Central Nervous System Acute Lymphoblastic Leukemia: role of Natural Killer Cells.

Liron Frishman-Levy¹,²,³, Avishai Shemesh³,², Allan Bar-Sinaï⁴, Chao Ma⁵, Zhenya Ni⁵, Shahar Frenkel⁶, Vera Muench⁷, Hilke Bruckmueller¹⁰, Christian Vokuhl¹¹, Klaus-Michael Debatin⁷, Cornelia Eckert⁸, Martin Stanulla⁹, Martin Schrappe¹⁰, Kerry S. Campbell¹², Ron Loewenthal¹³, Denis M Schewe¹⁰, Jacob Hochman⁴, Lueder H. Meyer⁷, Dan Kaufman⁵, Gunnar Cario¹⁰, Angel Porgador³ *, Shai Izraeli*¹,²

¹ Childhood Leukemia Research Section, Department of Pediatric Oncology and the Cancer Research Center, Edmond and Lily Safra Children Hospital and Sheba Medical Center, Tel Hashomer, Ramat Gan, Israel

² Department of Human Genetics and Biochemistry, Tel Aviv University, Tel Aviv, Israel

³ The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences and The National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer Sheva, Israel

⁴ Department of Cell and Developmental Biology, Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

⁵ Department of Medicine and Stem Cell Institute. University of Minnesota, Minneapolis, MN USA.

⁶ Ocular Oncology Service, Department of Ophthalmology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
7 Department of Pediatrics and Adolescent Medicine, Ulm University Medical Center, Germany
8 Department of Pediatric Oncology/Hematology, Charité Universitäts medizin Berlin, Berlin, Germany
9 Department of Pediatric Oncology, Hannover Medical School, Hannover, Germany
10 Department of Pediatrics, University Hospital Schleswig Holstein, Kiel, Germany
11 Department of Pediatric Pathology, University Hospital Schleswig Hostein, Kiel Germany
12 The Research Institute of Fox Chase Cancer Center, Philadelphia, PA, USA
13 Tissue Typing Laboratory and National Laboratory for Solid Organ Transplants Sheba Medical Center, Ramat Gan, Israel.

‡ L.F.L and A.S. contributed equally to this work

*Corresponding authors:
Shai Izraeli MD, Sheba Medical Center, Tel Hashomer, Ramat Gan 52621, Israel email sizraeli@sheba.health.gov.il Phone +972-3-5305943 Fax +972-3-5307419

Angel Porgador PhD, The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences and the National Institute of Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel. (T) 97286477283 (F) 97286477626 (E) angel@bgu.ac.il
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Key points

- Increased IL-15 expression in leukemic lymphoblasts is associated with activation of NK cells.
- CNS may be an immunological sanctuary protecting lymphoblasts from NK cell activity.
Abstract

Central nervous system acute lymphoblastic leukemia (CNS-ALL) is a major clinical problem. Prophylactic therapy is neurotoxic and a third of the relapses involve the CNS. Increased expression of interleukin 15 (IL-15) in leukemic blasts is associated with increased risk for CNS-ALL. Using in vivo models for CNS leukemia caused by mouse T-ALL and human xenografts of ALL cells we demonstrate that expression of IL-15 in leukemic cells is associated with the activation of natural killer (NK) cells. This activation limits the outgrowth of leukemic cells in the periphery but less in the CNS since NK cells are excluded from the CNS. Depletion of NK cells in NOD/SCID mice enabled combined systemic and CNS leukemia of human pre-B ALL. The killing of human leukemia lymphoblasts by NK cells depended on the expression of the NKG2D receptor. Analysis of bone marrow diagnostic samples derived from children with subsequent CNS-ALL revealed a significant high expression of the NKG2D and NKp44 receptors. We suggest that the CNS may be an immunological sanctuary protected from NK cell activity. CNS prophylactic therapy may thus be needed with emerging NK cell based therapies against hematopoietic malignancies.
Introduction

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. Involvement of the central nervous system (CNS) by ALL is a major clinical problem. CNS prophylaxis consisting of intra-thecal chemotherapy and/or cranial irradiation and high dose systemic chemotherapy, significantly reduced CNS recurrence. Yet, these therapies are associated with substantial acute and long term neurotoxicity. Moreover, CNS relapse remains a major therapeutic obstacle in ALL accounting for 30% of the relapses.

Despite its clinical importance, little is known about the mechanisms that cause CNS leukemia. We previously reported the association between increased expression of interleukin-15 (IL-15) mRNA in ALL blasts and increased risk for CNS involvement. Several studies suggest an oncogenic role of IL-15 in hematological malignancies. Williams et al. recently proposed that IL-15 enhances growth of leukemic cells in the growth-factors poor environment of the cerebral spinal fluid (CSF). Overall, however, the biological role of IL-15 in CNS-ALL remains unclear.

