Nutlin-3a enhances natural killer cell–mediated killing of neuroblastoma by restoring p53-dependent expression of ligands for NKG2D and DNAM-1 receptors

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Abstract

In this study, we explored whether Nutlin-3a, a well-known nontoxic small-molecule compound antagonizing the inhibitory interaction of MDM2 with the tumor suppressor p53, may restore ligands for natural killer (NK) cell-activating receptors (NK-ARs) on neuroblastoma (NB) cells to enhance the NK cell-mediated killing. NB cell lines were treated with Nutlin-3a, and the expression of ligands for NKG2D and DNAM-1 NK-ARs and the NB susceptibility to NK cells were evaluated. Adoptive transfer of human NK cells in a xenograft NB-bearing NSG murine model was assessed. Two datasets of NB patients were explored to correlate p53 expression with ligand expression. Luciferase assays and chromatin immunoprecipitation (ChIP) analysis of p53 functional binding on *PVR* promoter were performed. Primary NB cells were also treated with Nutlin-3a, and NB spheroids obtained from one high-risk patient were assayed for NK-cell cytotoxicity. We provide evidence showing that the Nutlin-3a-dependent rescue of p53 function in NB cells resulted in: (*i*) increased surface expression of ligands for NK-ARs, thus rendering NB cell lines significantly more susceptible to NK cell-mediated killing; (*ii*) shrinkage of human NB tumor masses that correlated with overall survival upon adoptive transfer of NK cells in NB-bearing mice; (*iii*) increased expression of ligands in primary NB cells and boosting of NK cell-mediated disaggregation of NB spheroids. We also found that p53 was a direct transcription factor regulating the expression of PVR ligand recognized by DNAM-1. Our findings demonstrated an immunomodulatory role of Nutlin-3a, which might be prospectively employed for a novel NK cell-based immunotherapy for NB.

Introduction

Neuroblastoma (NB), the most common extracranial tumor of childhood, is still challenging, with 3-yr. event-free survival lower than 40%, despite intensive multimodal therapies (1). Thus, new treatment strategies are warranted, mainly aimed to restore impaired antitumor immune responses against NB (2-4). Indeed, NB evades NK cell-mediated innate immunosurveillance through the downregulation of ligands for NK cell-activating receptors (NK-ARs)(3,5,6). The restoration of the expression of such ligands represents a strategic approach to boost NK cell-mediated antitumor responses against NB. Interestingly, whereas ULBP1, ULBP2, and ULBP3 ligands for the NKG2D receptor are known to be regulated by c-MYC and p53 transcription factors (7,8), no mechanism regulating the expression of ligands for DNAM-1 receptor have been reported so far. Of note, wild-type p53 is found in most NB cases, with a rare exception with relapse (9). However, p53 is functionally impaired due to *MYCN* amplification, one of the major NB prognostic factors (1), that upregulates not only p53 but also the p53-antagonist MDM2 (10-12). Accordingly, we previously found that the MYCN expression inversely correlated with expression of ligands for both NKG2D and DNAM-1 NK-ARs in NB (3). The BET-bromodomain inhibitor JQ1-dependent repression of MYCN leads to downregulation of both c-MYC and p53, resulting in the impaired expression of ligands for both NKG2D and DNAM-1 NK-ARs, thus rendering NB cell lines more resistant to NK cell-mediated killing (13).

Nutlins are nontoxic, small-molecule antagonists of p53-MDM2 interactions, known to restore p53-mediated cell cycle arrest and apoptosis in tumors (14). Among the MDM2-targeting compounds, Nutlin-3a has been mainly explored for its promising therapeutic potential in preclinical studies of leukemia, multiple myeloma, rhabdomyosarcoma (15-22), and NB cell lines (21,23-27). The clinical adoption of several Nutlin-3a analogues are currently under clinical investigation not only in hematological malignancies, but also in

several solid tumors such as sarcoma, glioblastoma, merkel cell carcinoma, small-cell lung cancer, and breast cancer (clinicaltrial.gov). The molecular characteristics of nontoxicity, cell permeability, and the wide-spectrum of antitumor functions render Nutlin-3a, and its analogues, optimal candidates for new therapeutic approaches in NB. In light of these evidences, we investigated whether the rescue of p53 activity by Nutlin-3a could enhance the expression of ligands for NKGD2 and DNAM-1 NK-ARs and, as a consequence, the NK cell-mediated recognition and killing of NB.

Materials and Methods

Patient samples

The study was conducted in accordance to Declaration of Helsinki. Bone marrow (BM) aspirates from 26 neuroblastoma (NB) patients diagnosed at onset in our institute and off therapy were used to isolate primary tumor cells for *in vitro* experiments with Nutlin-3a as described below. Tumor samples from 36 NB patients diagnosed in our institute were used for NanoString assays. For each patient, written informed parental consent and approval by the Ethical Committee of the Institution were obtained. Demographic, molecular, and histological features of the cases studied for *in vitro* experiments are detailed in Table 1. Staging and histological classification were performed according to the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Pathology Classification (INPC) (28,29), respectively. *MYCN* expression was evaluated following current guidelines (30) and together with *MDM2*, *TP53*, *MYC* and *ALK* gene status were measured by array CGH or IQ-FISH assays (as described in Table 1 and below). NB samples were stored in the BIT-NB Biobank of IRCCS (Gaslini, Genova, Italy) and obtained before treatment at the time of diagnosis. Tumor content was confirmed by local pathologists' reviews of hematoxylin and eosin stained tumor sections. Tumor DNA was extracted from fresh NB tissue using the MasterPure DNA Purification Kit (Epicentre-Illumina, Madison, WI), according to the manufacturer's instructions.

The correlation analysis between p53 and ligands for NK-ARs was evaluated in a cohort of 143 NB patients (Target 2018) available in cBioportal.org web site (www.bioportal.org). Bioinformatic analysis was performed by Transcription Factor Affinity Prediction (TRAP) website (trap.molgen.mpg.de)

NB cell lines, primary NB cells, NK cells, and reagents

Human NB cell lines were obtained as follows: SK-N-AS, SH-SY5Y, and SK-N-BE(2)c from the American Type Culture Collection (ATCC); LA-N-5 from the Leibniz-Institut DMSZ; SMS-KCNR from Children's Oncology Group Cell Culture. All NB cell lines were characterized by i) HLA class I typing by PCR-SSP sets (Genovision) according to the instructions of the manufacturer, and ii) array CGH (see below). The human erythro-leukemia cell line K562 was purchased from ATCC and used as a control target for NK cell functional assays. Cells were grown in RPMI 1640 medium supplemented with 10% FBS

(Thermo Fisher Scientific), 2 mM glutamine, penicillin (100 mg/mL), and streptomycin (50 mg/mL; Euro Clone S.p.a.). NB cell lines have been re-authenticated by BMR Genomics through PowerPlex Fusion System kit (Promega) and Applied Biosystem 3130XL (Life Technologies). Cell lines were kept frozen in liquid nitrogen, and after thawing, they were cultured for 2-3 weeks and passed 4-6 times before experimental use. All cell lines were routinely checked for the mycoplasma contamination prior the use.

Nutlin-3a (Cayman Chemical) was dissolved in DMSO (10 mM) and diluted in medium at the indicated doses for the *in vitro* experiments, or dissolved in ethanol (25 mg/mL) and diluted for the indicated concentration in 2-hydroxypropyl-β-cyclodextrin [HPβCD (SIGMA), 1 gr/10mL H_2O + 1% DMSO], which was also used alone as a vehicle control for *in vivo* experiments.

