Therapeutic effect of Lenalidomide in a novel xenograft mouse model of human Blastic NK cell lymphoma/blastic plasmacytoid dendritic cell neoplasm

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Running title: Development and treatment of the first BNKL orthotopic mouse model

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Key words: Blastic NK cell Lymphoma, NOD SCID IL2γ-/- mice, orthotopic mouse model, Lenalidomide, angiogenesis
Translational relevance

We propose, to our knowledge, the first orthotopic mouse model of Blastic NK cell lymphoma (BNKL). The development of this model could help in a better understanding of the biology of this malignancy. Moreover, since an effective treatment does not exist so far, we propose a possible treatment strategy with a metronomic administration of immunomodulatory drugs.

Abstract

Purpose: Blastic NK cell lymphoma/blastic plasmacytoid dendritic cell neoplasm (BNKL) is a rare and aggressive neoplasia characterized by infiltration of blast CD4+/CD56+ cells in the skin, the bone marrow (BM) and in the peripheral blood (PB). Currently, more efforts are required to better define molecular and biological mechanisms associated to this pathology. To the best of our knowledge, no mouse model recapitulated human BNKL so far.

Experimental Design: Primary BM cells from a BNKL patient were injected in NOD SCID IL2rγ-/- mice with the intent to generate the first BNKL orthotopic mouse model. Moreover, because of the lack of efficient treatments for BNKL, we treated mice with lenalidomide, an immunomodulatory and antiangiogenic drug.

Results: We generated in mice a fatal disease resembling well human BNKL. After lenalidomide treatment, we observed a significant reduction in the number of PB, BM and spleen BNKL cells. Tumor reduction parallels with a significant decrease in the number of circulating endothelial (CECs) and progenitors cells (CEPs), and CD31+ murine endothelial cells. In mice treated with lenalidomide, BNKL levels of active Caspase-3 were significantly augmented, thus demonstrating proapoptotic and cytotoxic effects of this drug in vivo. An opposite result was found for PCNA, a proliferation marker.

Conclusions: Our BNKL model might better define the cellular and molecular mechanisms involved in this disease, and lenalidomide might be considered for the future therapy of BNKL patients.
Introduction

Starting from 1994, some cases of tumors characterized by the expression of CD4 and CD56 in lymphoblastic cells were described \(^1\). This co-expression of CD4 and CD56, in absence of B, T and myeloid lineage suggested a NK cell origin. In 1999 Petrella et al defined this entity as a blastic NK cell lymphoma (BNKL), although the absence of specific markers suggest that it is difficult to clearly establish the origin of this neoplasm \(^2\). These tumors are characterized by frequent skin and lymph nodes involvement, bone marrow (BM) infiltration, and spleen enlargement. The majority of BNKL patients are aging adults, but the disease may occur in younger adults or in children. Progressive BNKL typically leads to the patient’s death within a few years after initial examination \(^3-4-5\).

Recently it was discovered that BNKL cells express antigens like CD123 (IL-3 receptor-\(\alpha\)) and BDCA-2 (blood dendritic cell antigen-2), providing evidence for a relationship with immature plasmacytoid dendritic cells (pDCs), and suggesting a possible derivation for this rare neoplasm. The expression of the lymphoid proto-oncogene TCL-1 demonstrates another immunophenotypic linkage between normal DCs and immature CD4+/CD56+ blasts \(^6\). To corroborate this data, different groups demonstrated that tumor cells were able to secrete interferon-\(\alpha\), suggesting that this malignancy is not only immunophenotypically consistent with immature DCs, but also maintains some of its functional characteristics \(^7-8-9\). In the recent WHO European Organization for Research and Treatment of Cancer classification of lymphoma, the previous BNKL terminology was replaced by Blastic plasmacytoid dendritic cell neoplasm \(^10\).

BNKL is characterized by a very poor prognosis, and the mean survival is less than two years \(^3-5\). So far, most of the reported BNKL cases were treated with conventional polychemotherapy. Complete remission was obtained rapidly, but in most cases a relapse occurred within a year \(^4-8\).