IL-15 displays pleiotropic functions by acting on various immune cells including natural killer (NK) cells. NK cells are a subset of lymphocytes of the innate immune system that play an important role in cancer immune-surveillance through their capacity to recognize and kill transformed cells without prior sensitization. NK cell-mediated cytotoxicity is regulated by the balance of signals transmitted by activating and inhibitory receptors upon conjunction with a target cell. The development, survival and activation of NK
cells are predominantly regulated by IL-15. Moreover, IL-15 was shown to augment NK cytotoxicity against tumor cells by up-regulating the expression of NKG2D and NKp44 receptors on NK cells as well as the expression of cytotoxic effector molecules.

Here, we describe experiments that demonstrate that NK cell activation by leukemic cells expressing IL-15 can lead to control of residual disease in the periphery but to a lesser extent in the CNS because of a lack of NK cell penetration into the brain. This could explain the association between IL-15 expression and CNS relapses of ALL and, importantly, suggests the need for CNS directed prophylaxis in emerging protocols of anti-leukemia therapies with NK cells.

**Materials and Methods**

**Cells**

018Z cells (see results) REH, Jurkat and NALM6 cell lines were maintained in RPMI medium supplemented with 10% FCS. T-25 cells were cultured in DMEM medium supplemented with 10% heat-inactivated horse serum.

**Constructs and generation of stable cell lines**

The murine IL-15 construct in which the signal peptide of IL-2 is fused to the mouse IL-15 cDNA, obtained from Prof. Hugh Brady, and subcloned into pCEFL expression vector. 018Z cells stably expressing luciferase and cherry fluorescent proteins were generated by transduction with the Cherry 2A luciferase_ pLNT/Sffv-MCS/ccdB provided by Prof. Vaskar Saha.
In vivo models

Experiments were performed in specific pathogen-free units and were approved by the institutional animal experiments committees. See results for detailed description of experiments. For imaging, mice were anesthetized with ketamine-xylazine or isoflurane and were intra-peritoneally injected with 150 mg/kg D-luciferin (Promega).

Bioluminescent imaging (IVIS, Caliper Life Sciences) of anesthetized mice was performed 10 min thereafter. A bioluminescent image was acquired for 1-min, medium binning, analyzed with Living Image software version 4.2 (Caliper Life Science) and quantified using a region of interest (ROI) set to total flux (photons/sec) or average radiance (photons/sec/cm²/sr). All images were set to the same scale based on the negative controls (mice with PBS injection).

Histopathology and Hematoxylin-Eosin staining of formalin fixed brain and eyes were performed by routine methods.

NK cell depletion

NK cells were depleted by intra peritoneal injections of 50µl rabbit anti-Asialo GM1 (Cedarlane Laboratories) one day before leukemia transplantation and then once every 5 days. To ensure NK depletion, mice were monitored weekly for peripheral blood NK cells by flow cytometry using NKp46 antibody.

Antibodies and fusion proteins

Antibodies used: Alexa647-conjugated anti-mouse CD335 (NKp46), eFluor450-conjugated anti-mouse CD69 (eBioscience), APC/PE-conjugated anti-human CD19, PE-conjugated anti-human CD14, FITC-conjugated anti-CD3, PE-conjugated anti-human CD45 (IQ Products), APC/PE/Pacific Blue-conjugated anti-human CD56, Pacific Blue-
conjugated anti-human CD16, APC-conjugated anti-human CD314 (NKG2D), Biotin-conjugated anti-mouse NKG2D, Strepaavidin-conjugated PE/APC, APC/PE-conjugated anti-mouse CD49b (DX5), APC/PE-conjugated anti-human CD336 (NKp44), BV421 mouse anti-human CD107a, PE anti-human HLA A/B/C (W6/32) 7-AAD (BioLegend), Purified mouse anti-human DNAM-1 (DX11), mouse anti-human NKG2D (#149810), mouse anti-human IL-15 mAb (R&D Systems), Isotype control mouse IgG1, mouse anti-human MICA, mouse anti-human MICB, mouse anti-human ULBP2 mAb, APC-conjugated F(ab9)2 goat anti-human IgG, FITC-conjugated F(ab9)2 goat anti-human IgG, APC-conjugated F(ab9)2 goat anti-mouse IgG (Jackson), Antibodies used for human cell surface labeling: PE-Cy5 conjugated CD10 mAb and ECD conjugated CD19 mAb (Beckman Coulter). The production of human Fc-fusion proteins NKp44, human NKp46, murine NKp46 (Ly94) and human and murine NKG2D was previously described37-39.

**Flow cytometry**

018Z cells were incubated on ice with various human Fc-fusion proteins (40 mg/ml) or labeled with anti-MICA, anti-MICB, or anti-ULBP2, washed and stained with APC- or FITC-conjugated anti-human-IgG or with APC- or FITC-conjugated anti-mouse -IgG. For analysis of cells prepared from hematopoietic organs red blood cell lysis was performed using RBC lysis buffer (BioLegend). Brains were homogenized in PBS followed by separation on a 40% Percoll (GE Healthcare) gradient to eliminate residual fat tissue prior to staining.

To reduce FcγII/IIIR-mediated antibody binding, 1µl of anti-mouse CD32/CD16 (mouse BD Fc block) was added to all samples prior to staining. Dead cells were excluded using
7AAD staining. Cells were analyzed on a Gallios flow cytometer (Beckman Coulter) or FACS Canto II (BD Biosciences) and data were analyzed using either Kaluza or FlowJo (Tree Star) software.

