This information is current as of May 29, 2022.

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*J Immunol* 2011; 186:5538-5542; Prepublished online 4 April 2011;
doi: 10.4049/jimmunol.1002254

http://www.jimmunol.org/content/186/10/5538

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/04/04/jimmunol.100225.4.DC1

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Cutting Edge: NKG2D-Dependent Cytotoxicity Is Controlled by Ligand Distribution in the Target Cell Membrane

Emily Martinez,* Joseph A. Brzostowski,† Eric O. Long,* and Catharina C. Gross*‡

Although the importance of membrane microdomains in receptor-mediated activation of lymphocytes has been established, much less is known about the role of receptor ligand distribution on APC and target cells. Detergent-resistant membrane domains, into which GPI-linked proteins partition, are enriched in cholesterol and glycosphingolipids. ULBP1 is a GPI-linked ligand for natural cytotoxicity receptor NKG2D. To investigate how ULBP1 distribution on target cells affects NKG2D-dependent NK cell activation, we fused the extracellular domain of ULBP1 to the transmembrane domain of CD45. Introduction of this transmembrane domain eliminated the association of ULBP1 with the detergent-resistant membrane fraction and caused a significant reduction of cytotoxicity and degranulation by NK cells. Clustering and lateral diffusion of ULBP1 was not affected by changes in the membrane anchor. These results show that the partitioning of receptor ligands in discrete membrane domains of target cells is an important determinant of NK cell activation. The Journal of Immunology, 2011, 186: 5538–5542.

Natural killer cells are a subset of cytotoxic lymphocytes that recognize and kill tumor cells and virus-infected cells (1). Lysis of target cells is a multistep process including adhesion of NK cells to target cells, synapse formation, polarization of cytolytic granules toward the target cells, and granule exocytosis (2). Whereas binding of LFA-1 on human NK cells to ICAM on target cells induces adhesion and polarization of lytic granules (3), degranulation is triggered by low-affinity FcγR CD16 or by synergistic combinations of coactivation receptors, such as 2B4 and NKG2D (4, 5).

NKG2D is a C-type lectin coactivation receptor expressed as a disulfide-linked homodimer on NK cells, NKT cells, and some T cells (6). In humans, NKG2D binds to stress-inducible members of the polymorphic MHC class I-related chain A/B (MICA/B) family and the multigene family of UL16-binding proteins (ULBP5; RAET1A-E). NKG2D ligands are expressed in multiple types of tumors and play an important role in immunosurveillance of cancer (7). However, by shedding NKG2D ligands from their cell surface, tumor cells may escape the antitumor response mediated by NKG2D (6).

Within the lipid bilayer, proteins and lipids are segregated laterally, leading to functional subcompartmentalization of the plasma membrane (8). Lipid rafts are membrane microdomains enriched in glycosphingolipids, sphingomyelins, and cholesterol. The role of membrane microdomains in promoting receptor-mediated lymphocyte activation has been well established (9, 10). Much less is known about how the distribution of receptor ligands on target cells affects lymphocyte function, although studies have suggested that it may be an important parameter for lymphocyte activation. For example, the cytoplasmic tail of CD80 (B7-1), a CD28 ligand expressed in APCs, is required for proper segregation of CD28 at the immunological synapse and for full T cell activation (11). Furthermore, although expression of MICA on resistant target cells could overcome MHC class I-dependent inhibitory signaling in NK cells, a truncated form of MICA lacking a potential acylation site could not (12), suggesting that NKG2D ligand distribution may play a role in overcoming NK cell inhibition.

In this study, we tested ULBP1 as a ligand to investigate the role of ligand distribution in NKG2D-dependent human NK cell activation. To do so independently of HLA class I ligands for inhibitory receptors, we expressed ULBP1 in a mouse cell line. A chimera consisting of the extracellular portion of ULBP1 and the transmembrane region of CD45 was generated. Its expression resulted in the localization of the normally GPI-linked ULBP1 from detergent-resistant membrane (DRM) fractions to detergent-soluble fractions. This redistribution of ULBP1 caused a reduction in cytotoxicity and degranulation...
by NK cells, implying a role for receptor ligand distribution in the activation of NK cell responses.