Primary NB cells were isolated from BM aspirates of NB patients by depleting $CD45^+$ cells with RosetteSep human CD45 depletion cocktail (cat# 15162, StemCell Technologies), followed by Ficoll-Paque Plus (Lympholyte Cedarlane) centrifugation, which allowed to negatively select primary NB cells with a purity of about 90% ($GD2^+$) as evaluated by flow cytometry. Primary NB cells were resuspended at $2x10^6$ cells/mL in RPMI (supplemented as above) with 20% FBS and cultured at 200 μL/well in 96-well flat-button plates. All primary NB cells grew in adherence with monolayer distribution, with the exception of those isolated from patient 1 (p1) which formed spheroids. After 5 days, medium was refreshed, and at 10-12 days cells, were treated with 2 μM of Nutlin-3a for 48 hours for *in vitro* experiments. The spheroids of p1 were split twice after 12 days of culture in 96-well flat-button plates, treated with 2 μM of Nutlin-3a for 48 hours or DMSO as control (four wells per condition), and evaluated using an optical microscope (Leica) before and after coculture with $200x10^3$ human NK cells/well (see below).

Human NK cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers with the RosetteSep NK-cell enrichment cocktail (cat# 15065, StemCell Technologies), followed by Ficoll-Paque Plus centrifugation, which allowed to negatively select NK cells with a purity of about 98% ($CD3^-CD56^+$) as evaluated by flow cytometry. NK cells were routinely checked, for the expression of NKG2D and DNAM-1 activating receptors, and NKG2A, KIR2DL1, KIR2DL3, and KIR3DL1 inhibitory receptors by flow cytometry. NK cells with purity greater than 90% and positive for all four inhibitory receptors were resuspended in NK MACS medium (Miltenyi Biotec) supplemented with NK MACS Supplement, human AB serum, and recombinant human IL2 (500 IU/mL;

PeproTech). NK cells were cultured at 37°C, split every three days, and used up to 20 days after isolation for both *in vitro* and *in vivo* experiments. All NK-cell function assays were performed in an alloreactivity setting (31,32).

Plasmid construction, transfection, and luciferase reporter assay

The *PVR* promoter regions spanning from nucleotide (nt) 879 upstream of the transcriptional start site (TSS) (corresponding to nt 622-1501 of the *PVR* promoter sequence) or nt 437 upstream of the TSS (corresponding to nt 1064-1501 of the *PVR* promoter, deleted for the predicted p53 binding sites, spanning from 879 to 437 upstream the TSS) were cloned into the pGL3-basic vector (Promega, Madison, WI, USA) by adding BglII and HindIII restriction sites upstream and downstream of the sequences, respectively (sequences and schemes of cloned *PVR* promoter regions in Supplementary Table S1). The plasmids were named as pGL3-*PVR* promoter (*PVR*-promoter) and pGL3-*PVR* promoter p53-deleted (*PVR* promoter p53-deleted), respectively. The pRL-TK vector (Promega, Madison, WI, USA) was used as an internal reference in the luciferase reporter system. An empty pGL3 (basic) was used as a negative control. Plasmids were transfected into LA-N-5 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The day before transfection, cells were seeded in 12-well plates. At 80% confluency, the plasmids were transiently transfected according to the manufacturer's protocol. For each well, 1 μg of the luciferase-containing plasmid [pGL3-*PVR* promoter (*PVR*-promoter) and pGL3-*PVR* promoter p53-deleted (*PVR* promoter p53-deleted)] and 0.07 μg of the renilla-containing plasmid pRL-TK vector (Promega, Madison, WI, USA) were used. All transfections were carried out in quadruplicate. After 24 hours, cells were lysed with passive lysis buffer (Promega, Maddison, WI, USA), and both renilla and firefly luciferase activities were measured by using a Triathler Multilabel Tester (Hidex), after incubation with the relative Dual Luciferase Reporter Assay System reagents (Promega, Maddison, WI, USA) according to the manufacturer's protocol. The luciferase activity relative to renilla was represented as mean±standard error of mean (SEM).

Xenograft neuroblastoma model and treatment of NB-bearing NSG mice

All animal experiments were performed in accordance with a protocol approved by Italian Ministry of Health and our institutional animal care. All *in vivo* experiments utilized 4-6-week-old female NSG (NOD.Cg-Prkdcscid IL-2rgtm1Wjl/SzJ) mice (Charles River

Laboratories Italia srl). Briefly, 5 x 10^6 cells LA-N-5 NB cells resuspended in 100 mL PBS were injected subcutaneously into flank of mice (33). NB-bearing NSG mice were used for both the *in vivo* experiments to evaluate the effectiveness of Nutlin-3a in inducing both ligand expression and NK cell-mediated antitumor activity. In the first experiment, 20 to 25 days after the injection of LA-N-5, when tumor masses reached about 50 mm^3, LA-N-5-bearing NSG mice were randomly divided in three groups (6 mice for each group): control group mice were injected intraperitoneally (i.p.) with vehicle for Nutlin-3a or HPβCD; experimental group mice were injected with 20mg/kg (mg of Nutlin-3a/mouse body weight) and 40mg/kg Nutlin-3a, every two days for 12 days. At the end of treatment, about 35 days after the LA-N-5 injection, tumor masses were harvested and used for photo acquisition and IHC analysis as described below. In another set of experiments, LA-N-5-bearing NSG mice were randomly divided into groups of 6 mice. For controls, mice were injected with HPβCD (i.p. as vehicle in place of Nutlin-3a) and with PBS (peritumoral as vehicle in place of NK cells; i.p. as vehicle in the place of IL-2). For treatments, LA-N-5-bearing NSG mice were divided into 5 groups (g): vehicles only (i.p. HPβCD and both i.p. and peritumoral PBS, g1); i.p. IL2 and vehicles (i.p. HPβCD and peritumoral PBS, g2); i.p. Nutlin-3a and vehicles (both peritumoral and i.p. PBS, g3); peritumoral human NK cells, i.p. IL2, and vehicle (i.p. HPβCD, g4); i.p. Nutlin-3a, peritumoral NK cells, and i.p. IL2 (g5). In the experimental groups g3 and g5, 20 to 25 days after the injection of LA-N-5, when tumor masses reached about 50 mm^3, mice were injected i.p. with Nutlin-3a (40 mg/kg) every two days for two weeks. In the experimental groups g4 and g5, 5 x 10^6 of NK cells were peritumorally injected after 48 hours of the first Nutlin-3a treatment and every 5 days for two weeks. In the experimental groups g2 and g5, IL2 ($10x10^3$ U/mouse) was injected i.p. the same day of NK cell injection and for another consecutive three days. Three treatment cycles were performed for each experiment. Tumor size was assessed every two days by caliper measurement. At the end of treatment, 55 to 60 days after LA-N-5 injection or 35 days after the start of treatment, tumor masses from g1, g2, g3, g4, and g5 mice were harvested and used for photo acquisition and IHC analysis as described below. For overall survival experiments, mice were kept alive for up to nearly 50 days or sacrificed when tumor masses reached almost 2 cm^3.