**Isolation and culture of primary NK cells**

Primary human NK cells were isolated from peripheral blood from healthy donors using a human NK cell isolation kit, which is based on the negative selection approach (RosetteSep, STEMCELL technologies). Purity (>85-90%) was confirmed by flow cytometry and cells were cultured with IL-2 (300 units/mL) in CellGro stem cell serum-free growth medium (CellGenix) supplemented with 10% heat inactivated human serum, 1 mM sodium pyruvate, 2 mM L-glutamine, MEM nonessential amino acids, 1% penicillin/streptomycin, 10 mM HEPES. Following in vitro culture with IL-2, total non-NK cell contamination level was well below 5% with no CD3+ cells.

**KIR and HLA genotyping**

KIR genotyping was performed with the KIR Genotyping SSP Kit (Invitrogen). HLA typing was performed utilizing LUMINEX-TM technology and Immucor Transplant Diagnostic (Stamford, CT) kits to obtain HLA A*, B* and C* loci typings at low/intermediate resolution.

**Cytotoxicity assays**

Cytotoxic activity of primary human NK cells against ALL cells was tested in 5-hr Killing assay as previously described\(^4^0\). For NKG2D blocking experiments, NK cells were pre-incubated for 1h at 4°C with 10μg/ml of anti-human NKG2D mAb (Clone 149810; R&D Systems, Minneapolis, MN) or with isotype control. 7AAD staining was applied to exclude dead cells. The percentage of specific cytotoxicity was calculated by:
Quantification of NKG2D (KLRK1) and NKp44 (NCR2) receptors and ligands mRNA in ALL bone marrow samples was done in the same set of patient originally used in the IL-15 report17 except for two lacking additional material. Informed consent was obtained and the protocol was approved by local and central ethical committees. All patients on ALL-BFM 2000 underwent diagnostic lumbar puncture and cerebrospinal fluid (CSF) examination at admission to the participating hospital, cranial MRI and inspection of the retina. Only CNS 1 patients (no blasts in CSF cytospins) were included as CNS negative controls. In all but one sample (70% blasts) the specimens contained more than 90% blasts. The expression level of the succinate dehydrogenase complex subunit A (SDHA) gene was used to normalize for differences in input cDNA. QuantiTect Primer Assays were used to measure mRNA abundances of the NKG2D (KLRK1) and NKp44 and SDHA genes (Qiagen). Melting curve analyses were performed to verify the amplification specificity. Each sample was tested in duplicate. The expression ratio was calculated as $2^n$, where $n$ was the $C_T$ value difference normalized by the $C_T$ difference of a calibrator sample.

Statistical analysis

Results of in vivo or in vitro experiments are shown by mean ± SE or SD respectively. Data were analyzed using GraphPad Prism 5 software and comparisons between groups were performed by paired or unpaired Student t test as appropriate. P value <0.05 was considered statistically significant. Comparison of NKG2D (KLRK1) and NKp44 expression between patient groups was performed applying the Mann-Whitney U test.
Results

Expression of IL-15 in mouse T-25 T-cell lymphoblasts increases mice survival associated with clinical symptoms for CNS involvement and activation of NK cells.

To test the hypothesis that increased expression of IL-15 will be associated with CNS leukemia we utilized the T cell leukemia/lymphoma model described by Hochman et al.\textsuperscript{41,42}. T-25 cells, not expressing endogenous IL-15, were transfected with an expression vector encoding murine IL-15 (designated T-25-IL15; Supplementary Figure-1) and transplanted intra-peritoneally into BALB/c neonates. Constitutive expression of IL-15 significantly enhanced the survival of mice (Figure-1A). Remarkably, mice injected with T-25-IL15 cells had pronounced clinical CNS symptoms (e.g. ataxia, side walk, spin when held by the tail) not observed in control mice (Figure-1B). The most dramatic manifestation was ocular involvement that was observed only in mice injected with T-25-IL15 (Figure 1C). Ocular involvement has been previously shown in this model and in humans with advanced CNS leukemia\textsuperscript{42-44}. Lymphoblasts isolated from the eye continue to secrete the IL-15 protein (Figure-1D).

Surprisingly, histopathology analysis of brains isolated from 25 mice (12 mice injected with T-25-IL15 cells and 13 control mice) close to their time of death revealed that both groups exhibited lymphoblastic infiltration of the subarachnoid space indicating that IL-15 is not necessary for blast entry into the CNS (Figure-1E,F). We therefore hypothesized that the IL-15 expressing cells activated NK cells that reduced the aggressiveness of the systemic leukemia allowing sufficient time for development of symptomatic CNS and ocular disease. Consistent with this hypothesis the percentage of peripheral activated...
blood NK cells (NKp46, CD69 positive) was increased significantly in mice transplanted with T-25-IL15 (Figure-1G and supplementary Figure-2).