**Materials and Methods**

**Cells**

Resting human NK cells were isolated from peripheral blood cells by negative selection using an NK cell isolation kit (Stem Cell Technologies). Freshly isolated resting NK cells (95–99% CD3−CD56−) were resuspended in IMDM (Invitrogen) supplemented with 10% human serum (Valley Biomedical) and used 1–2 d after isolation. Polyclonal IL-2-activated NK cells were cultured as described previously (3). P815 cells were cultured in IMDM supplemented with 10% heat-inactivated FBS (Thermo Scientific). For phospholipase treatment, P815-ULBP1 and P815-ULBP1-CD45TM cells were treated with 2 μl/ml phosphatidylserine-specific phospholipase C (PI-PLC) (Sigma) for 1 h at 37°C and 5% CO2, and surface protein levels were measured by flow cytometry using an R-PE-conjugated ULBP1 mAb (R&D Systems). The ULBP1 mAb was conjugated with a Phycollink R-PE kit (Prozyme).

**Transfection of P815 cells**

P815 cells were transfected with human ULBP1 or ULBP1-CD45TM (Supplemental Fig. 1) using the Bio-Rad Gene Pulser (10 μg of each DNA, 260 V, 960 μF). Transfected cells were selected in IMDM supplemented with 10% heat-inactivated FBS and 800 μg/ml Genetecin (Invitrogen), and subcloned. Different clones were tested for ULBP1 expression and in functional assays, and representative clones from each cell line were selected for further use.

**DRM preparation**

DRM preparation was performed as described previously (13), except that OptiPrep (Axis-Shield) was used instead of sucrose. Fractions 4–11 were separated on 12% SDS NuPAGE gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 h at room temperature and incubated with biotinylated goat anti-human ULBP1 Ab (R&D System) overnight at room temperature. After washing, the membrane was stained with IRDye 680-labeled streptavidin (LI-COR Biosciences) for 1 h at room temperature, and bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences). In the case of latrunculin A treatment, cells were incubated with either 0.3% DMSO carrier or 3 μl/ml latrunculin A (Calbiochem) for 40 min at 37°C and 5% CO2.

**Total internal reflection fluorescence microscopy**

Cell surface ULBP1 or ULBP1-CD45TM on P815 cells was fluorescently labeled with PE-conjugated ULBP1 mAb (R&D). For latrunculin A treatment, labeled cells were incubated for 40 min at 37°C and 5% CO2 with 0.3% DMSO carrier or 3 μl/ml latrunculin A before analysis by total internal reflection fluorescence (TIRF) microscopy. TIRF imaging and analysis was performed as described previously (14).

**Cellular assays**

Cytotoxicity assays were performed as described previously (15). Degranulation assays were performed as described previously (4) with minor changes. In brief, 2 × 105 NK cells were added to 4 × 106 P815 target cells in a total volume of 200 μl IMDM medium supplemented with 10% heat-inactivated FBS, 6 μg/ml monensin (Calbiochem), 20 μl/ml FITC-conjugated CD107a mAb (Becton Dickinson), and 20 μl/ml PE-conjugated CD56 mAb (Becton Dickinson). Cells were mixed and incubated for 1 h at 37°C and 5% CO2. Afterward, cells were spun down and expression of CD107a on CD56+ cells was determined by flow cytometry. In case of latrunculin A pretreatment of the target cells, cells were incubated with either 0.3% DMSO carrier (Sigma) or 3 μl/ml latrunculin A (Calbiochem) for 40 min at 37°C at 5% CO2 and washed extensively before use in the assay.

**Results and Discussion**

**Linking the extracellular portion of ULBP1 to the transmembrane region of CD45 changes its localization within the membrane**

To change the distribution of the NKG2D ligand ULBP1 within the plasma membrane, we generated a chimera consisting of the extracellular portion of ULBP1 and the transmembrane region of CD45 (ULBP1-CD45TM) (Supplemental Fig. 1). In brief, the extracellular portion of ULBP1 including the GPI anchor site (Supplemental Fig. 1B) was fused to the transmembrane region of CD45 that includes only two extracellular amino acids and four amino acids in the cytosolic portion for anchoring purposes (Supplemental Fig. 1B). The GPI-linked ULBP1 and the recombinant ULBP1-CD45TM were transfected into the mouse mastocytoma cell line P815, and clones with similar expression levels were selected (Fig. 1A). To verify that ULBP1-CD45TM had lost the GPI anchor, P815 cells expressing ULBP1 and ULBP1-CD45TM were treated with PI-PLC, which cleaves GPI anchors. ULBP1 was sensitive to PI-PLC, but ULBP1-CD45TM was not, indicating a loss of the GPI anchor in the chimera (Fig. 1A). The incomplete cleavage of ULBP1 (Fig. 1A), which could be caused by limited accessibility to phospholipase, is consistent with the low amount of ULBP1 shedding observed in other target cells (16).