Antibodies, flow cytometry, apoptosis, and Western blotting

The following antibodies for flow cytometry, with clones indicated in parentheses , were used : anti-GD2-Alexa Fluor-647 (14.G2a), anti-CD107a-FITC (H4A3), anti-CD3-Alexa Fluor-700 (UCHT1), anti-CD56-PE-Cy7 (B159), anti-CD45 (HI30), anti-NKG2D-BV605 (1D11) purchased from BD Biosciences; anti-DNAM-1-APC (11A8) purchased from Biologend; anti-TIGIT (MBSA43) purchased from eBioscience; anti-KIR2DL1/2DS1-PC5.5 (EB6B), anti-KIR2DL2/L3/S2-PE (GL-183) purchased from Beckman Coulter; anti-NKG2A-Alexa Fluor-700 (131411), anti-KIR3DL1-APC (DX9), anti-ULBP1-PE (170818), anti-ULBP2/5/6-PE (165903), anti-ULBP3-PE (166510), anti-MICA (159227), anti-MICB (236511), anti-TRAIL/R2-APC (17908), anti-CD155/PVR-PE (300907), anti-Nectin-2/CD112-APC (610603) purchased from R&D Systems, goat F(ab')2 Fragment anti-mouse IgG FITC (IM1619) purchased from Dako. All these antibodies were used at the concentration according to the manufacturer's protocol. Apoptosis of tumor cells was evaluated with APC-conjugated AnnexinV (BD-Pharmingen) and propidium iodide (PI) (Sigma-Aldrich), used at the concentration according to the manufacturer's protocol and analyzed by flow cytometry. Flow cytometry was performed on FACSCantoII and LSRFortessa (BD Bioscences) and analyzed by FlowJo Software.

The following antibodies for immunohistochemistry, with clones indicated in parentheses, were used: anti-PVR (NBP1-88131) purchased from Space, anti-Nectin-2 (62540) purchased from Abcam, and anti-NKp46/NCR1 (195314) purchased from R&D Systems. All these antibodies were used at the concentration according to the manufacturer's protocol.

Human whole-cell extracts were quantified by a bicinchoninic acid assay (Thermo Fisher Scientific), resolved on 8-10% SDS-PAGE, and electroblotted. Filters were probed with primary antibodies for 3 hours at room temperature or overnight at 4°, followed by goat anti-mouse HRP-conjugated IgG (cod 115-035-003, Jackson ImmunoReaserch) at a 1:10.000 milk dilution (34). The following antibodies for Western blotting, with clones and milk dilution, respectively, indicated in parentheses, were used: anti-MYCN (B8.4.B, 1:200), anti-p53 (FL-393, 1:200), anti-p21 (C-19, 1:200), anti-Actin (I-19, 1:1000), all from Santa Cruz Biotechnology; anti-MDM2 (2A10, 1:1000) from Calbiochem-Millipore.

Array CGH and IQ-FISH

DNA from human NB primary tumors were tested by high-resolution array comparative genomic hybridization (a-CGH). The tests involved the use of a 4x180K platform (Agilent Technologies, Santa Clara, CA) with a mean resolution of approximately 25 kb. A copy number variant was defined as a displacement of the normal value of at least 8 consecutive probes and the mapping positions refer to the Genome Assembly GRCh38/hg19 (UCSC Genome Browser, http://genome.ucsc.edu, Feb. 2009 release). The data were analyzed using the Genomic Workbench 7.0.40 software (Agilent), the altered chromosomal regions and breakpoints events were detected using ADM-1 mathematical algorithm (threshold 10), with aa 0.5 Mb window size to reduce false-positives. The quality of the test was assessed on the strength of the QCmetrics values. Polymorphisms (http://projects.trag.ca/variation/) were not included because they were considered normal variants. The data generated in this study have been deposited in the GEO database under the accession number GSE145341.

Interphase quantitative fluorescence *in situ* hybridization (IQ-FISH) (35-37) was performed on 4-μm-thick paraffin-embedded NB tissue sections from patient 5, 6, 8, 9, 10, 15, 18, 20, and 22. Dual-color FISH probes containing *MYCN* (2p24) and *LAF* (2q11) control probes (labeled green; Kreatech Biotecnology, Amsterdam, The Netherlands) were used to assess *MYCN* gene status (amplified, not amplified) as recommended by the International Neuroblastoma Risk Group Biology Committee. The samples were imaged using the fluorescence microscope Axio Imager M2 equipped with ApoTome System (Carl Zeiss, Oberkochen, Germany).

Quantitative mRNA expression

Total human RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific), and 1 μg was used to synthesize first-strand cDNA using the SuperScript II First Strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) reactions were performed using pre-validated TaqMan gene expression assays (Hs00197846_m1 for *PVR*), and QuantStudio 6 Flex Real-time PCR System machine from Applied Biosystems, Thermo Fisher Scientific. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, and $2^{-\Delta Ct}$ considered as expression, with *GAPDH* (Hs02758991_g1) as the endogenous control.

ChIP analysis

LA-N-5 cells were cultured in cell culture dishes and at 80% confluency, were treated with DMSO as control or with Nutlin-3a (2 μM) for 16 hours. The cells were then treated with

formaldehyde (1% final concentration) for 10 minutes at room temperature, by adding it directly to the culture dishes, to cross-link protein complexes to the DNA. The reaction was stopped by adding glycine to a final concentration of 0.125M for 5 minutes at 4°C. Cells were washed with cold PBS, scraped and lysed in L1 buffer [2 mM EDTA, 50 mM Tris-HCl (pH 8.1), 0.1% NP40, 10% glycerol, and cOmplete™, EDTA-free Protease Inhibitor Cocktail according to the manufacturer's instructions] for 20 minutes at 4°C in rotation. The lysates were homogenized using 15 dounce-homogenizer strokes and then centrifuged at 5000 rpm for 5 minutes at 4°C. Nuclear pellets were resuspended in L2 buffer (5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1% SDS, and cOmplete™, EDTA-free Protease Inhibitor Cocktail according to the manufacturer's instructions) and kept for 20 minutes at 4°C in rotation. Nuclear lysates were sonicated with a Vibra-Cell™ Ultrasonic Liquid Processors to obtain chromatin fragments of an average length of 200 to 500 bp and centrifuged at 10000 rpm for 10 minutes at 4°C. After determining DNA concentrations, each chromatin sample was divided into aliquots of 150 µg. The sonicated supernatant fractions were diluted 10-fold with dilution buffer [5 mM EDTA, 50 mM Tris-HCl (pH8.0), 0.5% NP40, 200 mM NaCl, and cOmplete™ EDTA-free Protease Inhibitor Cocktail according to the manufacturer's instructions]. For each condition, one aliquot for the specific antibody (anti-p53, BK9282S CST, EuroClone) and one aliquot for the IgG control (BK2729S CST, EuroClone) were incubated with 40 µL Protein A Sepharose (GE Healthcare), saturated with 3% BSA and 200 µg/mL salmon sperm, for 3 hours at 4°C on a rotating platform. The pre-cleared chromatin samples were centrifuged at 13000 rpm for 30 seconds and incubated with 5 µg of the respective antibody or IgG overnight with gentle rotation at 4°C. Immuno-precipitated samples were recovered by incubation with 50 µL saturated Protein A Sepharose (GE Healthcare) on a rotating platform for 3 hours at 4°C. Before washing, the supernatant of the IgG control was taken as an input sample. After extensive washing (5 minutes at 4°C in rotation and subsequent centrifugation at 3000 rpm for 2 minutes) with wash buffers [2 washes with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl; 2 washes with 0.1% SDS, 1% triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl; 1 wash with 0.25M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1); 1 wash with 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)], samples were eluted in elution buffer (100 mM NaHCO3, 1% SDS) at room temperature twice while vortexing for 15 minutes. After each elution, samples were centrifuged at 13000 RPM for 5 minutes, and the eluate was collected. The samples were