Enhanced control of peripheral ALL but not CNS leukemia in NK proficient NOD/SCID mice compared with NK deficient NOD/SCID/IL2Rγ null mice

To study another model for human CNS leukemia with physiological IL-15 levels we obtained 018Z ALL cells derived from a B-cell precursor ALL of a child with suspected CNS involvement that died during induction chemotherapy. These cells causes isolated CNS leukemia in NOD/SCID mice\(^4\). To examine whether the 018Z-induced CNS phenotype is associated with NK cells activation we utilized two strains of immune-deficient mice: NOD/SCID mice containing some functional NK cells\(^4\) and NOD/SCID/IL2Rγ null (NSG) in which there are no NK cells\(^4\). NOD/SCID and NSG mice were intravenously injected with \(10^7\) or \(5\times10^6\) 018Z cells respectively and sacrificed upon the appearance of clinical CNS symptoms or the development of severe clinical disease. The median survival of the NOD/SCID mice was 80 days following transplantation and five out of 10 developed hind limbs paralysis or ataxia. In contrast all NSG mice developed hind limb paralysis three weeks following transplantation (Figure-2A). Histopathological examination of brain sections showed subarachnoid CNS leukemia infiltration in both NOD/SCID and NSG mice (Figure-2B). Flow cytometry of human CD10+CD19+ leukemic cells revealed significant involvement of bone marrow, spleen and blood in the NSG mice but not in the NOD/SCID mice (Figure-2C). Similarly, enlarged spleens were present in NSG but not in NOD/SCID mice (Figure-2D). Thus
018Z cells cause isolated CNS leukemia in NOD/SCID but a more aggressive combined CNS and peripheral leukemia in the NK-deficient NSG mice.

To examine the general relevance of these observations we analyzed the leukemic phenotypes of primary ALL cells from patients' bone marrow in the two mouse strains (supplementary Figure-3). For both samples, the peripheral leukemic load was significantly lower in NOD/SCID while in both strains there was substantial CNS infiltration. To study the correlations with NK cells we analyzed additional seven xenografts of primary ALLs in NOD/SCID mice depicting combined CNS/peripheral leukemia. As shown in Figure-3A there was a positive correlation between the levels of IL-15 mRNA in the ALL blasts and the level of NK cells with one outlier (marked with a triangle in Figure-3A) in which an increase in NK cells was observed despite the absence of IL-15 expression. Interestingly, these ALL cells carried the t(1;19)(q23 p13) chromosomal translocation that is known to be associated with increased risk for CNS involvement. Increased NK cells (351/10^5 splenic cells) were also observed in another t(1;19)(q23 p13) NOD/SCID xenograft with CNS leukemia (Patient A in Supplementary Figure-3). Thus t(1;19)(q23 p13) may stimulate NK cells in an IL-15 independent manner. Importantly, increased NK cell counts were associated with decreased peripheral leukemia and increased CNS infiltration of xenografted leukemic cells in the NOD/SCID mice (Figure 3B-D).
018Z induced aggressive peripheral and CNS leukemia in NK-depleted NOD/SCID mice

To study directly the hypothesis that the leukemia was confined to the CNS in NOD/SCID mice because of the activation of NK cells in the periphery, we injected 018Z cells expressing both mCherry and luciferase reporter genes into NK cell-depleted and non-depleted NOD/SCID mice. NK cell depletion was achieved by i.p. injection of anti-Asialo GM1 twice a week. As depicted in Figure-4A, the percentage of NK cells following treatment with anti-Asialo GM1 was 0.5±0.1 compared to 8.5±1.2 in PBS injected control mice. NK cells remained depleted throughout the experiment.

Similar to the phenotype observed in NSG mice, NK cell-depleted NOD/SCID mice developed aggressive systemic disease characterized by reduced survival (Figure-4B, P=0.003, Log-Rank test), substantial weight loss (Figure-4C) and significant increase of leukemic blasts in the blood, bone marrow and brain (Figure-4 panels Di-iii). Bioluminescent imaging of mice 27 days following transplantation supported these findings (Figure-4E, F). Thus the increased burden of leukemia in the absence of NK cells was associated with an aggressive combined bone marrow and CNS phenotype.

Poor penetration of NK cells to the CNS in mice with CNS leukemia

Our hypothesis that NK cells regulate development of isolated CNS leukemia assumes that NK cells do not penetrate the CNS, even in the presence of CNS leukemia that may be associated with compromise of the blood-brain-cerebrospinal fluid barrier. We therefore injected NSG mice intravenously with 2*10^5 018Z McCherry/luciferase labeled leukemic cells. Six days later mice received either PBS or 10^7 human peripheral blood
NK cells (PBNK) intravenously. Mice were followed by bioluminescent imaging at day 0, 7, 13, and 20 after NK treatment and sacrificed at the time when the debilitating symptoms developed (Supplemental Figure-4). While mice that received this single dose of NK cell-treatment did have slower tumor progression, as quantified by bioluminescence (Figure-5A), this did not translate to increased survival (Figure-5B). At the end of experiment there was extensive infiltration of peripheral hematopoietic organs and of the CNS in both groups. Analysis of NK cell trafficking to specific tissues shows that human NK cells can be seen in the blood and peripheral tissues at Day 14 post-NK cell treatment (Figure-5C), however, no NK cells are seen in the CNS at Day 14 or at the end of experiment when the mice became moribund (Figures-5C,D).

