Ligands for natural killer cell–activating receptors are expressed upon the maturation of normal myelomonocytic cells but at low levels in acute myeloid leukemias

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Natural killer (NK) cell–mediated cytolytic activity against tumors requires the engagement of activating NK receptors by the tumor-associated ligands. Here, we have studied the role of NKG2D and natural cytotoxicity receptors (NCRs) in the recognition of human leukemia. To detect as-yet-unknown cell-surface molecules recognized by NCRs, we developed soluble forms of NKp30, NKp44, and NKp46 as staining reagents binding the putative cognate ligands. Analysis of UL16-binding protein-1 (ULBP1), ULBP2, and ULBP3 ligands for NKG2D and of potential ligands for NKp30, NKp44, and NKp46 in healthy hematopoietic cells demonstrated the ligand-negative phenotype of bone marrow–derived CD34$^+$ progenitor cells and the acquisition of cell-surface ligands during the course of myeloid differentiation. In acute myeloid leukemia (AML), leukemic blasts from approximately 80% of patients expressed very low levels of ULBPs and NCR-specific ligands. Treatment with differentiation-promoting myeloid growth factors, together with interferon-γ, up-regulated cell-surface levels of ULBP1 and putative NCR ligands on AML blasts, conferring an increased sensitivity to NK cell–mediated lysis. We conclude that the ligand-negative/low phenotype in AML is a consequence of cell maturation arrest on malignant transformation and that defective expression of ligands for the activating NKG2D and NCR receptors may compromise leukemia recognition by NK cells. (Blood. 2005;105:3615-3622)

Introduction

Natural killer (NK) cells are important effectors of the innate immune system involved in the clearance of virus-infected and tumor cells.[1] The activity of NK cells is regulated by receptors with opposing functions, triggering either inhibitory or stimulatory pathways.[2] Killer inhibitory immunoglobulin-like receptors (KIRs) recognize epitopes shared by groups of human leukocyte antigen (HLA) class 1 molecules, and adequate expression of appropriate KIR ligands protects healthy "self" cells against NK cell reactivity.[3] In the absence of this inhibitory pathway, targets become susceptible to NK-mediated lysis. The stimulatory pathway is dependent on engagement of activating receptors, NKG2D, and natural cytotoxicity receptors (NCRs), which transduce signals initiated by the triggering ligands present on infected or transformed cells.[4,5]

NKG2D is expressed by NK cells and by T cell receptor (TCR) $\alpha\beta^+CD8^+$ and TCR $\gamma\delta^+$ subsets of T cells.[6,7] It recognizes several cell-surface ligands belonging to major histocompatibility complex class I chain–related (MIC) and UL16-binding protein (ULBP) protein families in humans[8,9] and H60 and Rae1 in mice.[10-12] MICA and MICB are generally either absent or present at low levels on normal cells, but their expression increases in response to various forms of cellular stress. Up-regulation of MICA and MICB on many human primary tumors of epithelial origin indicates that these ligands mark tumor cells for immune rejection.[13] The ULBP family of NKG2D ligands was identified by the ligands' ability to bind the cytomegalovirus (CMV) glycoprotein UL16.[9,14] Engagement of NKG2D by ULBPs induces NK cell cytotoxicity against tumor targets.[15] The expression patterns of ULBP and MIC molecules in tumor cell lines differ,[9,16] likely reflecting nonredundant functions of these NKG2D ligands.

NCRs, which include NKp30, NKp44, and NKp46, are almost exclusively expressed by NK cells.[17] They function as the main activating receptors, and NCR density and NK-mediated cytolytic activity against tumor cells are correlated.[18] In virus-infected cells, viral hemagglutinins are recognized by NKp44 and NKp46.[19,20] However, no endogenous cellular NCR ligands have been identified as yet. Cross-linking of NCRs induces coupling of the signal-transducing adaptor molecules DAP12, CD3ζ, and FcεRIγ, whereas NKG2D in human cells associates with DAP10.[21,22] Use of distinct signaling components allows NKG2D to cooperate with NCRs, in particular on NCR^{dull} NK cells, for recognition and lysis of tumor cells.[23,24]

Because the susceptibility of tumors to NK-mediated lysis relies on a balance between inhibitory and triggering signals, through changes in the expression pattern and levels of receptors and their ligands, tumors can evade immune surveillance by NK cells.

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Submitted July 13, 2004; accepted December 22, 2004. Prepublished online as *Blood* First Edition Paper, January 18, 2005; DOI 10.1182/blood-2004-07-2585.

Supported by grants from the Swiss National Science Foundation (4046-058689 and 3100-067072.01) and from Basler Cancer League (7/2003).

The online version of this article contains a data supplement.

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Human gastrointestinal tumors and leukemic blasts shed the MIC ligands, which accumulate in serum, causing the down-regulation of NKG2D.[25-27] Low levels of NCRs have been described in acute myelogenous leukemia (AML), and the insufficiency of NCR-ligand interactions has been hypothesized as the underlying cause of the low susceptibility of leukemic blasts to lysis by autologous NK cells.[28] However, to date little is known regarding the role of various activating ligands in human leukemia.

The aim of the present study was to analyze the pattern and regulation of ULBP and NCR ligand expression in peripheral blood (PB) and bone marrow (BM) cells from healthy donors and from patients with AML. To identify the still-unknown NCR ligands and to study their cell-surface expression, we have developed recombinant soluble NKp30, NKp44, and NKp46 receptor dimers. The results demonstrate that leukemic blasts in most patients with AML are characterized by ULBP and NCR phenotypes negative for or low in ligand as a consequence of maturation arrest during the differentiation of myelomonocytic cell lineages. Low levels of triggering ligands may impair the clearance of leukemia by NK and T cells bearing the activating NKG2D and NCR receptors.