treated with 10 μg RNAse A (Sigma Aldrich) for 10 minutes at room temperature and incubated at 67°C overnight to reverse the protein-DNA crosslinking. In each sample, $NaHCO_3$ was neutralized with 6 μL Tris-HCl 1M (pH 6-7.5). After treatment with proteinase K (Sigma Aldrich) for 2 hours at 56°C, DNA was extracted with phenol-chloroform (Invitrogen), precipitated with 100% ethanol, and resuspended in 50 μL of distilled water. 5 ng of the immunoprecipitated, IgG, and input samples were used for PCR with specific oligonucleotides spanning the *PVR* promoter: PVR P 53BS 3F AGGCTGGTCTTGAACTCCTG and PVR P 53BS 3R CCATTGCGCCACTACACTAC. The reaction was performed in triplicate using 5 ng of DNA, GoTaq qPCR Master Mix (Promega), and the relative qPCR primer pair in the thermocycler, CFX Connect Real-time PCR detection system (Bio-Rad). The primer pair efficiency, the relative quantity of each immunoprecipitated and IgG (ΔC(t)) with respect to the input sample, and the standard error of the mean (SEM) of the relative quantity were determined with CFX ManagerTM software (Bio-Rad). The percentage of the relative quantity of each immunoprecipitated sample was normalized with respect to IgG and expressed as percentage of input chromatin (% input).

NanoString assay

Total RNA was extracted from 36 fresh frozen NB samples using the Total RNA Purification Plus Kit (Norgen, Biotek Corp. Thorold) and purified with the RNA Cleanup and Concentration kit (Norgen, Biotek Corp. Thorold). RNA concentration was measured with Nanodrop 2000 (Thermo-Scientific), while RNA integrity and purity was evaluated with the RNA Bioanalyzer kit (Agilent Technologies). Differential expression of immune-related gene transcripts was determined using The human NanoString PanCancer Immune Profiling assay (https://www.nanostring.com/products/gene-expression-panels/gene-expression-panels-overview/hallmarks-cancer-geneexpression-panel-collection/pancancer-immune-profiling-panel), was used to evaluate the expression of 730 immune-related genes and 40 housekeeping genes according to manufacturer's protocol (NanoString Technologies, Inc., Seattle, WA). Briefly, 8 μL of the NanoString detection probe containing mastermix was incubated overnight with 100 ng of total RNA for each NB sample. After hybridization, the samples were loaded into 12-stripe NanoString tubes and placed in the nCounter Prep-station (NanoString) for purification. Gene expression was measured on the NanoString nCounter Analysis System (NanoString Technologies). The

raw NanoString RCC files were preprocessed by R/Bioconductor library NanoStringNorm as previously described (38). The data were normalized for i) the variation of the technical assay with the geometric mean of internal positive controls, ii) the background count with the mean plus two standard deviations, and iii) the RNA content with the geometric mean of housekeeping genes, following the manufacturer's recommendations (39). The normalized expression values were transformed into log2 and used for statistical analysis as described (40).

Cytotoxicity and degranulation assay

NK-cell cytotoxic activity was tested by a standard 4-hour ^{51}Cr-release assay. Briefly, ^{51}Cr (Amersham International; 100 μCi [3.7 MBq]/1 × 10^6 cells)-labeled K562 or NB target cells (5 × 10^3) were mixed with human NK cells from healthy donors at different effector-target (E/T) cell ratios and incubated at 37°C. After 4 hours of incubation, 25 μL supernatant were removed, and the ^{51}Cr release was measured with a TopCount NXT beta detector (PerkinElmer Life Sciences, Boston, MA). All experimental groups were analyzed in triplicate, and the percentage of specific lysis by counts per minute (cmp) was determined as follows: 100 × (mean cpm experimental release - mean cpm spontaneous release)/(mean cpm total release - mean cpm spontaneous release). Specific lysis was converted to lytic units (L.U.) calculated from the curve of the percentage lysis. One lytic unit was defined as the number of NK cells required to produce 20% lysis of 10^6 target cells during the 4-hour incubation.

Degranulation assays were performed by coculturing human NK cells from healthy donors with K562 or NB target cells at 1:1 ratio for 3 hours in complete medium in the presence of anti-CD107a (diluted 1:100). In the last 2 hours, GolgiStop (BD Bioscence) at a 1:500 dilution was added. Cells were then stained with anti-CD56 and anti-CD45 (both diluted 1:50), and the expression of CD107a was evaluated by flow cytometry in the $CD56^+CD45^+$ subset. In the blocking experiments, NK cells were pretreated for 20 minutes with 25 μg/mL of neutralizing anti-NKG2D (149810, R&D Systems) and anti-DNAM-1 (DX11, BD-Pharmingen), at room temperature before coculture with target cells.

Immunohistochemistry (IHC) and live cell imaging

Tumor masses from LA-N-5-bearing NSG mice were formaldehyde-fixed, paraffin-embedded, cut into 3 μm sections and baked for 60 minutes at 56°C in a dehydration oven.

Antigen retrieval and deparaffinization were carried out on a PT-Link (Agilent Technologies) using the EnVision FLEX Target Retrieval Solution kits at high pH (Agilent Technologies) for both PVR and Nectin-2, as per the manufacturer's instruction. Slides were then blocked for endogenous peroxidase for 10 minutes with a peroxidase-blocking solution (Agilent Technologies) or the avidin/biotin blocking system (Thermo Fisher Scientific, Fremont CA, USA), according to the manufacturer's instructions and then incubated for 30 minutes with 5% PBS/BSA. Slides were subsequently incubated overnight at 4°C with primary antibodies against PVR (1:200), Nectin-2 (1:300), and NKp46 (1:100) and subsequently with secondary antibody coupled to peroxidase (Dako, ready to use) or streptavidin alkaline phosphatase (Dako, ready to use). Bound peroxidase or strepatividin were detected with diaminobenzidine (DAB) and EnVision FLEX substrate buffer containing peroxide (Dako) or with Fast Red chromogen substrate (Dako) solution, respectively. Tissue sections were counterstained with EnVision FLEX haematoxylin (Agilent Technologies), and immunostained slides were acquired using a Nikon microscope [eclipse E600=upright microscope and NanoZoomer S60 digital slide scanner (Hamamatsu)]. IHC density was obtained by evaluating integrated optical density by Color Deconvolution plugin through Image J, measured in independent slide images acquired with the same optical microscopic parameters such as magnification, light exposure, and acquisition time. Sections of normal human intestinal mucosa and human colon carcinoma were used as positive controls for PVR and Nectin-2, respectively (Suppl. Fig. S6A). As negative controls, slides were processed with the same procedure described above with the exclusion of the primary Ab incubation, before being acquired.

For time-lapse experiments, $5x10^5$ of LA-N-5 or SMS-KCNR NB cell lines were marked with 1 μM of CellTracker Deep Red (Thermo Fisher Scientific), cultured in cell dishes (Ibidi) ideal for live cell imaging and high-resolution microscopy, and treated with 2 μM of Nutlin-3a for 48 hours. Then cancer cells were incubated for 15 minutes with 1 μM of CellTracker Deep Red (Thermo Fisher Scientific), a fluorescent dye well suited for tracking of cellular movements, that is retained inside cells through several generations. After removing the CellTracker, human NK cells ($2x10^6$ cells/mL) from healthy donors as described above were then added to cultured cells, and dishes were immediately imaged. Time-lapse acquisitions were performed by a Leica TCS-SP8X laser-scanning confocal microscope (Leica Microsystems, Mannheim, Germany) using the 633 nm laser line of a tunable white light laser (WLL) source for CellTracker Deep Red excitation for cancer cell

imaging, and the phase contrast to visualize both tumor and NK cells. Confocal images were and acquired with a HC PLAPO CS2 20x objective (0,75 numerical aperture, Leica Microsystems). Z-reconstructions of serial single optical sections were obtained every 30 seconds and carried out with a 512x512 format, scan speed of 700Hz, a zoom magnification up to 1.3, and z-step size of 1 μm. Regions of interest (ROI) were manually created surrounding each group of fluorescent cancer cells in the maximum intensity projection (MIP) containing 12 distinct z planes of confocal images using the LAS X (Leica Microsystems) software. The area size variations (expressed in % values) of each cell group, measured before and after NK cell addition, were compared with area values of cells pre-treated with DMSO as control. Acquisition settings (lasers' power, beam splitters, filter settings, pinhole diameters and scan mode) were the same for all examined samples of each staining. Time-lapse microscopy was performed with a stage incubator (OkoLab, Naples, Italy) to maintain stable conditions of temperature, CO_2, and humidity during live-cell imaging.