Taken together, the NK depletion experiment in NOD/SCID mice (Figure 4) and the analysis of human NK cell distribution in NSG mice (Figure 5C) show that while NK cells appear to mediate anti-ALL activity in the periphery, the lack of NK cells in the CNS demonstrates this region remains a sanctuary against NK cell-mediated activity.

The B-cell precursor ALL line 018Z secretes endogenous IL-15 and is efficiently killed by primary NK cells via NK2GD

We next investigated the mechanisms of NK cells activity on 018Z human leukemic cells. Consistent with the previously reported association between IL-15 production by ALL cells and CNS involvement\textsuperscript{17,22}, 018Z secreted IL-15 (Figure-6A). As our previous \textit{in vivo} results indicated that 018Z leukemia growth was regulated by murine NK cells, we performed a standard killing assay in which 018Z were pre-labeled with CFSE and incubated with IL-2-activated primary human NK cells. Consistent with the mouse
experiments, 018Z cells were efficiently killed by human NK cells relatively to the resistance of another B cell precursor leukemia cell line (REH) that does not secrete IL-15 (Figure-6B). Thus, 018Z cells express IL-15 and are sensitive for NK cytotoxicity. This sensitivity could not be explained by reduction in HLA expression as 018Z and REH expressed similar levels of HLA class I (Figure-6C). As KIR/KIR-ligand disparity influences NK cell lysis of leukemic cells we genotyped the NK cell donor and target cells. KIR2DL1, KIR2DL2, and KIR2DL3 inhibitory receptors were expressed on the NK cells while both 018Z and REH expressed ligands for KIR2DL1 (Cw*04 and Cw*06) and in addition 018Z expressed HLA-Cw*07, a KIR2DL2/3 ligand (supplemental Figure-5). Thus despite an increased expression of inhibitory ligands, 018Z was susceptible to killing by NK cells. These results indicate that KIR/HLA mismatch is not a major factor contributing to the increased susceptibility of 018Z to cytotoxicity.

Therefore, we further investigated the expression of ligands for NK activating receptors. Since some cellular ligands for NK receptors (NKR) are yet unknown we characterized their expression by 018Z using Fc-fusion proteins of NKR. Flow cytometry was used to examine the binding of Fc-fusion proteins of human& mouse NKG2D, human NKp30, NKp44& NKp46, and mouse LY94 (NKp46 homolog) to putative ligands on the surface of 018Z cells. We found that 018Z cells express ligands recognized by both human and mouse NKG2D as well as ligands for human NKp44 receptor (Figure-6D, panels i to vi). We further examined the expression of known
specific human NKG2D ligands: MHC class I-related protein A and B (MICA/MICB) and the UL16 binding protein 2 (ULBP2) on 018Z cells. As shown in Figure-6D panel vii, 018Z cells express all three ligands with highest expression for ULBP2. Interestingly, ULBP2 was reported to be effectively recognized also by mouse NKG2D which could explain the in vivo sensitivity of 018Z to murine NK (Figure-4). As NKp44 is not expressed by murine NK, expression of ligands to NKp44 by 018Z did not contribute to its NK-mediated growth retardation in mice. For the human NK scenario, since the human NK resistant REH cell line (Figure-6B) expressed cell surface ligands to NKp44 but very low level of ligands to NKG2D (data not shown), we hypothesized that the higher sensitivity of 018Z to human NK cells was mediated by NKG2D. Indeed, utilizing blocking antibodies to NKG2D we show that the killing of 018Z is primarily mediated by NKG2D (Figure-6E). NEC2/DNAM-1 interaction was reported to be involved in pathway of acute lymphoblastic leukemia cell recognition by NK cells. However blocking the DNAM-1/PVR-nectin-2 recognition did not affect the lysis of 018Z by NK (Figure-6F). Together our experiments suggest that the increased sensitivity to lysis by NK of the IL-15 secreting 018Z is mediated by the NKG2D/NKG2D-ligands interaction.

**Expression of NK receptors and Ligands in primary pediatric ALL diagnostic bone marrow samples and association with CNS leukemia.**

To further establish the role of NK cells and its relevance to childhood ALL its association with CNS leukemia, we first examined the mRNA expression of NKG2D (KLRK1) and NKp44 (NCR2) receptors in ALL bone marrow samples originally used in the IL-15 report. Clinical and biological characteristics of samples analyzed are shown
in Table 1. Expression of both NKp44 and NKG2D was analyzed by qRT-PCR of cDNAs derived from bone marrow samples of patients who were 1) CNS negative at initial diagnosis with no CNS relapse (n=44); 2) CNS positive at initial diagnosis (n=21); 3) CNS negative at initial diagnosis with subsequent CNS relapse (n=21). As depicted in figure-7A, expression levels of NKG2D (KLRK1) were associated with CNS leukemia (when comparing group CNS positive vs. negative and CNS negative vs. CNS negative with subsequent CNS relapse, p=0.07 and p=0.03, respectively). Similarly, expression levels of NKp44 (NCR2) in bone marrow were significantly higher in CNS involving leukemia (p=0.004 and p=0.001, respectively, Figure-7B).