To test whether linkage to the transmembrane domain of CD45 changed the localization of ULBP1, we prepared DRM fractions from P815-ULBP1 and P815-ULBP1-CD45TM cells. Whereas ULBP1 was almost exclusively localized in the DRM fraction, the ULBP1-CD45TM protein was associated with the soluble fraction (Fig. 1B). Association of ULBP1 with the DRM fraction was even stronger than that of the DRM marker flotillin 1 (Fig. 1B). Therefore, linking the extracellular portion of ULBP1 to the transmembrane region of CD45 changed its localization from the DRM fraction to the detergent-soluble membrane fraction.

**Targeting of ULBP1 to the detergent-soluble membrane fraction reduces the sensitivity of target cells to lysis by NK cells**

It has been shown that redistribution of ICAM-2 on tumor cells via ezrin renders these cells more sensitive to lysis by NK cells (17). To test whether distribution of ULBP1 in either the
distribution and mobility were visualized by TIRF microscopy. To study these parameters, we labeled ULBP1 and ULBP1-CD45TM with a PE-conjugated Ab to ULBP1, and their clustering and lateral mobility within the plasma membrane were monitored. To investigate if detergent-soluble domains affected the extent of ULBP1 clustering, we measured the percentage of ULBP1 clusters in detergent-soluble and DRM fractions. We next tested how the segregation of ULBP1 into DRM and detergent-soluble membrane fractions affected the extent of ULBP1 clustering. DRM fraction or the detergent-soluble membrane fraction had any functional consequence for sensitivity to NKG2D-dependent cytotoxicity, P815-ULBP1 and P815-ULBP1-CD45TM cells were used as targets in a 2-h lysis assay (Fig. 2). Expression of ULBP1 on P815 cells rendered them more sensitive to lysis by primary, resting NK cells (A) or IL-2-activated NK cells (B) was measured in a 2-h assay. Bars indicate SD of independent experiments with three NK cell donors. Degranulation of freshly isolated resting NK cells (C) or IL-2-activated NK cells (D) in response to P815, P815-ULBP1, and P815-ULBP1-CD45TM cells was measured in a 1-h degranulation assay with CD107a mAb. Each symbol represents an individual donor among three (C) or five (D), which were tested in independent experiments. Paired t test was performed. *p ≤ 0.01.

FIGURE 2. Exclusion of ULBP1 from the DRM fraction of target cells reduces the cytotoxicity and degranulation of NK cells. Susceptibility of P815 (open circles), P815-ULBP1 (black circles), and P815-ULBP1-CD45TM (gray circles) cells by freshly isolated resting (A) or IL-2-activated NK cells (B) was measured in a 2-h assay. Bars indicate SD of independent experiments with three NK cell donors. Degranulation of freshly isolated resting NK cells (C) or IL-2-activated NK cells (D) in response to P815, P815-ULBP1, and P815-ULBP1-CD45TM cells was measured in a 1-h degranulation assay with CD107a mAb. Each symbol represents an individual donor among three (C) or five (D), which were tested in independent experiments. Paired t test was performed. *p ≤ 0.01.

Clustering and lateral diffusion of ULBP1 and ULBP1-CD45TM in the plasma membrane are similar

We next tested how the segregation of ULBP1 into DRM and detergent-soluble domains affected the extent of ULBP1 clustering and lateral mobility within the plasma membrane. To investigate these parameters, we labeled ULBP1 and ULBP1-CD45TM with a PE-conjugated Ab to ULBP1, and their distribution and mobility were visualized by TIRF microscopy. TIRF microscopy is a spatially limited, high-contrast technique that eliminates interference from bulk fluorescence, which may be present within cells, and selectively detects fluorophores proximal to and within the plasma membrane of cells on glass coverslips. Both ULBP1 and ULBP1-CD45TM were distributed into small clusters at the surface of P815 cells (Fig. 3A). Although individual ULBP1 and ULBP1-CD45TM proteins were labeled with a single PE-fluorophore, photobleaching characteristics (the presence of multistep bleaching events over long track length, data not shown) of fluorescent PE-labeled particles suggested that ULBP1 and ULBP1-CD45TM were observed primarily as clusters and not single molecules. Cluster analysis by fluo-
rescence intensity measurements revealed no significant difference in ULBP1 and ULBP1-CD45TM clusters, with a median intensity of 1283 and 1204, respectively (Fig. 3B). Therefore, the distribution of ULBP1 in different membrane domains did not have a detectable impact on the number of ULBP1 molecules per cluster. Furthermore, the cluster intensity for both molecules was homogenous (Fig. 3B). We conclude that the difference in sensitivity to NKG2D-dependent cytotoxicity is not due to a change in the number of ULBP1 molecules per cluster.

