK cells. We also demonstrate that by binding NKG2D, ULBPs activate multiple signal transduction pathways in primary NK cells.

Our findings that both soluble NKG2D-Fc and anti-NKG2D mAb M585 completely block binding of ULBP1, ULBP2, and ULBP3 to NK cells (Fig. 1, A and B) demonstrate that NKG2D is the ULBP counterstructure on primary human NK cells. Further evidence that NKG2D is the functional ULBP receptor on primary NK cells is that NKG2D-Fc blocks ULBP-induced signaling in these cells (Figs. 2D and 4D). Although the ULBPs and MICs all bind to NKG2D on NK cells, there appear to be some differences in the NKG2D epitopes used for binding. For example, while anti-NKG2D mAb M585 blocks binding of all five proteins to NK cells (Fig. 1B), mAb M580 partially blocks binding of ULBP2 (Fig. 1C) and MICA (M. Kubin, unpublished observations) but has no effect on the binding of ULBP1, ULBP3, and MICB. IL-15, IL-12, and IFN-α all up-regulate both NKG2D expression and ULBP binding to NK cells derived from coculture on irradiated RPMI 8666 cells (Fig. 1D). NKG2D is expressed on essentially all CD56+CD3− NK cells from freshly isolated PBMC (unpublished observations). Experiments are in progress to determine whether coculture of NK cells with irradiated RPMI 8666 cells influences the level of ULBP receptors expressed relative to freshly isolated NK cells.

Previous work has established the association of the membrane-bound signal transducer DAP10 with NKG2D (10). Studies in CD4-DAP10 chimera transfectants and pervanadate-treated NKG2D-DAP10 transfectants have shown that DAP10 is capable of recruiting the p85 subunit of PI 3-kinase (10, 11). In this study we found, using soluble versions of the natural ligands for NKG2D, that ULBPs induce phosphorylation and activation of PI 3-kinase as well as activation of the PI 3-kinase target, Akt, in NK cells (Fig. 4). Furthermore, the PI 3-kinase inhibitors wortmannin and LY294002 block ULBP-induced activation of Akt (Fig. 4C). Although several types of receptors are capable of activating Akt, these data are consistent with the hypothesis that, like MICA, ULBPs may bind to and signal through a DAP10-containing receptor complex. Future experiments will reveal whether NKG2D can partner with other adapters besides DAP10, and if the ULBPs bind to receptors other than NKG2D in different cell types. Another membrane adapter protein expressed in NK cells, DAP12, does not associate with NKG2D and does not contain a PI 3-kinase binding site (20). We are currently testing whether, in addition to NK cells, ULBPs bind to and activate other cells that express NKG2D, such as CD8+ αβ T cells, γδ T cells, and activated macrophages (6, 8).

We found that ULBPs and MICs induce a marked increase in intracellular calcium and that inhibition of PI 3-kinase with either LY294002 or wortmannin attenuates ULBP-induced calcium mobilization (our unpublished observations). Thus, PI 3-kinase activity is required to couple NKG2D/DAP10 to calcium mobilization.
In addition to calcium flux, ULBPs induce rapid tyrosine phosphorylation of several proteins (Fig. 2A). Although qualitatively similar, ULBP1 causes a more intense tyrosine phosphorylation pattern than ULBP2 (Fig. 2A). Furthermore, for all signaling events tested, ULBP1 and ULBP2 clearly induce stronger signaling responses than ULBP3. Similarly, we found that soluble ULBP1 and ULBP2 bind to NK cells from several different donors to a greater extent than does soluble ULBP3 (3). In addition, our preliminary BioCore analysis indicates that ULBP1 binds NKG2D most strongly and with the slowest off-rate, followed closely by ULBP2. ULBP3 shows significantly weaker binding and has a faster off-rate than ULBP1 and ULBP2. These observations are consistent with a model in which the strength of the ULBP-mediated signal correlates with the stability of the ligand-receptor complex. A recent study using soluble versions of NKG2D and the murine NKG2D ligands, RAE-1 and H60, found that RAE-1 and H60 each bind to NKG2D tightly, but that H60 binds to NKG2D with approximately 25-fold higher affinity (21). However, it should be noted that the NKG2D ligands are normally membrane-bound proteins, and it is possible that other cell surface proteins on both effector and target cells may contribute as accessories to the overall strength of the NKG2D/NKG2D ligand interaction.