Patients, materials, and methods

Healthy donors and patients

Healthy donors of PB (n = 22) and BM (n = 7) and patients with AML (n = 30) were enrolled in the study. Twenty-four patients had newly diagnosed, untreated AML, and 6 patients had relapses of the disease. The diagnosis and definition of AML subtypes M1 to M7 were based on morphologic, cytogenetic, and immunophenotypic criteria. The average blast content in PB was 57.5% plus or minus 4.9%. All control and clinical samples were obtained with informed consent, in compliance with the guidelines of the Ethical Committee of the University Hospitals of Basel (Switzerland).

Generation of soluble NCR dimers

To generate constructs encoding recombinant soluble (s) NCR receptors sNKp30, sNKp44, and sNKp46, polymerase chain reaction (PCR) primers were designed to amplify from RNA of a human activated NK cell line the truncated cDNA coding for the predicted extracellular region of each receptor[29-31] (Document S1, available on the *Blood* website; see the Supplemental Document link at the top of the online article). Amplified sequences were cloned into the plasmid vector pBluescript-Bir containing at the 3′ end of the cloning site an in-frame tag sequence encoding a BirA peptide recognized by the BirA1.4 monoclonal antibody (mAb) developed in our laboratory (G.D.L., unpublished data, February 2000). The tagged cDNAs were subcloned in the eukaryotic expression vector BCMGS-Neo[32] and transfected into J558 mouse myeloma cells by electroporation, and cells expressing the highest levels of recombinant protein were selected by intracellular staining and by Western blotting of culture supernatants using the tag-specific mAb.[33] sNCR dimeric complexes with the BirA peptide–specific mAb were purified by affinity chromatography on a Sepharose–protein G column with immobilized BirA1.4 mAb. The complexes were used as staining reagents in flow cytometry (see "Flow cytometry").

Flow cytometry

Fluorescence-activated cell sorter (FACS) staining was performed in 100 μL aliquots of fresh heparinized PB using fluorescein isothiocyanate (FITC)–, phycoerythrin (PE)–, allophycocyanin (APC)–, or peridin chlorophyll protein (PerCP)–conjugated mAbs against human CD3, CD14, CD19, CD33, CD34, CD45, or CD56 or appropriate isotype-matched control mAbs, followed by lysis of red blood cells with FACS lysis buffer (all from Becton Dickinson, San Jose, CA). For the analysis of erythrocytes, PB was diluted 1:1000 in phosphate-buffered saline (PBS), and 100-μL aliquots were stained with PE-conjugated mAb against human glycophorin-A (Becton Dickinson) without lysis. For the analysis of platelets, PB was centrifuged for 10 minutes at 85*g*. The supernatant was collected, platelets were pelleted by centrifugation for 10 minutes at 680*g*, and 100-μL aliquots were stained with FITC-conjugated mAb against human CD61 (Becton Dickinson) without lysis. For the analysis of granulocytes, cells were gated according to side and forward scatter. To analyze BM, mononuclear cells (MNCs) were obtained by Histopaque (less than 1.077 g/cm^3; Sigma, St Louis, MO) density gradient centrifugation. To measure ULBPs, staining was performed with unlabeled mAbs against ULBP1 (M295; immunoglobulin G1 [IgG1]; 10 μg/mL), ULBP2 (M311; IgG1; 20 μg/mL), and ULBP3 (M550; IgG1; 10 μg/mL),[9] followed by staining with FITC-conjugated goat anti–mouse IgG (Jackson ImmunoResearch, West Grove, PA). After blocking with mouse serum, cells were additionally stained with directly labeled lineage-specific mAbs. To measure putative NCR ligands, dimeric complexes of sNKp30, sNKp44, and sNKp46 were used as staining reagents (5-10 μg/mL) and control anti-BirA1.4 mAb, and the binding was revealed using FITC-conjugated goat anti–mouse IgG. ULBP and putative NCR ligand expression was quantified as the mean fluorescence intensity (MFI) ratio of values obtained with specific mAbs divided by values given by secondary goat anti–mouse IgG. Control stainings with isotype control mAbs—mouse IgG1, mouse anti–human CD80, mouse anti–human CD83, and mouse anti–human CD56 (all IgG1 from Becton Dickinson)—showed MFI ratios of 1.0 to 1.2. At least 100 000 events were acquired using FACSCalibur, and analysis was performed using CellQuest software (both from Becton Dickinson).

Cell culture and stimulation

CD34$^+$ cells were isolated from BM using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD34$^+$ cells (0.5 to 1 × 10^6) were cultured in 6-well plates containing 3 mL Iscove modified Dulbecco medium (IMDM) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum (FCS; all from Invitrogen, Carlsbad, CA) in the presence of flt3 ligand (FL) at 100 ng/mL, stem cell factor (SCF) at 100 ng/mL, and granulocyte-macrophage colony-stimulating factor (GM-CSF) at 20 ng/mL (all kind gifts of Amgen Inc, Thousand Oaks, CA) for 7 days. Interferon-γ (IFN-γ) at 100 U/mL (PeproTech, London, United Kingdom), interleukin-1β (IL-1β) at 20 U/mL (kind gift of Novartis, Basel, Switzerland), and lipopolysaccharide (LPS) at 10 ng/mL (*Salmonella abortus equi* NG420; kind gift of R. Landmann, Basel, Switzerland) were added for an additional 3 days, as indicated. AML MNCs containing more than 90% blasts were cultured with FL, SCF, and GM-CSF, without or with IFN-γ, as described earlier in this paragraph, for 4 days. Myeloid differentiation was determined by FACS using mAbs against CD34 and CD33. PB monocytes were purified using CD14 magnetic microbeads (Miltenyi Biotec) and were cultured at 1 × 10^6 cells/mL in IMDM/10% FCS in the presence of FL, SCF, and GM-CSF without or with IFN-γ for 3 days, followed by the FACS analysis of ULBP expression.