Because of treatment failure, it is urgent to investigate novel therapies for BNKL. However, no BNKL animal model has been described so far. Different strains of immunodeficient mice have been studied to generate models of haematological malignancies: NOD-SCID and NOD-SCID-related strains (NOD SCID \(\beta^2\) null –NSB-, NOD SCID IL2 \(\gamma^\gamma\) null –NSG-) have been the most widely used in these studies \(^11-14\). We have generated a BNKL model by injecting human BM cells from a BNKL patient.
in NSG mice. Human BNKL cells were able to generate in mice a disease that resembles the original characteristics of human tumor. We treated BNKL mice with lenalidomide, an immunomodulatory and antiangiogenic drug acting through multiple mechanisms \(^{15-23}\) and found a very promising preclinical activity in a prevention experiment. Moreover, lenalidomide administration induces a prolonged survival in a model of BNKL established tumor.
Materials and methods

Collection of patient cells and of normal controls

Bone marrow (BM) cells were collected from a patient with newly diagnosed BNKL after obtaining an internal review board approval and informed consent from the patient. Mononuclear cells isolated from the BM were separated by Bicoll Separating Solution (Biochrom AG, Berlin, Germany), suspended in RPMI 1640 (EuroClone, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Paisley, UK), 100 IU/ml of penicillin (EuroClone), 100 μg/ml streptomycin (EuroClone), 0.3 mg/l L-Glutamine (EuroClone) and frozen viably in FBS plus 10% CryoSure-DMSO (WAK-Chemie Medical GMBH, Steinbach, Germany). For some studies, normal NK cells from healthy blood donors were purified (>95% purity) with anti-CD56 beads using a commercially available MACS kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Animals and injection of human cells

NODSCID (NS), NODSCID β2 null (NSB) and NODSCID IL2γnull (NSG) were kindly donated by Dr. L. Shultz from the Jackson Lab (ME), bred and housed at in the animal facilities at IFOM-IEO campus (Milan, Italy), and at CIMA (Pamplona, Spain). All animals, housed in microisolator cages, were used between 6 and 8 weeks of age, and all procedures were carried out in accordance with the guidelines for animal experimentation of our Institutions, under an approved protocol. For induction of lymphoma in mice, 10 x 10⁶ low density mononuclear cells from the BM of the BNKL patient were injected intraperitoneally (ip) in non-irradiated mice. Secondary transplants were performed by injecting spleen cells of primary transplanted mice, and successively on tertiary and quaternary recipients.

Flow cytometric analyses

Human cell engraftment in the PB, spleen and BM was investigated by flow cytometry (FC). BM and spleen cells were isolated by dissociation of the organs before FC analysis. Briefly, cells were previously incubated with anti-mouse antibody CD16/CD32 (BD, Mountain view, CA) to block FcRγRII and RIII unspecific signal, and then stained with anti-human CD45, (clone 2D1, BD) and anti-mouse CD45 (30-F11, BD). Angiogenic response was measured by flow cytometry as described in...
detail by enumerating murine circulating endothelial cells (CECs) and progenitors (CEPs) in the PB. To exclude hematopoietic cells, anti mouse CD45 was used; murine CECs and CEPs were detected using the endothelial murine markers VEGFR2 (clone Avas12a1; BD) and CD117 (clone 2B8, BD). After antibodies incubation and red cell lysis, at least 1x10⁶ cells per sample were acquired by a FACSCalibur (BD). Seven-aminoactinomycin D (7AAD; Sigma-Aldrich, IL) was used to enumerate viable, apoptotic and dead cells. After the acquisition, appropriate gating was used to exclude dead cells, platelets and debris, and analyses were considered as informative when adequate numbers of events (i.e., > 50, typically 100–200) were collected in the enumeration gates. Percentages of stained cells were determined and compared to appropriate negative controls defined as nonspecific background staining.