Statistical analysis

Digital images of Western blots and IHC were analyzed by Image J (http://rsbweb.nih.gov/ij/index.html). Survival data are presented as Kaplan-Meyer plots and were analyzed using a log-rank (Mantel-Haenszel) method. For all data, statistical significance was evaluated by the two-tailed unpaired Student's *t*-test. Normalized values were analyzed for correlation by the regression analysis using GraphPad software. *P* values not exceeding 0.05 were considered to be statistically significant.

Results

Nutlin3a effects on ligands for NK-ARs and NB cell line susceptibility to NK cells

The treatment of p53-wild-type or p53-mutant NB cell lines, including both *MYCN*-amplified and *MYCN* non-amplified NB cell lines, with different Nutlin-3a concentrations for 48 hours induced a progressive up-modulation of p53, MDM2, and p21 only in p53-wild-type NB cell lines without affecting the MYCN expression (except in LA-N-5 cells at higher doses)(**Suppl. Fig. S1A-B**). As expected, highest doses of Nutlin-3a induced apoptosis, particularly in p53-wild-type NB cell lines (41)(**Suppl. Fig. S2A**). The rescue of p53 function

by pre-apoptotic Nutlin-3a concentration induced a significant upregulation of NKG2D receptor ligands ULBP1 and ULBP3 in *MYCN* non-amplified SH-SY5Y cells (**Suppl. Fig. S2B**), as well as NKG2D receptor ligands ULBP1, ULBP2/5/6, and ULBP3 and DNAM-1 receptor ligands PVR and Nectin-2 in both *MYCN*-amplified LA-N-5 and SMS-KCNR cells (**Fig. 1A, Suppl. Fig. S2C**), without affecting the expression of MICA and MICB in any NB cell line analyzed (**Suppl. Fig. S2D**). Conversely, the expression of ligands for NK-ARs did not change in the p53-mutant NB cell lines (**Suppl. Fig. S2B**).

Experiments were specifically designed to evaluate if the Nutlin-3a-dependent upregulation of ligands for NK-ARs on *MYCN*-amplified NB cells could improve NK cell-mediated recognition and killing. Results demonstrated that both LA-N-5 and SMS-KCNR NB cells became significantly more susceptible to NK cell-mediated recognition (**Fig. 1B-C**) and lysis (**Fig. 1D-E**) upon treatment with Nutlin-3a. Blocking experiments demonstrated that DNAM-1, more than NKG2D, was involved in the NK cell-mediated recognition of Nutlin-3a-treated NB cells (**Fig. 1F-G**). Time-lapse analysis of NK cell-mediated killing of both target cell lines showed that the tumor area of Nutlin-3a-treated NB cells underwent a significant gradual contraction, compared with the tumor area of DMSO-treated control cells, after 3 hours of coculture (**Suppl. Fig. S3A-B, Suppl. Video S1**). Nutlin-3a-treatment of NK cells did not affect the expression of either NK-ARs or the TIGIT inhibitory receptor, known to compete with DNAM-1 in the binding of the PVR ligand (42)(**Suppl. Fig. S4A-B**), nor did it affect NK-cell degranulation in response to NB target cells (**Suppl. Fig. S4C**). Collectively, these data indicated that Nutlin-3a-mediated p53 rescue increased the susceptibility of NB cells to NK cell-mediated killing by inducing the expression of ligands for both NKG2D and DNAM-1 receptors on p53-wild-type NB cells.

P53 is a direct transcription factor for PVR activating ligand

Whereas p53 is known to be a transcription factor for *ULBP1* and *ULBP2* by specifically linking p53-responsive elements to related gene introns (8), whether p53 could act as a transcription factor directly binding the gene promoters of ligands for NK-ARs has not yet been reported. First, we analyzed the possible correlation between p53 and ligands for NKG2D (MICB, ULBP1, ULBP2, and ULBP3) or DNAM-1 (PVR and Nectin-2) in 143 NB patients (Target 2018, www.bioportal.org). *TP53* expression significantly correlated with *PVR* expression, but not with the other ligands analyzed (**Suppl. Fig. S5A**). Bioinformatic analysis by the TRAP website revealed six putative p53 binding sites on the *PVR* promoter, but only one on the *ULBP3* promoter and none on the other ligand promoter tested (**Suppl. Fig. S5B-C**). These data suggest that the p53-mediated induction of the other ligands was independent of the direct p53 binding to the related promoters and requires further studies. The significant correlation between *TP53* and *PVR* expression was confirmed in 36 NB patients by NanoString gene analysis (**Fig 2A**). Nutlin-3a significantly induced time-dependent *PVR* mRNA expression in p53-wild-type NB cell lines compared with DMSO-treated control cells (**Fig. 2B**). To validate the potential functional binding of p53 to the *PVR* promoter, we performed a dual luciferase reporter assay using two constructs, one containing a *PVR* promoter region including six putative p53-binding sites and the other deleted of the four putative p53-binding sites furthest away from the transcriptional start site, as predicted by bioinformatics analysis (**Suppl. Fig. S5C**). The deletion of the putative p53-binding sites resulted in significantly reduced luciferase activity, suggesting that p53 might bind to chromatin on the *PVR* promoter, contributing to *PVR* expression (**Fig. 2C**). Last, ChIP assays using primers encompassing the *PVR* promoter region including the higher affinity p53 putative binding site revealed by TRAP, showed that Nutlin-3a favored p53 binding to the *PVR* promoter (**Fig. 2D**), thus indicating that p53 may function as a direct transcription factor for *PVR.*

Nutlin-3a upregulates the expression of ligands for NK-ARs *in vivo*

In order to evaluate the efficacy of Nutlin-3a *in vivo*, we performed murine xenograft experiments (**Fig. 3A**). IHC analyses in sections of NB tissues, isolated from LA-N-5-bearing NSG mice, showed a significant increase of both PVR and Nectin-2 expression in tumor masses from mice that had been treated with pre-apoptotic doses of Nutlin-3a (24) compared to tumor masses from control mice treated with the Nutlin-3a vehicle HPβCD (**Fig. 3B-C**), without affecting both growth and apoptosis rates **(Suppl. Fig. S6B)**.