Cytotoxicity and IFN-γ release assays

To analyze the killing of AML blasts, a standard chromium release assay was used. Target cells (2 × 10^6) were labeled with 9.25 MBq $Na_2^{51}CrO_4$ (Amersham, Little Chalfont, United Kingdom) for 2 hours at 37°C. NK effector cells were purified from normal PB by an immunomagnetic NK-negative selection kit (Miltenyi Biotec) and were stimulated with IL-2 (100 U/mL; kind gift of Novartis) and phytohemagglutinin (1 μg/mL; Murex Biotech, Dartford, United Kingdom) in the presence of irradiated allogeneic MNCs.[34] In blocking experiments, target cells were preincubated with 10 μg/mL anti–major histocompatibility complex (anti-MHC) class 1 mAb (W6/32; American Type Culture Collection, Manassas, VA) for 10 minutes before assay. Target and effector cells were incubated for 4 hours at 37°C. Maximum chromium Cr 51 (^{51}Cr) release was determined with target cells lysed in 1% Triton-X. Percentage of cytotoxicity was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Experiments were performed in triplicate.

A calcein-acetyoxymethyl (calcein-AM)–based assay was used to determine the NK cell cytotoxicity against THP-1 cells and K562-L cells

subcloned in our laboratory. Target cells (2×10^6) were labeled in 1 mL serum-free M199 medium (Invitrogen) with 10 μM calcein-AM (Calbiochem, La Jolla, CA) for 30 minutes at 37°C. The cytotoxicity test was performed in triplicate; spontaneous and maximal calcein release were determined with 6 wells. In blocking experiments, targets were preincubated with a mixture of sNKp30, sNKp44 and, sNKp46, as indicated. Calcein-AM release was measured using a Spectramax Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA) (excitation 485 nm, emission 530 nm). Percentage of lysis was calculated with the same formula as that used for the ^{51}Cr assay.

To analyze the release of IFN-γ, 5×10^4 purified NK cells were cocultured with THP-1 cells at the effector-target ratio of 1:1 for 42 hours in the presence of IL-2 (100 U/mL). IFN-γ was measured in supernatants using enzyme-linked immunosorbent assay (ELISA) reagents (kind gift of C. Heusser, Novartis). In blocking experiments, anti-NKG2D mAb, sNKp30, sNKp44, sNKp46, or anti-BirA1.4 mAb was added, as indicated.

Real-time reverse transcription–PCR (RT-PCR)

PB-derived $CD19^+$ B cells, $CD3^+$ T cells, and $CD14^+$ monocytes were purified by FACS sorting (more than 98% purity). Total RNA (2 μg) was isolated using Trizol, and reverse transcription was performed with SuperScript II (both from Invitrogen). The cDNA was amplified in duplicate with primers for ULBP1 (Hs00360941_m1), ULBP2 (Hs00607609_mH), and ULBP3 (Hs00225909_m1) and control primers for hypoxanthine phosphoribosyl transferase (HPRT) (Hs00355752_m1; all from Applied Biosystems, Foster City, CA). Amplification (40 cycles; 95°C for 15 seconds, 60°C for 1 minute) was monitored using the TaqMan MGB probe labeled with 6-FAM dye and nonfluorescent quencher on the ABI Prism 7000 Sequence Detection System.

Statistical analysis

The nonparametric Mann-Whitney *U* test was used to evaluate the statistical differences between ULBP and NCR ligand level expression by leukemic blasts and residual healthy cells in AML patients.

Results

ULBPs are expressed by B lymphocytes and myeloid subpopulations of PB cells

ULBP transcripts were found in normal PB and BM cells,[9,35] but expression of ULBP by these cells had not been detected. We analyzed cell-surface ULBP1, ULBP2, and ULBP3 in subpopulations of PB cells from healthy donors (Figure 1A; Table 1). All 3 ligands were highly expressed by B cells but were not detectable on T and NK cells. Among myeloid lineages, erythrocytes were negative; platelets expressed ULBP2 at MFI ratios up to 9.6 and low levels of ULBP1 and ULBP3. On monocytes and granulocytes, ligand levels were highly variable among several tested healthy donors. ULBPs were not detectable or were detectable at very low levels (MFI ratios less than 2.0) in monocytes from 8 of 22 donors and in granulocytes from 10 of 18 donors, whereas they were well pronounced in other donors. ULBP1 was always present at the highest levels, with MFI ratios up to 9.5, and ULBP2 was always present at the lowest levels, with MFI ratios not more than 5.2 (Table 1). To explain this variability in ULBP expression among cell populations from different donors, ULBP-negative $CD14^+$ monocytes were purified and stimulated with the activators of monocyte functions. IFN-α, IL-1β, tumor necrosis factor (TNF)–α, and LPS used singly or in combination were not effective for any of the ULBPs (data not shown). There was, however, a significant selective up-regulation of ULBP1 in response to the myeloid growth factors (FL, SCF, GM-CSF) and a further increase by IFN-γ (Figure 1A), indicating that the ULBP expression level measured on freshly isolated cells might have reflected the cell activation state in vivo. Monocyte-derived dendritic cells, both immature and mature, were negative for all 3 ULBPs (data not shown). These experiments showed that ULBP molecules are expressed on the surfaces of B cells, platelets, monocytes, and granulocytes from most donors. Apart from platelets, which express the highest levels of ULBP2, the ligand that predominates in normal PB is ULBP1.