**Echography**

Spleen’s areas before and after treatment were measured by echography. A Vevo770 microimaging system (Visualsonics Inc., Toronto, Canada) with a single element probe of center frequency of 30 MHz (RMV 707B) was used for all the examinations. The transducer has a focal depth of 12.7 mm, lateral resolution of 115 μm, and an axial resolution of 55 μm. Precise and repeatable control over the position of the two dimensional image plane was obtained with a Vevo Integrated Rail System II.

Mice were anesthetized and hair was removed from the area of interest to reduce imaging artefacts in the ultrasonographic examination. Afterwards, mice were placed on a heated table and examined in supine position (slightly right decubitus) to assess the spleen. Warmed ultrasound gel was placed on the abdomen. All imaging was performed in 2-D plane (cine loops B mode) of the spleen. Real time imaging was performed with a frame rate of 55 MHz with a 10x10 mm yield of view. The organ was evaluated by multiple sagital scans of the spleen and the images were measured in duplicate (sagital and transverse diameter). All the settings were kept constant throughout the experiment and the echographies were performed by the same person to reduce the variability.

**RNA isolation, quantitative RT-PCR and microarray analyses**

Total RNA was isolated from samples with Trizol (Invitrogen). For microarray analysis, Trizol followed by a further isolation with RNeasy (Qiagen) was used. Quantitative real time RT-PCR was performed using standard conditions to quantify mRNA levels of human VEGF-A, IL-6, VEGFR1, Ve-Cadherin and CXCL12 mRNA.
in order to identify angiogenesis-related genes in BNKL as compared to normal NK cells. For microarray analysis, RNA samples were processed for this hybridization onto Affymetrix chips (Affymetrix Human Gene 1.0 ST). After normalization and transformation of the signal into Log2 values, expression data in BNKL was compared to data from the healthy NK sample and results are shown as fold-change. Hybridizations of the samples and bioinformatics analyses were conducted by the Genomics and Bioinformatics core facility at CIMA, University of Navarra.

**Immunohistochemistry and quantification**

Tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned (5 μm in thickness). Slides were stained with H/E. For immunohistochemistry, slides were deparaffinised, hydrated through graded alcohols, and incubated for 10 min with 3% H₂O₂ in water to quench the endogenous peroxidase activity. An antigen retrieval method was used for detection of the antibodies. Slides were incubated with goat normal serum in TBS (0.01 M Trizma Base, 0.1M NaCl, pH 7.36) for 30 min at room temperature (RT). Dilutions of primary antibodies were as follows: 1:400 for anti-PCNA (Proliferating cell nuclear antigen, Clone PC10, Dako); 1:200 for Caspase-3 (Cleaved Caspase-3 Asp 175, Cell Signaling); 1:20 for CD31 (Clone SZ31, Dianova); 1:100 for hVEGF (Santa Cruz biotechnology). Primary antibodies were incubated at 4°C overnight or for 1 h at RT in the case of CD31. Tissues were washed in TBS and incubated with the appropriate secondary antibody. Afterwards, slides were incubated for 30 min with the EnVision™ anti-rabbit detection system (Dako). Peroxidase activity was carried out with DAB (3,3'-diaminobenzidine, Dako). Finally, slides were counterstained with hematoxylin, dehydrated, and mounted. For quantifications, 30 random images (x200) per experimental group were captured with a microscope (Leica, Wetzlar, Germany) equipped with the Analysis™ software. Positive cells were quantified with Image J (Java image processing program developed by NIH, National Institutes of Health). Measurements are given as relative area occupied by positive signals with respect to the reference area.

**Drug and schedule**

Lenalidomide was generously provided by Celgene Corporation (Summit, NJ, USA). The drug was prepared fresh daily just before gavaging with the concentration of
50 mg/kg/day, dissolved in 0.5% carboxymethyl-cellulose, 0.25% tween 80, to avoid drug hydrolysis in the aqueous medium.