The lateral movement of labeled ULBP1 and ULBP1-CD45TM particles recorded by TIRF microscopy was tracked automatically using an algorithm developed for MatLab software (19), which was further modified to refine particle positioning with a two-dimensional Gaussian fit (20). Short-range mean square displacements were determined from positional coordinates of particles tracked for five frames (over 160 ms) (20) and were linearly dependent on time under all conditions measured, consistent with a simple diffusion model for this range of movement. Short-range diffusion coefficients were then determined for thousands of particles in multiple cells and graphed either in cumulative probability plots (also known as cumulative distribution function) to represent the frequency of diffusion coefficients for the entire population of tracked particles (Fig. 3C), or median scattered plots (Supplemental Fig. 2A). Each one of several thousand particles is represented as a separate point in the cumulative probability plot. This type of graph can visually resolve small differences between samples even when extensive overlap occurs. ULBP1 clusters displayed a high lateral mobility at the surface of P815 cells, with a median diffusion coefficient of 0.122 μm²/s. Lateral mobility of ULBP1-CD45TM, with a median diffusion coefficient of 0.075 μm²/s, was reduced compared with ULBP1. Single-particle tracking experiments have shown that diffusion rate at the plasma membrane is reduced when proteins associate with lipid rafts (21) or with protein complexes (22). The distribution of ULBP1-CD45TM in the detergent-soluble membrane fraction may have resulted in intermolecular interactions that reduced ULBP1 mobility even further than the association of ULBP1 with DRM domains.

To test whether the lateral mobility of ULBP1 and ULBP1-CD45TM was controlled by the actin cytoskeleton, we tracked mobility on P815 cells treated with either DMSO carrier alone or 3 μM latrunculin A (Fig. 3D, Supplemental Fig. 2B). Whereas the mobility of ULBP1 did not change after treatment with latrunculin A, the mobility of ULBP1-CD45TM increased from a median diffusion coefficient of 0.065 μm²/s to a diffusion coefficient of 0.093 μm²/s, which was close to the mobility of ULBP1 (0.118 μm²/s) after treatment (Fig. 3D). A higher dose of latrunculin A (10 μM) did not increase the mobility of ULBP1-CD45TM any further and had no effect on the mobility of ULBP1 (data not shown).

Previous work from our group has shown that immobilization of ICAM on target cells, rather than its clustering, promotes proper LFA-1-dependent conjugate formation and granule polarization in primary NK cells (14). To test whether changes in lateral diffusion of ULBP1 were responsible for the difference in sensitivity to lysis by NK cells (Fig. 2), we took advantage of the similar lateral diffusion of ULBP1 and ULBP1-CD45TM after treatment with latrunculin A. If lateral diffusion of ULBP1 was the main determinant of the functional difference (i.e., greater ULBP1 mobility leading to increased NKG2D-dependent degranulation), treatment of the target cells with latrunculin A should equalize the response to P815-ULBP1 and P815-ULBP1-CD45TM cells. As seen earlier in the absence of DMSO (Fig. 2), in the presence of DMSO, ULBP1 induced significantly greater degranulation than ULBP1-CD45TM (Fig. 3E). Treatment of P815 cells with latrunculin A did not equalize the response induced by ULBP1 and ULBP1-CD45TM: degranulation induced by ULBP1 did not change, but that induced by ULBP1-CD45TM was reduced even further (Fig. 3E). Therefore, NKG2D-dependent cytotoxicity is controlled by the distribution of NKG2D ligands into separate membrane domains, independently of the number of ligand molecules per cluster, and of the lateral mobility of clusters in the plasma membrane.