Nutlin-3a enhances NK cell-mediated killing of *MYCN*-amplified NB in tumor-bearing mice

Next, we asked whether the increased expression of ligands for NK-ARs by Nutlin-3a treatment could be effective in improving NK-cell activity against NB in NB-bearing NSG mice. The combination of Nutlin-3a treatment, human NK-cell transfer, and IL2 boosting (43) (**Fig. 4A**) induced a significant reduction of the tumor growth compared with the various controls, including treatments with HPβCD or PBS (used as both Nutlin-3a and IL2 or NK-cell vehicles, respectively) with or without IL2 (**Fig. 4B-C, Suppl. Fig. S7**) or NK cells and IL2 (**Fig. 4B**, last panel). IHC of NB tissues, isolated from the five groups of LA-N-5-bearing NSG mice, showed a significant increase of both PVR and Nectin-2 expression only in mice treated with Nutlin-3a compared to other groups, thus excluding an immunomodulatory effect on ligand expression by IL2 and/or NK cells in combination with vehicles (**Suppl. Fig. S8A-B**). These data suggest that Nutlin-3a was able to boost NK cell-mediated killing of NB in an *in vivo* animal model. The combined treatment of Nutlin-3a and NK cells significantly improved mouse overall survival, leading to a 50% survival at 50 days of monitoring (**Fig. 4D**).

Subsequent experiments were performed to assess if the reduced tumor size and improved survival of Nutlin-3a/NK cell-treated NB-bearing mice were associated with an increase of tumor-infiltrating NK cells. NK cells, detected by the expression of the NK cell-activating receptor NKp46 by IHC, were predominantly present both in peritumoral and internal zones of tumor masses from NB-bearing mice treated with the combination of Nutlin-3a and NK cells, and had a significantly higher number than that seen in NK cell-treated mice (**Fig. 4E-F, Suppl. Fig. S9**). This finding indicated that the immunomodulatory effect of Nutlin-3a on NB cells promoted a higher infiltration and retention of NK cells in the tumor masses. These data were consistent with the evidence that the combined treatment of Nutlin-3a and adoptive transfer of NK cells may efficiently control the tumor growth in NB-bearing NSG mice (**Fig. 4A-D**).

Nutlin-3a enhances the susceptibility of primary NB spheroids to NK cell-mediated killing

Finally, to investigate the immunomodulatory effect of Nutlin-3a on human primary NB cells, tumor cells were isolated from bone marrow (BM) aspirates of 26 NB patients (**Table 1**). Both the number of tumor cells and the ability to grow in culture were found to directly correlate with a poor prognosis, as evaluated by the stage, subtype, *MYCN* or *ALK* status, and patient follow-up (**Table 1**). From 18 of the 26 patient BM samples, we obtained a number of tumor cells greater than 1 x 10^6/mL. Tumor cells of 8 patients grew in monolayer adherence similarly to NB cell lines, and NB tumor cells obtained from patient 1 [p1, with metastatic disease and *MYCN* and *ALK* amplification (**Fig. 5, Table 1**)] formed spheroids. Nutlin-3a significantly enhanced the expression of ligands for both NKG2D and DNAM-1 receptors in primary NB cells compared with DMSO-treated control cells (**Fig. 5A, Suppl. Fig. S10**). NK-cell functional assays with spheroids obtained from p1 showed that, after 16 hours of cococulture with NK cells, the Nutlin-3a-pre-treated NB spheroids were significantly more

disaggregated than the DMSO-pre-treated spheroids, as evaluated by optical microscopic analysis (**Fig. 5B-C**). In Nutlin-3a-pre-treated spheroids cocultured with NK cells, tumor cells showed significantly enhanced apoptosis compared with DMSO-pre-treated spheroids cocultured with NK cells (**Fig. 5D**). Altogether, these data indicated that Nutlin-3a significantly upregulated ligands recognized by NK-ARs in primary NB tumor cells, thus boosting the NK cell-mediated killing of tumor spheroids obtained by a particularly aggressive form of NB.

Discussion

NK cells play a crucial role in NB immunotherapy, as evaluated in both preclinical and clinical studies (44,45). However, NB adopts several immune evasion strategies, such as the downregulation of both MHC class I and ligands for NK-ARs, thus rendering NB cells resistant to both T and NK cells, respectively (3,5,6,44). The identification of anti-cancer drugs having additional advantages of immunomodulatory effects, such as the induction of ligands for NK-ARs, remains challenging (46). We previously showed that the expression of the activating ligands for NKG2D and DNAM-1 is inversely correlated with that of MYCN in high-risk NB patients (3). We also found that JQ1 treatment, although able to efficiently downregulate the expression of MYCN, fails to upregulate activating ligands, thus making NB cell lines refractory to NK cell-mediated recognition (13). Of note, none of the chemotherapeutic drugs tested, commonly used in the clinical treatment of NB, showed such effects on different NB cell lines (47). Therefore, in the search of more efficient and less toxic therapeutic approaches (48,49), new strategies are needed to support and enhance the NK cell-based immunotherapy of NB.

Herein, we provided evidence of the immunomodulatory effects of Nutlin-3a, which lead to significantly increased NK cell-mediated killing and shrinkage of p53-wild-type NB cells, evaluated *in vitro* and *in vivo* in a murine model. Mechanistically, this effect occurred through the Nutlin-3a-mediated rescue of p53 function, resulting in a significant induction of NKG2D receptor ligands ULBP1, ULBP2/5/6, and ULBP3 and DNAM-1 receptor ligands PVR and Nectin-2 on NB cells. We also demonstrated that p53 was a direct transcription factor for PVR ligand.

Several Nutlin-3a analogues are currently under clinical investigation in various types of tumors (clinicaltrial.gov web site). Nutlin-3a is non-toxic for normal cells (50,51), thus appearing as a suitable tool for tumor therapy. Interestingly, pediatric tumors are very often p53-wild-type at diagnosis (52), and therefore are potential therapeutic targets for Nutlin-3a-based treatment (20). Although Nutlin-3a has been reported to induce both pro-apoptotic effects and cell-growth arrest in tumor cells (16-18,20,23), including NB (21,23-27), its immunomodulatory activity at low doses, as revealed in this study, had never been reported.

We found a significant correlation between p53 and PVR expression at the transcriptional level in NB patients, whereas no correlation was detected with the other ligands, except a trend for ULBP3. By using two different approaches, namely luciferase reporter assays and ChIP assays, we demonstrated that p53 was a direct transcription factor for the *PVR* ligand, by specifically binding the related gene promoter. Whereas p53 is already known to act as a transcription factor for *ULBP1* and *ULBP2* ligands by binding p53-responsive elements to related gene introns (8), no information is known about the regulatory mechanism of p53 on *Nectin2*. However, given the lack of correlation with p53 at the transcriptional level, as well as the absence of p53 binding sites on the ligand promoter according to bioinformatic prediction, it is conceivable that the induced expression of the other ligands, other than PVR, was independent on the direct p53 binding to the related promoters and that p53 may act

indirectly through other target genes. In this context, Nutlin-3a is known to induce not only cell cycle arrest and apoptosis in p53 wild-type NB cells, but also to trigger premature cellular senescence and neuronal differentiation (23), conditions that may contribute to induced expression of ligands for NK-ARs (53). Of note, cell cycle arrest and senescence are known to be triggered by the expression of *p21*, a p53 target gene (54). Consistently, we found that the functional rescue of p53 at pre-apoptotic doses of Nutlin-3a resulted in a significant accumulation of p21 in NB cell lines. Therefore, we can hypothesize that Nutlin-3a-induced p21 expression may trigger an early phase of cell growth arrest, thus contributing to enhance the expression of activating ligands.

In the clinical setting, the concentration of Nutlin-3a or its analogues can vary according to the drug pharmacokinetic distribution and clearance, mechanisms that occur in specific tissues following drug administration (55-57). Therefore, in addition to inducing cell cycle arrest and apoptosis at cytotoxic concentrations, Nutlin-3a can have an immunomodulatory effect at pre-apoptotic concentrations, depending on the drug distribution, thus strengthening its clinical use, and that of its analogues, for the treatment of cancer. In conclusion, our work indicated that Nutlin-3a treatment with the adoptive transfer of NK cells might constitute an effective combinatorial strategy for NK cell-based immunotherapy to treat p53-wild-type tumors, such as NB.