**Luminex xMAP® Technology analysis**

PB samples were collected at mice sacrifice. An aliquot was kept to obtain serum, after blood centrifugation at 1500 rpm for 15 min at 4°C. Samples were aliquoted and stored at -80°C. Defrosted serum samples were analyzed with an xMAP multiplex immunobead assay technology (Luminex, Corp., Austin, TX) to simultaneously quantify different mouse cytokines (IL6, TNFα, VEGF); a second kit was used to quantify human TNFα. The xMAP technology is based on binding of the proteins of interest to antibodies linked to microspheres with an internal spectral code specific for each protein. The amount of the bound proteins is determined by a second antibody labelled with a fluorescent dye. 25 µl of each serum sample were assayed in duplicate in a 96 well microtiter filter plate by Milliplex™ MAP human (Millipore Co., Billerica, MA), according to the manufacturer’s instructions. The potential biomarkers were selected according to previous publications describing the role of these molecules in lenalidomide clinical activity. Measurements were performed in a Luminex™ IS100/200 instrument. Concentrations of each protein were assessed according to standard calibration curves, analyzing the median fluorescent intensity (MFI) data with the 5-parameter logistic curve-fitting method.

**Statistical analysis**

Statistical differences between groups were examined using the Student’s *t* test for unpaired data for parametric variables, and the Mann-Whitney *U* test for unpaired non-parametric variables. Normality was analyzed with the Shapiro-Wilks test. Data were analyzed with the SPSS statistical software (version 15.0 for Windows SPSS). *P* values lower than 0.05 were considered as statistically significant. Time-to-event variables were assayed with the Kaplan-Meier test (GraphPad Prism version 5.00).
**Results**

**Patient clinical diagnosis**

The patient, a male of 52 years of age at the time of diagnosis, was referred to our Cancer Center because of multiple, palpable, enlarged lymph nodes, a skin lesion near the left nipple, and night sweating. A CAT scan described a variety of mediastinal, axillary and abdominal enlarged lymph nodes with diameters ranging from 10 to 25mm and an enlarged spleen. After the surgical removal of the skin lesion, a diagnosis of BNKL was made according to the histological and immunophenotypic standards and characteristics described in the WHO classification system. At diagnosis, immunophenotypic analysis was CD45+, CD4+, CD56+, CD7+, CD19-, CD3-, CD14-, CD33+, CD13-, MPO-, Tdt- (Supplementary Figure 1A). This immunophenotypic analysis showed 80% of BNKL blast cells in the BM and 70% in the peripheral blood (PB). Patient karyotype was:

44,XY,+der(1)t(1;21)(q21;q22),t(2;7)(q23;p13),der(9)t(9;13)(p21;q21),-13,-15,der(15)t(15;19)(p?13;q15),-21 [28]/ 46,XY [2].

In order to define molecular alterations occurred in this neoplasm, a microarray analysis was performed to compare gene expression profiles of BNKL with healthy NK cells. Using 2-fold change criteria, a total number of 3075 genes (for BM) and 2799 genes (for PB) were found in comparison with normal NK cells, thus showing numerous differences in the transcriptomes between these samples. However, comparison of transcriptomes in both malignant samples (PB and BM) revealed a very similar profile (with variation in only 233 genes among them). Many of the genetic alterations found in the malignant samples were related to cell cycle, growth factors, TNF- and INF-cell regulation, apoptosis, immunoglobulins, angiogenesis and lymphocyte-related cell signalling. According to the gene expression results, TGF-β and JAK/STA pathways seemed to be profoundly altered in this neoplasm. A brief list of relevant deregulated genes was selected based on their possible involvement in BNKL and is shown in Table 1.