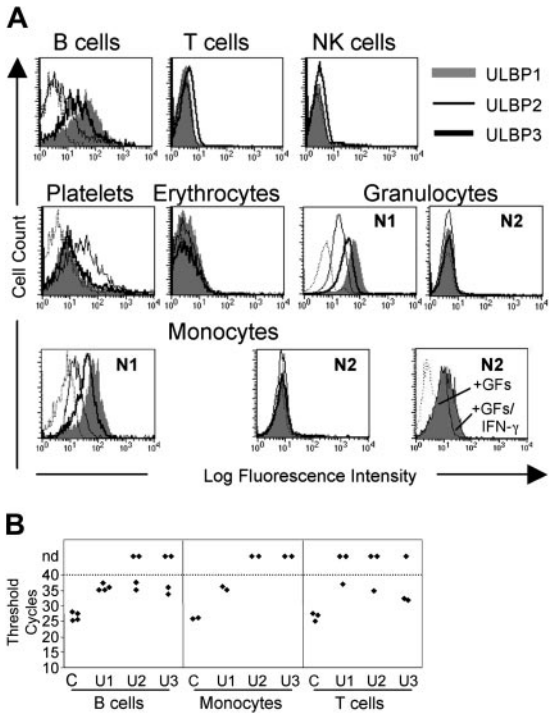

Figure 1. ULBP molecules are expressed by PB cell subpopulations of healthy donors. (A) FACS analysis of PB cells from healthy donors after staining with mAbs for ULBP1 (shaded curve), ULBP2 (thin solid line), or ULBP3 (bold solid line) or with secondary FITC-labeled goat anti–mouse IgG alone (dotted line). PB cell subpopulations, indicated above each histogram, were distinguished with mAbs specific for lineage markers. Analyses of monocytes and granulocytes in 2 healthy donors with ULBP-positive (N1) and ULBP-negative (N2) phenotypes are shown. Also shown is the expression of ULBP1 by N2 monocytes stimulated with growth factors (GFs; FL, SCF, GM-CSF) or GFs with IFN-γ. (B) Real-time RT-PCR analysis of ULBP1 (U1), ULBP2 (U2), and ULBP3 (U3) mRNA levels in B cells, monocytes, and T cells purified from 2 to 4 donors, as indicated by the number of diamonds. Dotted line marks the upper limit of 40 amplification cycles. Threshold cycles indicates number of cycles at which the amount of PCR product passed the threshold of detection; C, control mRNA of HPRT; nd, not detectable.

Quantitative real-time RT-PCR demonstrated that ULBP mRNAs in PB cells are generally of low abundance (Figure 1B). ULBP1 transcripts could be detected in B cells and monocytes of all donors, with 9.3 ± 0.9 and 9.8 ± 0.7 more amplification cycles than control HPRT transcripts. ULBP2 and ULBP3 transcripts were detectable in B cells from some donors but were below the detection limit of 40 PCR cycles in monocytes. Some T cell preparations, although phenotypically always ligand negative, expressed ULBP mRNAs. These data indicate that the expression of ULBP mRNA does not always predict cell-surface ligand levels in PB cell subpopulations, as has also been described with hematopoietic cell lines.[9]

AML blasts express low levels of ULBPs and NCR ligands

Our next goal was to compare the expression of ULBPs and NCR ligands by normal myeloid cells and AML blasts. Given that no

Table 1. Expression of ULBP proteins by PB cell subpopulations

Subpopulation	Mean MFI ratios ± SEM (range)		
	ULBP1	ULBP2	ULBP3
B cells	21.7 ± 2.8 (7.3-57.6)	10.4 ± 1.7 (4.3-23.5)	17.2 ± 4.3 (6.3-90.6)
Monocytes	3.4 ± 0.5 (1.1-9.0)	2.1 ± 0.3 (1.1-5.2)	2.4 ± 0.3 (1.0-6.1)
Granulocytes	2.8 ± 0.6 (1.0-9.5)	1.9 ± 0.3 (1.0-3.8)	2.1 ± 0.3 (1.0-5.2)
Platelets	2.0 ± 0.2 (1.8-2.5)	7.8 ± 1.6 (4.7-9.6)	3.7 ± 0.2 (3.4-4.1)

Analysis was performed with PB samples from 3 healthy donors for platelets and from 10 to 22 healthy donors for all other cell types.

information is available to date about the ligands of NCRs, we generated dimers of recombinant proteins corresponding to extracellular domains of NKp30, NKp44, and NKp46 and used them in the binding studies. To examine the specificity of these reagents, the dimers were first used to stain THP-1 and K562-L cell lines (Figure 2A). Complexes of sNKp30, sNKp44, and sNKp46 bound to THP-1 cells (MFI ratios of 3.0-6.5) but not to K562-L cells. Next, we examined the ability of dimers to interfere with the recognition and killing of THP-1 and K562-L cells by NK cells (Figure 2B). The sNCR cocktail strongly inhibited the killing of THP-1 expressing high levels of putative NCR ligands, whereas it was not effective with K562-L.

The specificity of ligand recognition by the recombinant dimers was further confirmed by measuring the IFN-γ release by NK cells cocultured with THP-1 cells (Figure 2C). sNKp30, binding to a putative NCR ligand with the highest level of expression on THP-1 cells, strongly inhibited the release of IFN-γ in synergy with antibodies blocking the NKG2D receptor and with sNKp44 and sNKp46 dimers, whereas the control α-BirA1.4 mAb had no effect on the efficient production of IFN-γ by activated cells.