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Author's Contributions

L.C. conceived the study, coordinated the project and designed the experiments; L.C. and I.V. interpreted the data; I.V., V.L. and M.C., performed western blotting, flow cytometry and NK cell activity experiments; P.I., E.F., C.N. and L.D.M. provided critical tools and performed *in vivo* experiments; O.M. and D.F. performed NanoString experiments; I.V. and C.B. performed q-PCR and ChIP experiments; C.B. performed luciferase assay; O.M. performed IHC experiments; M.O. and A.P. performed Array CGH and IQ-FISH analysis; L.C., V.L. and S.P. performed live-cell imaging experiments; A.C., A.P. and F.L. provided patient samples and clinical information; F.L., V.B., D.F., V.P., R.B. and L.M. discussed the results and provide critical comments; L.C. wrote the manuscript; all authors critically revised and edited the paper.

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Patient number	Age	INSS stage	INRG stage	Subtype[$]	Differentiation grade[#]	Tumor site	*MYCN*	*MDM2*	*TP53*	*MYC*	*ALK*	Tumor cell number/ml of BM	Primary tumor cell growth	Follow-up	Disease state[γ]
p1	2y 5m	4	M	NB	SD	Retroperitoneum	amp	sc	gain	sc	amp	$3{,}5x10^6$	yes	dead	-
p2	3y	4	M	NB	SD	Adrenal gland	amp	sc	sc	sc	sc	$2{,}1x10^6$	yes	dead	PR
p3	3y	4	M	NB	SD	Adrenal gland	amp	sc	sc	sc	sc	$1{,}7x10^6$	yes	alive	PR
p4	3y 9m	4	M	NB	SD	Adrenal gland	gain	sc	sc	sc	gain	$1{,}8x10^6$	yes	alive	PR
p5	2y 8m	4	M	NB	SD	Retroperitoneum	gain*	nd	nd	nd	nd	$2{,}0x10^6$	yes	dead	PR
p6	4y 2m	4	M	NB	SD	Adrenal gland	gain*	nd	nd	nd	nd	$1{,}4x10^6$	no	alive	PR
p7	2m	4	M	NB	SD	Thoracic cavity	sc	sc	gain	delete	sc	$1{,}7x10^6$	no	alive	CR
p8	3y 7m	4	M	NB	SD	Thoracic cavity	sc*	nd	nd	nd	nd	$3{,}0x10^6$	yes	alive	PR
p9	8y	4	M	NB	SD	Adrenal gland	sc*	nd	nd	nd	nd	$0{,}7x10^6$	no	dead	PR
p10	2y 9m	4	M	NB	SD	Adrenal gland	sc*	nd	nd	nd	nd	$1{,}5x10^6$	no	alive	PR
p11	15y	4	M	NB	SD	Retroperitoneum	nd	nd	nd	nd	nd	$0{,}6x10^6$	no	alive	CR
p12	5y 1m	4	M	NB	SD	Adrenal gland	nd	nd	nd	nd	nd	$0{,}6x10^6$	no	alive	PR
p13	5y 6m	4s	Ms	GNBL	nodular	Retroperitoneum	ssc	gain	sc	sc	sc	$1{,}0x10^6$	no	alive	PR
p14	3m	4s	Ms	NB	SD	Adrenal gland	gain	gain	gain	gain	gain	$1{,}7x10^6$	yes	alive	PR
p15	7m	3	L2	NB	SD	Adrenal gland	gain*	nd	nd	nd	nd	$1{,}5x10^6$	no	alive	PR
p16	1y 4m	3	L2	NB	SD	Retroperitoneum	gain	sc	gain	sc	gain	$1{,}2x10^6$	no	alive	PR
p17	1y 9m	3	L2	NB	SD	Retroperitoneum	sc	sc	sc	sc	sc	$2{,}0x10^6$	no	alive	PR
p18	5y 5m	2A	L2	NB	SD	Thoracic cavity	sc*	nd	nd	nd	nd	$0{,}3x10^6$	no	alive	PR
p19	9m	2B	L2	NB	SD	Adrenal gland	sc	sc	sc	gain	sc	$0{,}7x10^6$	no	alive	PR
p20	2y 10m	1	L1	GNBL	nodular	Neck	amp*	nd	nd	nd	nd	$2{,}2x10^6$	yes	alive	PR
p21	4y	1	L1	NB	SD	Thoracic cavity	sc	gain	sc	sc	sc	$1{,}4x10^6$	no	alive	PR
p22	9m	1	L1	NB	SD	Sacrococcgeal	sc*	nd	nd	nd	nd	$0{,}8x10^6$	no	alive	PR
p23	2y 1m	1	L1	NB	SD	nd	nd	nd	nd	nd	nd	$1{,}1x10^6$	no	alive	PR
p24	7m	1	L1	NB	SD	Adrenal gland	sc	sc	sc	sc	sc	$0{,}6x10^6$	no	alive	PR
p25	4y 1m	1	L1	GNBL	nodular	Retroperitoneum	nd	nd	nd	nd	nd	$1{,}6x10^6$	yes	alive	PR
p26	3y 7m	1	L1	not	not classified	Thoracic cavity	nd	nd	nd	nd	nd	$0{,}5x10^6$	no	alive	PR

Table 1: Diagnostic characteristics and status of *MYCN*, *MDM2*, *TP53*, *MYC*, and *ALK* in NB patients

INRG, International Neuroblastoma Risk Group; INSS, International Neuroblastoma Staging System

*evaluated by FISH array

[$]NB, neuroblastoma; GNBL, ganglioneuroblastoma

[#]SD, scarcely differentiated

[γ]CR, complete remission; SRD, stable residual disease; AD, progressive disease

amp, amplified; sc, single copy; nd, not determined

Figure legend

Fig. 1: Nutlin-3a renders NB cell lines more susceptible to NK cell-mediated killing by upregulating the expression of ligands for NK-ARs.

(A) Summary of five independent flow cytometry analyses of surface expression of the indicated ligands for NK-ARs in NB cell lines left untreated (DMSO, grey bar) or treated with Nutlin-3a (red bar) at 2 μM for 48 hours. Mean ± SD; ***p<0.001. (B) Degranulation of human $CD45^+CD56^+CD3^-$ NK cells from healthy donors, measured as CD107a cell surface expression upon stimulation with LA-N-5 or SMS-KCNR left untreated (DMSO) or treated with Nutlin-3a at 2 μM for 48 hours. K562 cells were used as a positive control. The percentage of $CD107a^+$ NK cells is indicated. A representative experiment out of the ten performed is shown. (C) Summary of the degranulation of NK cells isolated from ten healthy donors. Dots, percentage of $CD107a^+$ NK cells; horizontal bars, mean; **p<0.01. (D) DMSO and Nutlin-3a-treated LAN-5 and SMS-KCNR cell lines were used as targets for NK cells at the indicated E:T ratios in a standard 4-hour ^{51}Cr-release assay. Mean±SD; ***p<0.001 (E) Summary of cytotoxic assay of NK cells isolated from healthy donors. Specific lysis was converted to L.U. 20%. Dots, L.U. 20% of the effector/target unpaired tested; horizontal bars, mean; **p<0.01. (F) Anti-NKG2D and anti-DNAM-1 neutralization experiments in a degranulation assay of NK cells pre-incubated with one or both neutralizing anti-NKG2D and anti-DNAM-1 and stimulated with LA-N-5 or SMS-KCNR left untreated (DMSO) or pretreated with Nutlin-3a at 2 μM for 48 hours. K562 cells were used as a positive control. The percentage of $CD107a^+$ NK cells is indicated in each dot plot. A representative experiment of NK cells isolated from one healthy donor out of six performed is shown. (G) Summary of the neutralization experiments showing degranulation of NK cells isolated from six healthy donors. Dots, percentage of $CD107a^+$ NK cells; horizontal bars, mean; *p<0.05, **p<0.01, ***p<0.001. *P*-value, (two-tailed unpaired Student's *t*-test).