To further measure the angiogenic profile of BNKL, quantitative RT-PCR analyses for angiogenesis-related genes was performed. Results showed that VEGF-A, IL-6, VEGFR1, Ve-Cadherin and CXCL12 mRNA were over-expressed in BM BNKL cells compared to normal CD56+ cells purified from healthy blood donors (Supplementary Figure 1C).
The patient has received several lines of chemotherapy including CHOP, ESHAP, and an autologous stem cell transplant after BEAM. After failure of these therapies, the patient is currently undergoing a stem cell transplant from an unrelated donor after reduced-intensity conditioning.

**Development of a xenograft mouse model of human BNKL**

Two months after BNKL cell injection the number of human BNKL cells circulating in the murine PB begun to rise above the detection limit of the FC procedure used (0.1%). The phenotype and morphology of human cells in NSG mice overlapped with that observed in the patient (Figure 1A-C). Near by day 100, BNKL-related symptoms were observed (animals were hunch-backed and ruffled coat), and mice began to die. At necropsy, mice had splenomegaly (Figure 1D) and enlarged lymph nodes. Tumor cell invasion of mouse tissues such as BM (Figure 1C, bottom panel), spleen (Figure 1D, right panel) and lymph nodes (Figure 1E) can be appreciated by IHC. This underlines again the typical tropism of lymphoma cells to spleen, as diagnosed in the original patient. BNKL cells were found by FC in PB (11%), BM (35%) and in the spleen (68%).

In secondary, tertiary NSG mice transplants, obtained by injection of a cell suspension of mice spleen, the BNKL engraftment kinetic and the time of symptom onset and death were not significantly different (Supplementary Figure 2A-B).

On day 100, BNKL-related symptoms and lymphoma cells were observed in 70% (7/10), 67% (4/6) and 100% (13/13) of NS, NSB and NSG mice, respectively. These data suggest, as previously demonstrated in AML models \(^{25}\), that NSG mice are more prone to the engraftment of human haematological malignancies compared to other NOD SCID-related strains. It is relevant to mention that culture with different types of feeder layer (such as irradiated BM or HK follicular dendritic cell line \(^{27}\)) or with conditioned supernatant obtained by bladder carcinoma cell line 5637 \(^{28}\), were unable to sustain the growth of these cells. Therefore, our orthotopic mouse model is a unique in vivo model to study this disease.

**Therapeutic effect of lenalidomide in the human BNKL mouse model**

So far, lenalidomide has been used to treat hematological neoplastic diseases such as multiple myeloma, myelodysplastic syndromes and lymphoma \(^{15-16, 29-30}\); with some
encouraging evidence of possible clinical activity in colorectal carcinoma. We therefore decided to test this drug in our model of human BNKL. Two weeks after the primary cells injection mice were divided in two groups; a group was treated daily for 3 months by gavage with lenalidomide (Lena group); another group was administered with vehicle, a solution of carboxymethyl-cellulose/tween 80, used to dissolve the drug (control group). To monitor engraftment during treatment, we performed successive blood collections from the tail vein and observed the presence of human neoplastic BNKL cells by FC. As Figure 2A shows, human engraftment was significantly reduced in the treated group compared with the control group. The low levels of engraftment present before treatment in Lena group decreased during treatment (Figure 2A). In agreement with these data, also spleen echographies (taken at day 83 after BNKL cell injection, and after 69 days of treatment) revealed a significant reduction in spleen areas of Lena group compared with control and healthy groups (Figure 2B).

At sacrifice, the first macroscopic difference we observed, in the lenalidomide treated mice, was a reduction of the splenomegaly with respect to the control group (Figure 2C). As observed in H/E staining, spleens of treated mice did not show any alterations and are comparable to healthy control spleens (Figure 2C-D,1D). Lenalidomide improved significantly the survival (p = 0.0002) of treated mice compared to control mice that dead by day 100 (Figure 3C). We evaluated PB, BM and spleen BNKL cell engraftment by FC (Figure 3A). BNKL cells were below the detection limit in PB, BM and spleen in the Lena group, while the mean of the percentages of BNKL engraftment in control mice spleen was about 68%. Moreover, since NK cells express TNFα, and lenalidomide inhibits TNFα production, we decided to evaluate serum values; in the Lena group, cytokine levels were significant decreased with respect to the control group (p = 0.046, Figure 3B). As it occurs for hTNFα, we observed the same behavior for hVEGF, highly expressed by BNKL cells and reduced after treatment (Figure 3D).