We then extended these findings to the protein level and tested the cell surface expression of NKG2D and NCR2 proteins in the immune cells within those primary ALL BM samples with additional material available (six CNS negative and 3 CNS positive samples). Although NCR2 mRNA level significantly differ in primary ALL BM samples, we could not detect significant cell surface expression of the NCR2 protein in the subset analyzed. In contrast, significant enhancement of cell surface expression of the NKG2D protein was observed in T, NKT and NK cells in BM diagnostic samples from CNS positive patients (Figure-7C). In accordance the qPCR analysis of the mRNA of these specific samples manifest enhancement of KLRK1 mRNA as well as of IL-15 mRNA (Figure-7D).

Enhanced expression of NKG2D ligands by pediatric B-ALL cells was previously reported, particularly for ULBP1 and ULBP3\(^56\). We thus assessed mRNA expression
levels of the specific NKG2D ligands in these ALL BM samples. Interestingly, ULBP1 and ULBP3 mRNA expression levels were enhanced in the CNS negative as compared to the CNS positive samples (Figure-7D). ULBP3, and to a lesser extent ULBP1, were shown to be secreted in exosomes and thus could facilitate inhibition of NKG2D-mediated NK function. We therefore analyzed ULBP1, ULBP2 and ULBP3 in the entire set of 68 diagnostic BM samples and calculated the (ULBP1+ULBP3/ULBP2) mRNA expression ratio per each ALL BM sample. Figure-7E clearly shows that the ratios in the CNS negative group were significantly higher than the ratios in the CNS positive and CNS relapse groups (note that the Y axis scale is logarithmic). Thus, high expression of the NKG2D receptor in infiltrating NK cells coupled with lower expression of inhibitory NKG2D ligands in bone marrow at the time of diagnosis of ALL correlated with CNS leukemia.

Discussion

The occurrence of CNS leukemia is largely explained by neurotropism of lymphoblasts and the poor penetration of anti-leukemic drugs into the CNS. Here we demonstrate that the CNS is also an immunological sanctuary for ALL cells.

We have sought a mechanistic explanation to our previously reported association between IL-15 expression in ALL blasts and the increased risk for CNS relapse of ALL. Consistent with the known regulation of NK cells by IL-15, we observed that the expression of IL-15 in cell lines and in primary ALL cells was associated with activation of NK cells. Our data suggest that NK cells do not directly regulate leukemic blast
penetrance and survival in the CNS. Rather neurotropism is caused by *endogenous*
properties of ALL cells. For example William et al suggested that IL-15 may be directly
involved in the survival and proliferation of cells in the CNS\(^2\). Similarly Krause et al
showed that MER kinase may regulate the CNS tropism of t(1;19) ALL\(^4\). Our studies of
mouse and human cell lines and primary ALL cells demonstrate that NK cells activated
by IL-15 or other mechanisms\(^5\) inhibit systemic peripheral leukemia but fail to enter
the brain and control the CNS leukemia.

Recently, there has been a significant progress in NK cell-based therapies of acute
leukemias. In addition to exploiting mismatch of NK inhibitory receptors during stem cell
transplantation\(^6-\), direct treatment of ALL with allogeneic or autologous NK cells has
been recently proposed\(^5,\). Our findings may be important for emerging NK based
therapies of acute leukemias suggesting the need for additional prophylactic CNS
directed therapy.

A role for autologous NK cells in ALL surveillance has been suggested by genetic studies
indicating that possessing more NK activating KIR genes reduces the risk of developing
leukemia\(^7\). Interestingly increased NK sensitivity associated with increased NK ligands
expression has been recently demonstrated in BCR-ABL ALLs, a leukemia that is highly
associated with CNS involvement\(^5\). Our mechanistic studies imply a major role for
NKG2D in NK surveillance of endogenous ALL cells. We observed increased mRNA
and protein expression of NKG2D on NK cells from diagnostic bone marrow of ALL
patients with CNS involvement. This increase may be caused by IL-15 or alternatively
by reduction in the secretion of “decoy” ULBP ligands\(^5,\).
This is the first report suggesting that NK cells activity against ALL is excluded from the CNS and it raises multiple questions. For example, should CNS prophylaxis be added to leukemia therapeutic protocols with NK cells? Can increased NKG2D and a lower ratio of ULBP1+ULBP3/ULBP2 expression in diagnostic marrow be translated into biomarkers for CNS relapse and for sensitivity to autologous NK cells? These and other questions emerging from our discoveries need to be tested in prospective clinical trials.

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Authorship Contribution

LFL SI, GC and MS initiated the project. LFL, AS, ABS, ZN, LHM, KMD DK, JH, DMS, AP and SI planned experiments. LFL, AS, ABS, CM, ZN, VM, SF, HB, CV, KSC, RL, DMS, LHM, GC, performed and analyzed experiments. LHM, KMD, DMS, GC, DK, AP and SI further analyzed experiments. CE, MS, MSc, and GC provided and analyzed patients’ data. The primary manuscript was written by LFL, AS, LHM, DMS, DK, AP and SI and further edited by all authors.