The molecular basis for the change in NKG2D-dependent responses when ULBP1 is moved to a different membrane environment is still unknown. This change could be relevant, as the related NKG2D ligand ULBP2 is expressed as both a GPI-linked form and a transmembrane form (23). However, expression of the transmembrane form of ULBP2 on the NK-sensitive CHO cell line had a similar small enhancing effect on sensitivity to lysis by NK cells, as expression of both forms (23). Potential cis interactions of ULBP1 with cell surface proteins are different in the DRM domains than in the rest of the plasma membrane. The slower mobility of ULBP1-CD45TM, as compared with ULBP1, and the recovery to a similar lateral mobility after inhibition of F-actin suggest that ULBP1-CD45TM interacts with molecules tethered to the cytoskeleton. Receptor ligands on target cells may often exist within the context of larger protein complexes, and the role of these complexes in ligand recognition should be given greater consideration. The overall extent of basal ULBP1 clustering, before contact of target cells with NK cells, was virtually identical for ULBP1 and ULBP1-CD45TM. Nevertheless, despite a similar number of molecules per cluster, the molecular and biophysical properties of ULBP1 and ULBP1-CD45TM clusters may be different because of the unique properties of membrane subdomains. Such differences may change the interaction of receptor NKG2D with its ligands at immunological synapses, the organization of which plays an important role in lymphocyte responses (24). Distribution of ULBP1 either within or outside of DRM domains may also affect trogocytosis, a process by which cell surface proteins are transferred between target cells and lymphocytes (25–27). Whether intercellular transfer of a ligand for an activation receptor from a target cell to an effector cell leads to amplification of the response or to desensitization of the receptor is unclear. It would be interesting to investigate whether the distribution of ULBP1 in different membrane domains has an impact on its transfer to NK cells.

Our data suggest that ligand distribution into distinct membrane domains in general may play an underappreciated role in the activation of NK cells. Given the potential of tumor cells or virus-infected cells to alter ligand distribution at the plasma membrane and to escape immune responses, it will be important to investigate how the distribution of other ligands impacts the activation of lymphocytes.
Acknowledgments
We thank M. Vales-Gomez and H. Reyburn for advice and comments and
M. Peterson and M. March for discussion.

Disclosures
The authors have no financial conflicts of interest.

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Supplemental Legends

Supplemental Figure 1:  Design of a chimeric protein containing the extracellular portion of ULBP1 and the transmembrane region of CD45.  A, The ULBP1 signal for the GPI anchor was replaced by the transmembrane region of CD45. GPI-linked ULBP1 partitions into the detergent-resistant membrane (DRM) fraction, whereas CD45 is localized in the detergent-soluble membrane fraction.  B, Amino acid sequence of ULBP1 (top) and of the fusion with the CD45 transmembrane region (bottom). The highlighted Gly (G) in ULBP1 is the site of attachment of the GPI anchor. The highlighted Pro Arg (PR) linker is encoded by a SacII restriction site. To generate those constructs a full-length ULBP1 cDNA in the pRmHa3 Drosophila expression vector (5) was amplified with forward primer 5'-GAATTCGCCACCATGGCAGCGGCCGCCAGC and reverse primer 5'-AGATCTCATCTGCCAGCTAGAATGAAGC, cloned into the vector pCR2.1-TOPO, and verified by sequencing. The insert was excised with SpeI and XbaI and cloned into the XbaI site of expression vector RSV.5neo, GenBank M83237. To fuse the extracellular portion of ULBP1 to the transmembrane region of CD45 the ULBP1 cDNA was amplified with the same forward primer and the reverse primer 5' - CGCGGGGCCTGGGGCCAGAGGGTG, which included a SacII restriction site at the 3' end. The coding sequence of the CD45 transmembrane region (CD45TM) and a few flanking amino acids was synthesized as two complementary oligonucleotides (IDT, Coralville, IA) and cloned into SacII and AgeI digested vector pcDNA6/V5-His Version B (Invitrogen, Carlsbad, CA). It was then digested with SacII and EcoRI and ligated to the cDNA for the extracellular region of ULBP1. The ULBP1-CD45TM fusion sequence was verified by sequencing, shuttled through pBluescript KS+ to gain XhoI and XbaI sites, and cloned into the SalI-XbaI sites of RSV.5neo.

Supplemental Figure 2:  Median scattered plots of ULBP1 and ULBP1-CD45TM diffusion coefficients.  A, The movement of ULBP1 and ULBP1-CD45TM particles labeled with PE-conjugated Ab to ULBP1 was tracked by capturing TIRF images at 35 frames per second for 100 frames. Diffusion coefficients of ULBP1 (blue) and ULBP1-CD45TM (green) particles are shown in median scattered plots. Plots represent one representative experiment out of 7. B, Movement of DMSO-treated P815-ULBP1 (blue) and P815-ULBP1-CD45TM (green), and Latrunculin A-treated P815-ULBP1 (red) and P815-ULBP1-CD45TM (aqua) cells was tracked by TIRF as described above. The plot represents 1 out of 8 independent experiments. Two-tailed Mann-Whitney Test was performed. (*** indicates a p value {less than or equal to} 0.0001.
A

μm²/sec

ULBP1

ULBP1-CD45TM

***

B

μm²/sec

ULBP1+DMSO

ULBP1+LatA

ULBP1-CD45TM+DMSO

ULBP1-CD45TM+LatA

ns

***