Although lenalidomide has numerous putative mechanisms of action which in particular involve the immune system, it also exerts a demonstrated antiangiogenic effect as well. To evaluate the antiangiogenic effect in our model, levels of CECs and CEPs were quantified at mice sacrifice. A significant decrease was found in CECs and CEPs levels in treated mice compared to untreated ones (Figure 4A). Moreover, spleens and BM IHC analysis of CD31+ mouse endothelial cells showed significant increased levels in the control and healthy groups compared to the Lena
group (Figure 4B, Supplementary Figure 2C). Since VEGF and IL6 are molecules directly involved in angiogenesis we decided to evaluate their levels in serum samples collected at mice sacrifice. We obtained significant differences between control and tumor bearing mice for IL6 (p = 0.049) and VEGF (p = 0.03). However, IL6 and VEGF levels were similar between treated and untreated groups (Figure 4C).

It has been described that lenalidomide might have a direct cytotoxic activity against tumor cells 15. In this regard, IHC revealed that levels of active Caspase-3 were significantly augmented in Lena group spleens compared to healthy spleen and control group (untreated tumor-bearing mice). This finding demonstrates the in vivo proapoptotic effect of lenalidomide (Figure 5A). Moreover, an opposite result was found for PCNA, a marker of proliferation (Figure 5B), thus indicating that treatment might inhibit cell proliferation and cause cell cycle arrest.

To verify that lenalidomide was able not only to prevent tumor development, but also to reduce engraftment in established tumors, we performed another experiment starting the treatment 2 months after BNKL cell injection. Mice survival was significantly increased in treated mice compared with tumor-bearing untreated animals (p = 0.0004) (Figure 6A). When all the untreated mice were sacrificed evaluation of human engraftment showed a significant reduction in the Lena group compared with control group (Figure 6B).
Discussion

BNKL is a malignancy with an aggressive clinical course and no effective clinical options. We developed what, to the best of our knowledge, should be considered the first preclinical orthotopic model of human BNKL. Tumor cells were able to grow in NSG mice and consistently replicate a pathology that reflects immunophenotypically and histologically the original cells injected. Human cells found in 3 mouse compartments (PB, BM and spleen) had a BNKL phenotype. Moreover, we observed involvement of lymph nodes and splenomegaly, which are typical characteristics found in BNKL patients at diagnosis. The course of the pathology in mice was aggressive: after injection, BNKL cells could be found often at 2 months, lymphoma manifestations occurred rapidly within the third month and mice did not survive more than about 100 days. Our study also shows for the first time (to our knowledge) a transcriptomic analysis of BNKL, which may serve to better understand the disease and to identify potential future targets for therapy. Many genes have been found deregulated in this neoplasm in comparison to healthy NK cells. Of note, genes related to activation of TGF-ß, JAK/STAT, and TNF/INF signalling cascades seem to play a role in this malignancy. Interestingly, topoisomerase II-a (Tpo2a), a therapeutic target for many solid and haematological tumors is highly increased in BNKL.

We could reproduce this model not only by injecting primary cells derived from the patient, but also by injecting human cells obtained by mouse spleen dissociation. We did not observe any particular changes in BNKL growth kinetics in secondary and tertiary recipients nor differences in cell antigens expression, and tumor aggressiveness. Moreover, it is relevant to mention the lack of BNKL cells ability to grow in vitro, under different conditions; they are able to replicate only in vivo. This represents a limitation of the model that hinders molecular characterization of the cells and testing antitumor compounds in vitro. Our orthotopic model is therefore, a unique in vivo model to study this disease.