Conflict of interest disclosure

The authors declare no actual or potential conflict of interest.
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Table 1. Characteristics at initial diagnosis of 44 CNS negative patients with acute lymphoblastic leukemia, 21 CNS positive patients, and 21 patients subsequently relapsing with CNS involvement.

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<td>16 (76.2)</td>
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<td>3 (14.3)</td>
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<td>50,000 - &lt; 100,000</td>
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<td>19 (90.4)</td>
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<td>5 (23.8)</td>
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\( P^a \chi^2 \) or Fisher’s exact test;

\( ^b \) white blood cell.
Figure Legends

Figure 1. Constitutive expression of IL-15 in T-25 lymphoma cells increases survival of mice and is associated with clinical CNS symptoms. (A) Kaplan-Meier survival curve of 7-day old BALB/C mice injected with either T-25-IL15 (n=27) or T-25 cells (n=28). (P<0.0001, Log-rank test). (B) Kaplan-Meier curve depicting the accumulated rate of CNS symptoms. Mice were monitored every day for clinical symptoms for CNS involvement including spin when held by tail, ataxia and side-walk. These symptoms were present only in the T-25-IL15 group. (C) Representative picture of a T-25-IL15 injected mouse with ocular involvement. Note the opacity of the left eye (arrow) caused by accumulation of lymphoma cells in the left anterior chamber. (D) IL-15 secretion was measured by ELISA in cells derived from the eyes of T-25-IL15 injected mice (n=2). T-25-injected mouse served as control. (E, F) H&E staining of brain sections derived from T-25 (up) and T-25-IL15 injected mice (down) showing infiltration of lymphoma cells in the sub-arachnoid space. Histopathology analysis revealed that there was no difference in the number of mice with CNS involvement between both groups. (G) T-25-IL15 injected mice exhibited significant increase in peripheral blood NK cells. Values presented as means ± SE (p=0.01, t-test).

Figure 2. 018Z human Pre-B ALL cells induce isolated CNS leukemia in NOD/SCID mice and combined CNS/peripheral leukemia in NSG mice. (A) A Kaplan-Meier survival curve of NOD/SCID (n=10) and NSG (n=4) mice injected intravenously with 018Z cells (p=0.001; Log-rank test). (B) Representative H&E staining
of brain sections from NOD/SCID (down; n=10) and NSG (up; n=3) mice presenting leukemia infiltration in the sub-arachnoid space. (C) Flow cytometry analysis of peripheral leukemia percentage in the blood (i), bone marrow (ii) and spleen (iii) using antibodies for CD10 and CD19. Values are means ± SE, (p<0.0001; Unpaired t-test). (D) Representative pictures of spleens taken from NSG (left) or NOD/SCID (right) mice.

Figure 3. Associations of NK-cells infiltration in spleens of human ALL-bearing NOD/SCID with levels of IL-15 mRNA expression and with leukemic infiltrates of CNS and peripheral organs. (A) Linear regression analysis of splenic mouse NK cells (DX5 and NKG2D positive cells per 10^5 splenic cells) as a function of IL-15 mRNA expression in infiltrating human ALL cells (determined by qRT-PCR, values normalized to beta actin mRNA levels^{72,73}). The outlier (gray triangle) is a xenograft from a patient with t(1;19) positive ALL demonstrating significant NK cells accumulation despite no IL-15 expression. (B-D) Increasing splenic NK-cell recruitment is significantly associated with increased infiltration of human ALL cells in the recipient’s central nervous system (B) and with lower systemic leukemia burden in spleen and peripheral blood (C, D). (Spearman correlation).

Figure 4. Depletion of NK cells reduced survival of mice and enhanced peripheral and CNS leukemia. NOD/SCID mice were i.p. injected with either anti-Asialo GM1 (n=13) or sterile PBS (n=11) then transplanted with 10^7 cells i.v. (A) Confirmation of NK depletion by Flow cytometry analysis of peripheral NK cells in blood samples from mice injected with anti-Asialo GM1 compared with PBS controls (p<0.0001). (B) Kaplan-
Meier survival curve depicting reduced survival of the NK-Depleted mice (p=0.003; Log-rank test). (C) Reduced weight of NK depleted mice on the day of sacrifice (p=0.0005, t-test). (D) Flow cytometry analysis of 018Z-cherry positive cells in the blood (i), bone marrow (ii), and brain (iii). Data presented are from three independent experiments. Values are means ± SE. statistical analysis was performed by t-test. (E) Bioluminescent imaging of mice 27 days following transplantation with 018Z cells expressing the luciferase reporter; NK depleted mice (left) and PBS injected mice (right). (F) Quantification of the images at panel E by Living Image Software; The leukemic load represented as counts per minute (CPM; p=0.004; t-test).

Figure 5. In vivo antitumor responses by expanded human NK cells in 018Z-engrafted NSG mouse model.