We found that ULBPs cause a strong phosphorylation of STAT5 at tyrosine residues that correlate with its activation (Fig. 4C). STAT5 exists as two closely related proteins, STAT5a and STAT5b (22). Studies in STAT5-deficient mice indicate that STAT5a is required for lactogenesis (23), whereas STAT5b is essential for NK cell-mediated proliferation and cytotoxicity (24). Both STAT5 proteins are expressed in primary NK cells, and studies are underway to determine which form(s) is activated by ULBPs. STAT5 activation is typically mediated by JAK2; however, STAT5 can be directly activated by some receptor tyrosine kinases (25, 26), and Src-dependent mechanisms for STAT5 activation also exist (27). We found that ULBPs induce phosphorylation of JAK2 (Fig. 2B) but do not appear to activate c-Src (C. L. Sutherland, unpublished observations). Experiments are in progress to determine whether JAK2-specific inhibitors block ULBP-induced activation of STAT5 and ULBP-induced NK cytotoxicity against tumor targets.

The serine/threonine kinase, Akt, promotes cell survival in response to various growth and survival factors (28). We found that ULBPs and MICs stimulate phosphorylation of Akt in a pathway involving PI 3-kinase (Fig. 4C). In contrast, we did not detect ULBP-induced activation of NF-κB, an antiapoptotic transcription factor that is a target of Akt (29, 30). Whether ULBPs provide a survival signal to NK cells requires further study. However, this concept is consistent with our unpublished findings that ULBPs up-regulate messages for several antiapoptotic factors, including a caspase inhibitor, API2/cIAP2, and a Bcl-2 homolog, Bcl2A1.

ULBPs activate the ERK MAP kinase pathway (Fig. 3) but have no apparent effect on JNK or p38. Our results suggest that transcription factors targeted by ERK, such as Elk-1, c-Fos, and Ets-1,
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FIGURE 5. ULBP-induced cytokine and chemokine production is dependent on PI 3-kinase. NK cells were cultured in the presence of IL-15 (50 ng/ml) for 20 h, washed, and pretreated with the PI 3-kinase inhibitor, wortmannin (0.2 μM, final concentration), or DMSO vehicle for 30 min. Cells were then treated with ULBP2-LZ (1 μg/ml), IL-12 (1 ng/ml), or the combination of ULBP2-LZ and IL-12 in the presence of wortmannin, PD98059, or DMSO vehicle for an additional 36 h. Supernatants were analyzed for the presence of GM-CSF, MIP-1β, and IFN-γ by ELISA as described in Materials and Methods. CD40L-Liz control protein (1 μg/ml) did not induce GM-CSF, MIP-1β, or IFN-γ production. The mean and SD are shown for an experiment performed in triplicate that is representative of independent experiments with three different donors.

may be activated by ULBPs and mediate functional responses in NK cells. ERK2 plays a pivotal role in NK cytotoxicity, presumably by controlling mobilization of perforin and granzyme B toward target cells (17). Whether ERK2 mediates ULBP-induced NK cytotoxicity remains to be tested. Our finding that MEK1/2 and ERK1/2 phosphorylations are blocked in PI 3-kinase-inhibited NK cells (Fig. 3) is consistent with a recent report that PI 3-kinase is the key upstream regulator of ERK in the NK cytolytic process (31). Our data showing that the MEK1/2 inhibitor, PD98059, weakly inhibits production of GM-CSF, MIP-1β, and IFN-γ (Fig. 5) suggests that the ERK pathway may play a small role in mediating ULBP-induced cytokine production.

We have previously shown that ULBPs synergize strongly with IL-12 for production of IFN-γ by NK cells (3). While IL-12 enhances ULBP-induced phosphorylation of STAT5 (Fig. 2c), it is unclear whether the STAT5 pathway contributes to IFN-γ production in this system. In addition to modulating IL-12-initiated signals, the ULBPs may function with IL-12 by activating signaling pathways that are distinct from but complimentary to those activated by IL-12. For example, in this study we found that ULBPs induce phosphorylation of Akt (Fig. 4c), whereas IL-12, either alone or in combination with ULBPs, has no effect on Akt phosphorylation (C. L. Sutherland, unpublished observations). Furthermore, PI 3-kinase inhibitors block both ULBP-induced phosphorylation of Akt (Fig. 4c) and IFN-γ production induced by costimulation with ULBPs and IL-12 (Fig. 5). A recent paper shows that Akt provides the CD28 costimulatory signal that augments TCR-initiated production of IFN-γ (32). Although additional studies remain to be performed, our data are consistent with the hypothesis that, like the CD28 pathway, Akt provides the CD28 costimulatory signal that augments TCR-initiated production of IFN-γ.