We were also able to demonstrate that preclinical BNKL treatment with lenalidomide results in an effective antitumor activity. Lenalidomide exerts antitumor activity through multiple pathways, and this drug is currently used in a variety of neoplastic diseases. In our BNKL model we saw a significant difference in tumor engraftment between control and Lena groups in a prevention experiment. Once mice were sacrificed, none of the 3 compartments analyzed (PB, BM and spleen) in treated
mice were positive for the presence of BNKL cells. Because of the lack of a mature immune system in NSG mice, NK deficiency, and lack of γδ of IL2, we could affirm that the action of lenalidomide in our model does not involve these mechanisms. It is likely that impairment of angiogenesis is playing a role in this model. Indeed, thalidomide and its derivatives, such as lenalidomide, reduce levels of angiogenic factors such as VEGF and bFGF. The antiangiogenic effect of thalidomide has been demonstrated in a variety of xenograft models. In keeping with these results we have found a significant reduction in the number of CECs and CEPs and of murine endothelial cells. However, our current model can not rule out that the reduction in angiogenic cells was simply due to a lower (or absent) tumor growth. Since lenalidomide has been shown to alter cytokines production, we measured hTNFα serum levels and we observed downregulation after treatment in our model (Figure 3B). Moreover, we measured IL6 and VEGF serum levels and we obtained significant differences between control and tumor bearing mice. However, we did not find a decrease in either IL6 or VEGF levels after administration of lenalidomide, in spite of the sharp decrease in tumor growth and vascularization. It was previously shown that anti-angiogenic drugs may sustain the expression of VEGF and other angiogenic factors due to drug-induced hypoxia. It is possible then that lenalidomide is causing a similar effect in this model.

Interestingly, in mice treated with lenalidomide, levels of active Caspase-3 were significantly augmented, thus demonstrating proapoptotic and cytotoxic effects of this drug in vivo. Lenalidomide has also been shown to inhibit proliferation in Burkitt's lymphoma cell lines and MDS cells. In agreement with these findings we observed a decrement in PCNA expression, suggesting a possible induction of cell cycle arrest.

In summary, we have developed the first preclinical model for BNKL, a rare and aggressive neoplastic disease with no effective treatment. Our model will serve to better define the tumor biology and the molecular mechanisms involved in this pathology. Administration of Lenalidomide may offer a therapeutic option for BNKL patients.
Acknowledgements

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References


Figure 1: Development of a xenograft mouse model of human BNKL
(A) FC analysis of the BNKL patient at the time of diagnosis. BM cells are evaluated for CD45 (APC), CD4 (FITC) and CD56 (Per). (B) Representative dot plots of mouse BM. BNKL cells are conserving the original CD45+CD4+CD56+ phenotype. (C) Representative NKL morphology at the time of diagnosis stained with May Grünwald Giemsa (upper panel, x400), and H/E mouse BM cells (bottom panel, x400). (D) Representative images of the enlarged spleen (right) found in NSG mouse injected with BNKL human cells, compared with the healthy control spleen (left), 100 days after injection, with respective representative spleen histologic slides stained with H/E show spleen involvement in tumor-bearing mice compared with healthy controls (x200). (E) Representative enlarged lymph node found in BNKL mice (x40, x200)

Figure 2: Treatment of xenograft model of BNKL with Lenalidomide
(A) Representative human BNKL engraftment kinetics (investigated in PB by flow cytometry through the presence of human CD45+CD4+CD56+ cells) in mice treated (Lena group) or untreated (control group). Treatment started at day 14 after BNKL cells injection. The graphic on hCD45+ kinetics of the Lena group is also represented in the smaller graph above using a lower scale. This representation shows a total absence of BNKL cells at day 100 after injection. After 69 day of therapy, engraftment differences between treated and untreated group are statistically significant (Lena p= 0.045).
(B) Average spleen area evaluated by echography confirms in vivo the reduction of the spleen after 69 days of lenalidomide administration. The area was calculated by multiplying the dimension of the sagittal axis by the transverse axis, measured by echographic technique (Lena vs control, p= 0.038). (C) Representative images of spleens of the control and Lena treated group at the end of experiment. (D) Histological analysis of the spleen shows differences between the two groups (x200). As observed on the right panel, the spleen of untreated mice is packed with BNKL cells, whereas that of treated mice resembles a normal spleen (similar to image of healthy control in Figure 1D).