To examine ULBP and NCR ligand expression in AML, PB cells from 30 AML patients were analyzed according to side and forward scatter and staining with mAb against CD45. ULBPs and NCR ligands were found at varying levels on the monocytes and granulocytes of patients with CD45^{bright} (Figure 3), resembling the variability of ULBP cell-surface density observed with healthy donors (Table 1). Although the ULBP-low phenotype of CD45^{bright} cells was observed in only 6 of 30 patients, ULBP expression by CD45^{dim} blasts was very low, with a MFI ratio of less than 2.0 in as many as 23 patients in this cohort (Figure 3). In addition, in 3 of the remaining 7 patients, only 6% to 37% of all blasts were ULBP-positive. We also found an MIC-negative/low phenotype in 6 of 9 analyzed patients, although antibodies used in our study did not permit the distinction between MICA and MICB ligands (data not shown). As with ULBPs, CD45^{dim} AML blasts displayed very low sNKp30, sNKp44, and sNKp46 dimer-binding capacity (MFI ratio of less than 2.0) in 10 of 12 analyzed patients. These results demonstrated that AML blasts in most patients displayed negative/low surface levels of ligands for NKG2D and NCRs. Analysis of

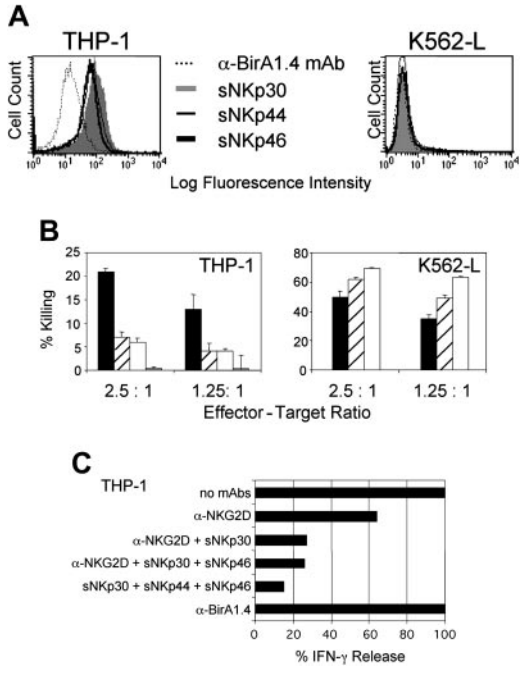

Figure 2. Soluble NCR dimers recognize THP-1 cells and inhibit their killing and IFN-γ release by NK cells. (A) FACS analysis of THP-1 and K562-L cells stained with sNKp30 (shaded curve), sNKp44 (thin solid line), and sNKp46 (bold solid line) or anti-BirA1.4 mAb (dotted line) and with secondary FITC-labeled goat anti–mouse IgG. (B) Inhibition of NK cell–mediated killing of THP-1 cells by sNKp30, sNKp44, and sNKp46 dimers. A calcein release–based cytotoxicity assay was used to determine the lysis of THP-1 and K562-L cells by NK cells at the indicated effector-target ratios. The killing assay was performed in triplicate in the absence of sNCRs (■) and in the presence of a mixture of sNKp30, sNKp44, and sNKp46 at 5 μg/mL (▨), 20 μg/mL (□), or 50 μg/mL (▤) of each dimer. Mean ± SEM values are shown. (C) Inhibition of IFN-γ release by NK cells cocultured with THP-1 cells, using anti-NKG2D mAb (20 μg/mL) and sNKp30, sNKp44, and sNKp46 (10 μg/mL, each) or anti-BirA1.4 mAb (10 μg/mL) in the indicated combinations. In the absence of blocking reagents (no mAbs), 100% IFN-γ release corresponded to 8.67 ± 3.5 ng/mL, as determined in 3 experiments.

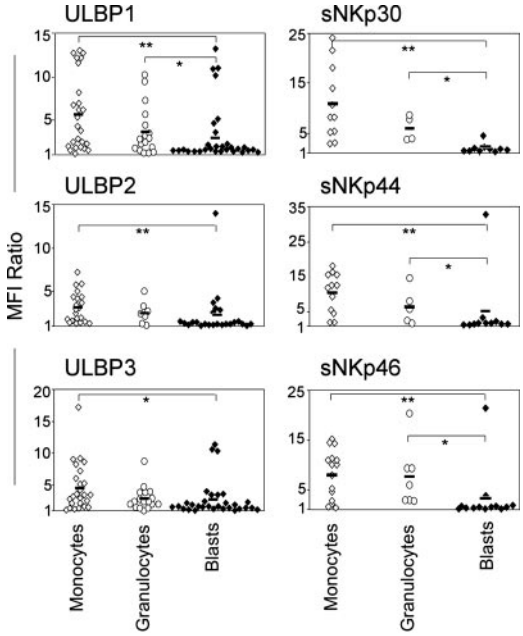

Figure 3. Low ULBP and NCR ligand expression in AML. PB samples from AML patients were stained with mAbs against lineage-specific markers and anti-CD45 mAbs to distinguish CD45^{dim} leukemic blasts from CD45^{bright} residual normal monocytes and granulocytes. ULBP1, ULBP2, and ULBP3 (left column) were measured with specific mAbs on monocytes (◇), granulocytes (○) and blasts (◆). Binding of sNKp30, sNKp44, and sNKp46 dimers (right column) to monocytes (◇), granulocytes (○), and blasts (◆). Mean values are indicated as horizontal bars. $^*P < .05$; $^{**}P < .005$.

ligand expression in terms of AML subtype revealed that 5 of 6 patients with monocytic M5 and 1 of 2 patients with M7 had ULBP-positive blasts. Among other subtypes, only 1 patient with M1 had ULBP-positive blasts, which, however, constituted only 20% of his blast population (not shown). Although investigation with a larger group of patients is needed before a conclusion can be made about the association between NKG2D ligand expression and the subtype of myeloid leukemia, these findings suggest that ligand expression by myeloid blasts is more frequent when leukemic transformation takes place at later stages of differentiation.