The concept that NKG2D can function as a costimulatory receptor is extended by the recent finding that NKG2D can serve, in the absence of CD28, as an alternative costimulator of TCR-dependent T cell activation (9). Moreover, the parallel between the CD28 and NKG2D signaling pathways is further strengthened by the finding that NKG2D can activate the PI3-K/Akt signaling pathway (33). Our data further extend the analogy between NKG2D/DAP10 and CD28 signaling.

Studies are in progress to further define the function of the ULBPs in immune surveillance. At present, several findings suggest that ULBPs, like MICs, play an important role in the activation of innate immunity. For example, ULBPs induce NK cells to produce multiple cytokines and chemokines, including GM-CSF, IFN-γ, TNF-α, MIP-1β, and I-309 (1, 3). IFN-γ and TNF-α are important anti-viral cytokines (34), whereas GM-CSF, MIP-1β, and I-309 probably function in vivo to recruit and activate NK cells, macrophages, and other components of both innate and adaptive immunity. Besides inducing cytokine and chemokine production, ULBPs stimulate potent NK cytotoxicity against tumor targets (1, 3).

FIGURE 6. ULBPs engage NKG2D/DAP10 and trigger multiple signal transduction pathways in human NK cells. ULBPs bind to a heterocomplex consisting of the C-type lectin NKG2D and the signal transducing adapter protein DAP10. ULBP-NKG2D/DAP10 interaction triggers calcium mobilization and activation of the PI3-K/Akt, MEK/ERK, and JAK2/STAT5 signaling pathways. Costimulation of NK cells with ULBPs and IL-12 greatly enhances the production of multiple cytokines and chemokines. The PI3-K pathway appears to play a central role in transmitting NKG2D/DAP10 signals, since ULBP-induced calcium mobilization; activation of MEK, ERK, and Akt; and cytokine/chemokine production are all attenuated by the PI 3-kinase inhibitors wortmannin and Ly294002.
Further investigation is required to understand how both ULBP expression and activation are regulated in normal cells and tumors. ULBP transcripts are expressed in a wide range of normal and tumor tissues (1). At the protein level, ULBPs are expressed in a variety of cell lines (1), whereas MIC expression is up-regulated on some epithelial tumors (2, 7). Murine ligands for NKG2D, the RA-E-1 and H-60 proteins, are induced on some tumors (35, 36), and their ectopic expression on tumor cell lines mediates potent rejection of MHC class I-bearing tumors in vivo (37, 38). Given these recent findings, it is tempting to speculate that, similar to RA-E-1 and H-60 in the mouse, ULBPs and MICs may stimulate tumor immunity in humans.

MIC expression is known to be up-regulated on HCMV-infected cells (9). How ULBP expression and ULBP and MIC functions are affected by pathological conditions requires further study. We have shown previously that the HCMV glycoprotein, UL16, blocks ULBP and MIC binding to NK cells, and ULBP-induced NK cell activation (1, 3). Both NK and T cells play important roles in controlling CMV infection (39, 40). Thus, another area of interest is to examine how UL16 masking of NKG2D ligands may protect a virus-infected cell against immune recognition.

In summary, we found that ULBPs bind to the NKG2D/DAP10-Akt signal transduction pathways. By phosphorylating different substrates and regulating different transcription factors, JAK2, ERK, and Akt may mediate the effects of ULBPs on NK cell activation (Fig. 6). This study is the first, to our knowledge, that characterizes the signaling pathways induced by engagement of NKG2D by its ligands. NKG2D is emerging as an important activating receptor that bridges the gap between innate and adaptive immunity and that can act as a costimulatory molecule in a similar manner as CD28. Understanding the mechanisms by which ULBP/NKG2D interactions activate immune effector cells may have implications for immune surveillance against pathogens and tumors.

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References