Figure 3: Engraftment comparison between control mice and treated mice
(A) At sacrifice, human engraftment in the control and Lena groups was evaluated by FC in mouse PB, BM and spleen. BNKL cells were below the detection limit in three compartments of the treated group, while the percentage of BNKL cells in control mice
spleen was about 68%. Differences between the control group and the treated group in the three compartments were significant (PB p= 0.010; BM p= 0.020; Spleen p= 0.021).

(B) Serum human TNFα values evaluated at mice sacrifice. Differences between the two groups (control-Lena) were significant (p = 0.046).

(C) Lenalidomide treated mice show a significant higher survival compared to control mice that die by day 100 (p= 0.0002).

(D) hVEGF high level expression is observed in control spleen compared to treated mice (x400, p < 0.0001)

**Figure 4: Lenalidomide antiangiogenic action**

(A) At sacrifice, the number of mouse CECs and CEPs per μl decreases significantly (CEC: p= 0.009; CEP: p= 0.030) as a consequence of the administration of lenalidomide. These results are paralleled by spleen IHC evaluation of CD31+ murine endothelial cells. Significant (p < 0.0001) differences were observed between the control and the Lena group or the healthy group (B).

(C) Mouse serum IL6 and VEGF evaluation in the three groups.

**Figure 5: Immunohistochemical analysis of lenalidomide action in BNKL in vivo**

Quantifications of active Caspase-3 (A) and PCNA (B) measurements are given as relative area occupied by positive signals with respect to the reference area. Significant differences (Caspase-3: p < 0.0001; PCNA: p < 0.0001) are observed between the control group and the Lena or healthy groups. In panels below, representative IHC images of active Caspase-3 (x1000) and PCNA (x400) of spleen in the three groups of study are shown.

**Figure 6: Lenalidomide effect on established tumors**

(A) Kaplan Mayer survival curves of control and Lena groups. Lenalidomide administration when tumor was established (2 months after BNKL cells injection) significantly improved mice survival (p = 0.0004). (B) At 50% survival of control mice (day 80), significant differences are found between PB engraftment of treated and not treated mice (p = 0.007). Eight mice per group are used.
Figure 1
Figure 2
**Figure 3**

A. PB

- % hCD45
- Control vs Lena

B. hTNFα

- pg/ml
- Control vs Lena

C. Estimated survival probability (%)

- Control vs Lena
  - Time (day)
  - p=0.0002

D. hVEGF

- Healthy, Control, Lena
- % relative area

- Healthy vs Control vs Lena
Figure 4
Figure 5

A) Caspase-3

- Healthy
- Control
- Lena

B) PCNA

- Healthy
- Control
- Lena

* * *
Figure 6

**Figure 6**

**A** Estimated survival probability (%)

- Control
- Lena

\[ p = 0.0004 \]

**B** % hCD45+

- Control
- Lena

**Figure 6**

**A** Estimated survival probability (%)

- Control
- Lena

\[ p = 0.0004 \]

**B** % hCD45+

- Control
- Lena

**Figure 6**
Table 1. Selected list of genes deregulated in BNKL in comparison with normal NK cells.

<table>
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<th>Gene name</th>
<th>Fold change BM</th>
<th>Fold change PB</th>
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**Adhesion and angiogenesis**

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Therapeutic effect of Lenalidomide in a novel xenograft mouse model of human Blastic NK cell lymphoma/blastic plasmacytoid dendritic cell neoplasm

Alice Agliano, Ines Martin-Padura, Paola Marighetti, et al.

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