ULBP1 and NCR ligands are up-regulated on myelomonocytic differentiation of BM progenitors

To investigate further whether ligand levels may be related to the differentiation stage of myeloid cells, we examined ULBP and NCR ligand expression by normal BM $CD34^+$ hematopoietic progenitors and precursors committed to the myeloid lineages (Figure 4A-B). Early $CD34^+CD33^-$ progenitors and $CD34^+CD33^+$ cells displayed very low surface levels of all ULBPs and NCR-specific ligands (MFI ratio of less than 2.0). $CD34^-$ myeloid cells—both $CD33^+CD14^-$ and mature $CD33^+CD14^+$ monocytes present in the BM—expressed the ULBPs, with a strong prevalence of ULBP1 compared with ULBP2 and ULBP3, and displayed all the NCR ligands on their surfaces. These results demonstrate that cell-surface ULBPs and NCR ligands become expressed on the loss of the early hematopoietic marker CD34 and on the acquisition of the myeloid markers CD33 and CD14.

To confirm that ligand expression is up-regulated on myelomonocytic differentiation, we purified BM-derived $CD34^+$ cells and cultured them in the presence of myeloid growth and differentiation factors, SCF, FL, and GM-CSF. After 10 days, cultures consisted of approximately 90% $CD34^-CD33^+$ cells. Myeloid cells generated in vitro from the ligand-negative hematopoietic progenitors expressed ULBP1 and NCR ligands (Figure 5). The surface expression of these ligands was further up-regulated with the addition of IFN-γ, also used in combination with LPS and IL-1β. Expression of ULBP2 and ULBP3 was not enhanced under these conditions. These results show that ULBP1 and ligands for NKp30, NKp44, and NKp46 are induced by myeloid growth and differentiation factors and in response to IFN-γ, whereas signals up-regulating the expression of ULBP2 and ULBP3 on $CD33^+$ cells remain unknown.

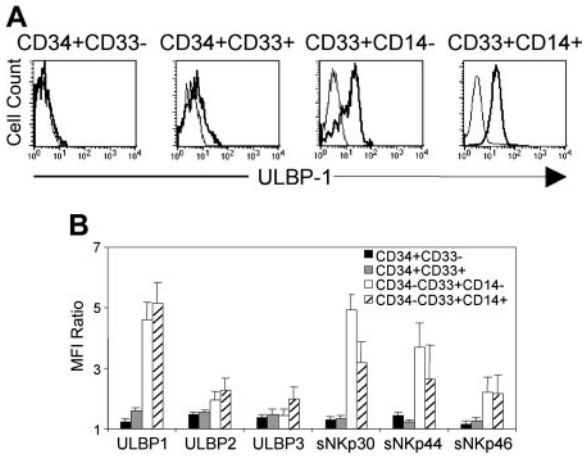

Figure 4. ULBP and NCR ligand expression is low on $CD34^+$ cells and well pronounced on myeloid progenitors in normal BM. ULBP expression was determined by FACS in the indicated BM subpopulations. (A) BM staining with ULBP1-specific mAbs (bold line) or control secondary FITC-labeled goat anti–mouse IgG alone (thin line). (B) BM staining (from 3 healthy donors) with mAbs to ULBP1, ULBP2, ULBP3 and to sNKp30, sNKp44, and sNKp46 dimers. ■ indicates $CD34^+CD33^-$; ▨, $CD34^+CD33^+$; □, $CD34^-CD33^+CD14^-$; and ▨, $CD34^-CD33^+CD14^+$ cell populations.

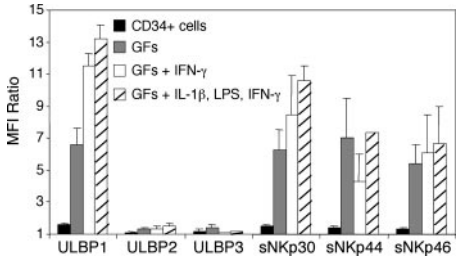

Figure 5. ULBP and NCR ligand surface expression increases on myeloid differentiation of normal BM progenitor cells in vitro. FACS analysis of ULBP1, ULBP2, and ULBP3 surface expression and sNKp30, sNKp44, and sNKp46 binding by freshly isolated $CD34^+$ cells (■) and after 7 days of culture with growth factors (GFs; SCF + FL + GM-CSF; ▨), GFs together with IFN-γ (□) or together with IL-1β, LPS, and IFN-γ (▨) for an additional 3 days. Results represent the mean ± SEM of 3 BM samples.