Mice were intravenously injected with 2x10^5 018Z tumor cells and imaged by bioluminescence after 6 days to confirm tumor establishment. Then mice received either PBS (n= 11 mice) or 1x10^7 human PBNK cells (n= 12 mice) at day 0 with IL-2 and IL-15 daily for the first week and IL-2 every other day for three more weeks. (A) Bioluminescent analyses indicate significant delay in tumor growth in mice treated with PBNK cells at Days 7 and 20. (B) Kaplan-Meier survival curve of NK cell-treated and control mice. This survival data utilizes 7 mice from PBS-treated group and 8 mice from PB-NK cell treated group. (C) NK cell engraftment was evaluated by flow cytometry. Mice blood, brain, bone marrow, spleen, lung and liver tissues were collected from 4 mice at day 14 after PBNK treatment. NK cell survival/engraftment was evaluated by flow cytometry for CD45+CD56+ cells in the live cell population. (D) Analysis of NK
cells (CD45+CD56+ cells) in blood and CNS at end of experiment when the remaining animals became moribund (n=7 in each group). These studies demonstrate lack of penetration of NK cells into the CNS with significantly decreased NK cells in the CNS as compared to peripheral blood and tissues. All statistical analyses were performed by Mann Whitney test in Prism 4.0.

Figure 6. 018Z cells are efficiently killed by primary NK cells through interaction with NKG2D receptors. (A) IL-15 levels (ELISA) in the medium of various ALL cell lines after overnight incubation at 37°C. (B) 018Z and REH cells were incubated for 5 hours with human primary NK cells at various effector to target (E:T) ratios. Specific killing of 018Z cells was significantly higher than that of REH cells for all E:T ratios. * p<0.001, ** p<0.0001 (t-test). (C) HLA-A/B/C expression on REH (black line) and 018Z cells (dashed line). (D) Binding of Fc-fusion proteins of NK receptors to 018Z cells. 018Z cells were incubated with various human Fc-fusion proteins of NK receptors followed by labeling with goat anti-hIgG (black line). (i) hNKG2D-Ig, (ii) mNKG2D-Ig, (iii) NKp44-Ig, (iv) hNKp46-Ig, (v) mNKp46-Ig, (vi) NKp30-Ig. Non-specific binding was detected by staining with human Fc-protein (filled gray). (vii) Expression of the hNKG2D ligands on 018Z cells. Purified mouse anti MICA (black line), MICB (dotted), ULBP2 (dashed) followed by labeled goat anti-mIgG (Filled Gray). Results are representative of three independent experiments. (E/F) Killing assay of 018Z cells by IL-2 activated human primary NK cells with or without blocking antibodies for NKG2D and DNAM-1, respectively. Pretreatment of NK cells with blocking antibodies for NKG2D
significantly reduced the killing of 018Z cells. Values are shown as means ± SD of triplicates. *p<0.00001 (t-test, no mAb or isotype control compared to anti hNKG2D).

**Figure 7. Expression of NKG2D and NKp44 receptors and ligands on NK cells and its association with CNS leukemia.** Box plots demonstrating the positive association between the expressions of (A) NKG2D/KLRK1 and (B) NKp44/NCR2, respectively, and CNS involvement in leukemia in the entire set of 86 primary BM samples. (C) Geometric mean fluorescence intensity (GeoMFI) of human NKG2D protein expression on T cells (CD45+/CD3+/CD56+/CD19−), NKT cells (CD45+/CD3+/CD56+/CD19+), NK cells (CD45+/CD3+/CD56+CD19−), and CD45+/CD3−/CD56−/CD19+ cells in a subset of six CNS negative and three CNS positive primary BM samples. (D) qRT-PCR of IL-15, NKG2D/KLRK1, MICA, MICB, ULBP1, ULBP2, ULBP3 in the same set as (C). mRNA values are normalized to SDHA expression; (E) Association of (ULBP1+ULBP3/ULBP2) mRNA ratio and CNS involvement in leukemia in the entire set of BM samples.

Data shown were derived through qRT-PCR after reverse transcription using RNA of bone marrow mononuclear cells at initial diagnosis. The line within the box plots of (A), (B) and (E) corresponds to the median value, the star the mean value, the box length to the interquartile range, and the lines emanating from the box (whiskers) extend to the smallest and largest observations; outliers are indicated; a.u.= arbitrary units. Statistical analyses were done applying Student t-test, 2-tailed.
Figure 2

(A) Percent survival over time for NOD/SCID and NSG mice. 

(B) Histological comparison of tissues from NOD/SCID and NSG mice. 

(C) Analysis of lymphoblasts in blood, bone marrow (BM), and spleen from NOD/SCID and NSG mice. 

(D) Images of blood samples from NSG and NOD/SCID mice, showing different volumes.

Figure 2
Figure 6
Central nervous system acute lymphoblastic leukemia: role of natural killer cells

Liron Frishman-Levy, Avishai Shemesh, Allan Bar-Sinai, Chao Ma, Zhenya Ni, Shahar Frenkel, Vera Muench, Hilke Bruckmueller, Christian Vokuhl, Klaus Michael Debatin, Cornelia Eckert, Martin Stanulla, Martin Schrappe, Kerry S. Campbell, Ron Loewenthal, Denis M. Schewe, Jacob Hochman, Lueder H. Meyer, Dan Kaufman, Gunnar Cario, Angel Porgador and Shai Izraeli