Up-regulation of NKG2D and NCR ligands on AML blasts in vitro

Next, we investigated whether FL, SCF, GM-CSF, and IFN-γ can up-regulate the triggering ligands on AML blasts in vitro. After 4 days in culture, 70% to 90% of $CD14^-$ blasts acquired the CD14 surface marker characteristic of monocytic differentiation (data not shown). ULBP1 and NCR ligand expression were not increased on blasts from patient A but were consistently higher from patients B and C with ligand levels that were initially low or high (Figure 6A). As already found with normal cells, ULBP2 and ULBP3 remained unchanged on leukemic blasts. Next, we tested whether an increase in ULBP1 and NCR ligand levels raises the susceptibility of AML blasts to NK cell–mediated cytolysis (Figure 6B). Killing rates of

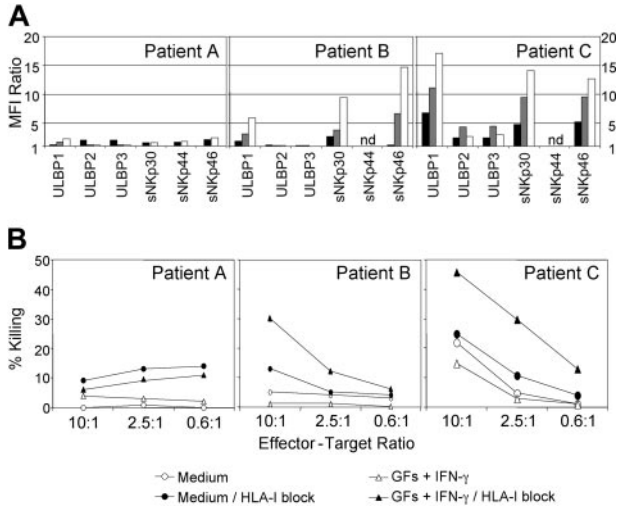

Figure 6. Up-regulation of ULBP and NCR ligands increases the susceptibility of AML blasts to NK cell–mediated cytotoxicity. (A) Up-regulation of ULBP and NCR ligands on AML blasts from 3 patients, as determined by FACS analysis of blasts cultured for 4 days in medium only (■), with GFs (FL + SCF + GM-CSF; ▨) or GFs + IFN-γ (□). (B) Killing of blasts cultured for 4 days without (medium; circles) or with GFs + IFN-γ (triangles) was determined without (open symbols) or with (closed symbols) blocking of HLA class 1 molecules with mAb (10 μg/mL), as indicated. NK cells derived from healthy donors were used at the indicated effector-target ratios. The HLA status of donor and target cells revealed the KIR ligand incompatibility in patient A and at least 1 matching HLA allele in patients B and C (Cw1/Cw3 and Cw3/Cw7, respectively).

blasts from leukemic patients with ligand-negative and ligand-positive blasts were 5% and 20%, respectively. The sensitivity to killing was decreased after treatment with FL, SCF, GM-CSF, and IFN-γ, most likely because of a protective effect of HLA class 1 molecules that were up-regulated 2- to 3-fold in response to IFN-γ (data not shown). However, when blasts were preincubated with antibodies preventing interactions of HLA class 1 ligands with the inhibitory receptors, the killing of blasts treated with growth factors and IFN-γ was markedly enhanced to approximately 30% and 45%, respectively, in the 2 responding patients. In the group of 5 patients included in such analysis, the killing response was variable, but the increase was consistent, ranging from 1.5- to 6-fold. These results indicate that enhanced levels of ligands for the activating receptors NKG2D and NCR can increase the recognition of AML blasts by NK cells.

Discussion

Rapid disease progression in acute leukemia and the high incidence of relapses after treatment with high-dose chemotherapy or transplantation of allogeneic hematopoietic stem cells[36,37] suggest that leukemic blasts can escape recognition by the immune system. To explain the poor immunogenicity of malignant blood cells, we examined the expression of ligands for NK cell–activating receptors in human AML. We found that leukemic transformation is frequently associated with an absence or with low cell-surface density of ligands for NKG2D and NCRs, which may render the blasts insensitive to recognition and killing by NK cells.

Staining of cells with mAbs specific for ULBP1, ULBP2, and ULBP3 and binding of soluble NCRs that reflects the distribution of putative NKp30, NKp44, and NKp46 ligands demonstrated that ligand levels on leukemic blasts were very low in most patients with AML. ULBP levels were not detectable or were low (MFI ratios of 1.0-2.0) in 23 (77%) of 30 patients, as were NCR ligands in 10 (83%) of 12 patients. This confirms and extends findings in a recent report of the ULBP- or MICA/B-negative/low primary leukemic blasts in 12 or 9 of 15 AML patients.[27] A low density of ligands triggering the NKG2D and NCR receptors in AML, together with the previously reported prevalence of the NCR^{dull} phenotype of NK cells in AML,[28] implies that interactions between activating receptors and their ligands may be insufficient to elicit strong cytolytic responses against the leukemic blasts.

Unlike leukemic cells, the residual monocytes and granulocytes in most patients with AML expressed ULBPs and showed binding of soluble NCRs. Accordingly, we found cell-surface ULBPs on subpopulations of PB cells from healthy donors, including B cells, platelets, monocytes, and granulocytes, whereas the ligands were absent on T cells, NK cells, dendritic cells, and erythrocytes. Cell-surface expression of these ligands by normal PB cells has not been documented thus far, though ULBP transcripts have been detected in these cells.[9,35] Using real-time RT-PCR we detected ULBP1, ULBP2, and ULBP3 transcripts in B cells, monocytes, and T cells, but their levels were low and did not fully correlate with the cell-surface density of individual proteins. A discordance in expression of ULBPs at mRNA and protein levels was also seen with tumor cell lines,[9] suggesting that ULBP expression is regulated at a level other than transcription. The function of ULBPs and NCR ligands on normal resting blood cell populations is not known. Their presence is unlikely to confer susceptibility to attack by autologous NK cells because normal PB cells are protected from cytolysis by the expression of HLA class 1 molecules specific for the inhibitory KIR receptors. Previous studies have demonstrated that NK cells can interact with B cells and monocytes, causing the proliferation and production of cell type–specific cytokines.[38-41] Adhesion molecules such as CD11a and CD54, as well as CD40 and CD40 ligand, were implicated in these interactions, and it remains to be seen whether ULBPs and NCR ligands expressed by normal blood cells function as costimulatory molecules for cells bearing cognate activating receptors.

Levels of NKG2D receptors can be up-regulated by the exposure of NK cells to IL-15,[14,42] whereas NCR levels are stable in culture and are not influenced by cytokines.[18] Expression of the NKG2D ligands MICA and MICB increases in response to various forms of cellular stress, including diseases such as cancer and rheumatoid arthritis.[13,43] Heat-shock transcription elements present in the promoter regions of their genes[44] are thought to be responsible for the induction of MIC molecules but have not been implicated in regulating the expression of ULBPs. Except for the up-regulation of some of the NKG2D ligands in CMV-infected cells,[45,46] signals regulating the expression of ULBP and the as-yet-unknown NCR ligands have never been described. Here, we have shown that IFN-γ is a strong stimulus up-regulating cell-surface levels of ULBP1 and NCR ligands on leukemic blasts and normal monocytes. This effect is reminiscent of the IFN-γ–dependent up-regulation of several costimulatory and adhesion molecules in monocytes and AML cells.[47,48] ULBP2 and ULBP3 levels were influenced neither by IFN-γ nor by other activators of monocyte function, including IFN-α, IL-1β, TNF-α, and LPS, and the nature of the signals regulating the expression of these ligands remains unknown.

Our results, showing that the levels of triggering ligands are usually lower on transformed cells than on normal myeloid cells, indicate a pattern of ligand expression opposite that of epithelial cells, which express higher levels of MICA and MICB on transformed cells than on healthy cells.[13] This expression pattern may reflect differences in the physiologic role of the triggering ligands. On the one hand, MICA and MICB are stress-induced ligands and likely participate in a protective response to cell insults. On the other hand, ULBP and NCR ligand expression are associated with late differentiation, and it is tempting to speculate that these ligands facilitate productive interactions between bone marrow–derived mature cells and NKG2D- and NCR-expressing lymphocytes. These interactions might result in the prompt local release of soluble factors.

Importantly, the expression of ULBPs and NCR-specific ligands increases during hematopoietic cell differentiation. In healthy human BM, early $CD34^+$ progenitors are ligand negative, whereas $CD34^-$-committed myeloid progenitors carrying CD33 and CD14 markers display the ligands on their surfaces. Consistent with this, growth factor–induced myeloid differentiation of $CD34^+$ cells in vitro is accompanied by the acquisition of the activating ligands. In AML, ligand surface density appeared to correlate with the hematopoietic differentiation stage at which leukemic transformation took place because ligand-positive blasts prevailed in patients with monocytic M5 and megakaryocytic M7 AMLs, which affect more mature progenitors. ULBP-negative or ULBP-low blasts were found in AML of subtypes M1 to M4, affecting early stages of hematopoiesis, confirming previous results in 15 M1 to M4 AMLs.[27] Growth factor–induced myeloid differentiation of leukemic blasts was associated with up-regulation of the ligands, arguing further that the ligand-low phenotype in AML is related to early maturation arrest during malignant transformation.

The accepted paradigm assigns a major role to NK-activating receptors in tumor surveillance. To explain the poor susceptibility of leukemia blasts to cytolysis by autologous NK cells, in vivo selection for transformed clones with low levels of triggering ligands has been hypothesized as a mechanism allowing tumor escape from recognition by NK cells.[28] The second evasion mechanism described in leukemia is based on shedding of MIC ligands for the NKG2D receptor.[27] Our results speak for a third mechanism of immune evasion associated with the multistep differentiation of hematopoietic cells. The ULBP and NCR ligand–deficient phenotypes of leukemic blasts, resulting from myeloid maturation arrest at early stages of blood cell differentiation, may have important consequences in preventing NK cell stimulation.

The capacity of NK cells to kill leukemic cells has been demonstrated in patients with AML receiving haplotype-mismatched stem cell transplants.[49-51] The incompatibility between the recipient's HLA class 1 antigens and the KIR repertoire expressed by donor NK cells is thought to eliminate the inhibitory interactions and to expose tumor cells to graft-derived NK cells. In this case, recognition of triggering ligands on the surfaces of leukemic blasts by killer cells carrying NKG2D and NCRs likely confers susceptibility to NK-mediated lysis. Our results show that ULBP1 and NCR ligand expression can be increased by treatment with differentiation-promoting myeloid growth factors. Clinical use of G-CSF concurrently with chemotherapy has improved long-term survival in patients with AML,[52] and it may be speculated that the observed reduced incidence of relapses is related to the enhanced susceptibility of cytokine-treated blasts to killing by the immune effector cells. Clinical experience with haplotype-mismatched transplantation has opened the possibility to select donors of stem cells and of NK cells[50] with appropriate HLA class 1 mismatches most suitable for tumor clearance. The NK cell compartment after transplantation reconstitutes rapidly,[49,53] and our results suggest that the up-regulation of triggering ligands on AML blasts by in vivo application of growth factors represents a complementary strategy that might help to recognize and eliminate residual leukemic cells.

Acknowledgments

We thank Andreas Buser, Mario Bargetzi, Alois Gratwohl, and André Tichelli for AML samples, Sandrine Meyer-Monard for help with HLA typing, Gabriel Baltariu for preliminary experiments, and Uwe Siegler for help with the cytotoxicity assays.

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