Fig. 2: p53 acts as a direct transcription factor on *PVR* promoter.

(A) Correlation between p53 and PVR expression from NanoString analysis performed in 36 NB patients from our institute. R^2 is shown. (B) LA-N-5 cells were treated with 2 μM of Nutlin-3a for the indicated timepoints or with DMSO as control. Total mRNA was isolated, and *PVR* mRNA evaluated by qPCR. Summary of four independent experiments is shown as mean±SD; **p*<0.05, ***p*<0.01. (C) Dual luciferase reporter assay performed with two constructs, one containing a region of the *PVR* promoter spanning from nucleotide -879 to the transcriptional start site containing six putative p53-binding sites, and the another spanning from nucleotide -437 to the transcriptional start site and deleted of the four putative p53-binding sites furthest away from the transcriptional start site, as shown in Suppl. Fig S5C and in Suppl. Table S1. The empty vector (pGL3-basic) was used as a negative control. Schematic representation of different constructs used is shown. Summary of four experiments of relative luciferase activity of different promoter fragments shown as mean±S.E.M; **p*<0.05. Data were normalized with respect to the renilla activity. *P*-value, compared with *PVR* promoter and *PVR* p53-deleted promoter (two-tailed unpaired Student's *t*-test). (D) Binding of p53 to p53-binding site in the *PVR* gene promoter in DMSO and Nutlin-3a-treated LAN-5 cells by ChIP assay. The amount of immunoprecipitated chromatin bound by either isotype control (IgG) or p53 (IP) was quantified by real-time PCR with primers amplifying a specific *PVR* gene promoter region. The sequence and the position of this region is reported. Specific signals were set relative to those obtained for the chromatin input. Summary of three ChIP independent experiments is shown as mean±SD; **p*<0.05. *P*-value, compared with DMSO and Nutlin-3a-treated NB cell lines (two-tailed unpaired Student's *t*-test).

Fig. 3: Nutlin-3a induces the expression of PVR and Nectin-2 in NB xenografts.

(A) LA-N-5-bearing NSG mice were treated intraperitoneally (i.p.) with vehicle (CTRL) or Nutlin-3a at 20mg/kg (drug dose/mouse body weight) or 40mg/kg doses every two days for two weeks. Groups of five NSG mice for each condition were assessed. Mice were sacrificed at the end of the treatment, and tumor masses were formaldehyde-fixed and paraffin-embedded. Blocks were cut into 3-µm sections. Tissues slides from vehicle, Nutlin-3a at 20mg/kg and at 40mg/kg masses were mounted on the same slide and stained for antibodies tested in control tissues (Suppl. Fig S6A). Images were acquired by optical microscope, and density measured by ImageJ software. (B) Representative PVR and Nectin-2 staining in LA-N-5 tissues from NSG mice. Nuclei are counterstained with hematoxylin (blue). Original magnification, x20. Scale bar, 30 mm. (C) IHC density was measured in ten independent areas on four independent slides for each condition, and the mean±SD of integrated density are reported in the histogram. *P*-value, compared with vehicle and Nutlin-3a-treated NB cell lines (two-tailed unpaired Student's *t*-test), $**p<0.01$, $***p<0.001$.

Fig. 4 Nutlin-3a boosts NK cells against NB cell lines *in vivo*, thus controlling tumor growth and improving mouse survival.

NSG mice were injected subcutaneously into the right flank with LA-N-5 cell line (6 mice per group) and treated with vehicles (g1); vehicles plus IL2 (g2); Nutlin-3a and vehicles (g3); vehicles and peritumoral NK cells plus i.p. IL2 (g4); Nutlin-3a, NK cells plus IL2 (g5). (A) Treatment scheme. (B) Tumor size was monitored by caliper, and the tumor growth curve generated for each mouse. Mean tumor growth for each group are indicated by black dashed lines. The orange bar indicates the threshold relative to tumor size mean of vehicle group. Mean tumor size of Nutlin-3a combined with NK cells plus IL2 (g5) compared to that of the NK cells plus IL2 group (g4) is shown as mean±SD; $**p<0.01$, in

the last graph. A representative of three independent experiments is reported. (C) Tumor masses explanted at day 35 of treatment for g1-g5. Images of one representative experiment of three are reported. (D) Overall survival of mice treated as in A. Mice were sacrificed when tumor masses reached almost 2 cm^3. A representative of three independent experiments is reported. Mean±SD; ***p<0.001 (E) Representative examples of NKp46 staining in LA-N-5 tumors treated as indicated. Red, NKp46-expressing cells. Nuclei were counterstained with hematoxylin (blue). Original magnifications, x20. Scale bars, 30 μm. Details of representative slides are reported (whole representative slides in Suppl. Fig. S8). (F) Summary of tumor-infiltrating NK cells quantified by ImageJ on five slides of three independent masses is reported as mean±SD; **p<0.01. *P*-value, (two-tailed unpaired Student's *t*-test).

Fig. 5: Nutlin-3a induces the expression of ligands for NK-ARs in primary NB cells, thus rendering spheroids more susceptible to NK cell-mediated lysis.

(A) Primary NB cells obtained from BM aspirates of nine NB patients were treated with 2 μM of Nutlin-3a, or DMSO as control, for 48 hours, and the surface expression of ligands for NK-ARs was analyzed by flow cytometry. Dots, representing the mean of three independent staining for each independent primary NB cells, are reported; horizontal bars, mean; **p<0.01, *** p<0.001. Some peculiar diagnostic characteristic of these NB patients are reported in the associated table. (B) Primary NB cells obtained from patient 1 (p1) were grown in culture as spheroids and were treated with three different doses of Nutlin-3a (2, 4, and 6μM), or DMSO as control, for 48 hours and evaluated by optical microscopy. After Nutlin-3a treatment, 200 x 10^3 NK cells/well were added and after 16 hours the spheroids were re-analyzed by optical microscopy. The spheroid diameter was analyzed by ImageJ. Representative images of spheroids treated with Nutlin-3a or DMSO before and after the

addition of NK cells are reported. The major diameter of spheroids is indicated by red bars and the values (microns) are reported in each panel. Original magnifications, x20. Scale bars, 100 μm. (C) Summary of spheroid major diameters evaluated in 230 spheroids; horizontal bars, mean;*** $p<0.001$. (D) Spheroids were harvested and stained for AnnexinV and PI to evaluate the apoptotic state before and after NK cell addition by flow cytometry. The mean percentage of cells in the early phase of apoptosis ($AnnexinV^{+}PI^{-}$, blue column), in the late phase ($AnnexinV^{+}PI^{+}$, orange column), of dead cells ($AnnexinV^{-}PI^{+}$, gray column), and alive cells ($AnnexinV^{-}PI^{-}$, green column) for each condition analyzed in four independent wells, is reported in the stacked histogram; ***$p<0.001$. *P*-value, (two-tailed unpaired Student's *t*-test).

Figure 1

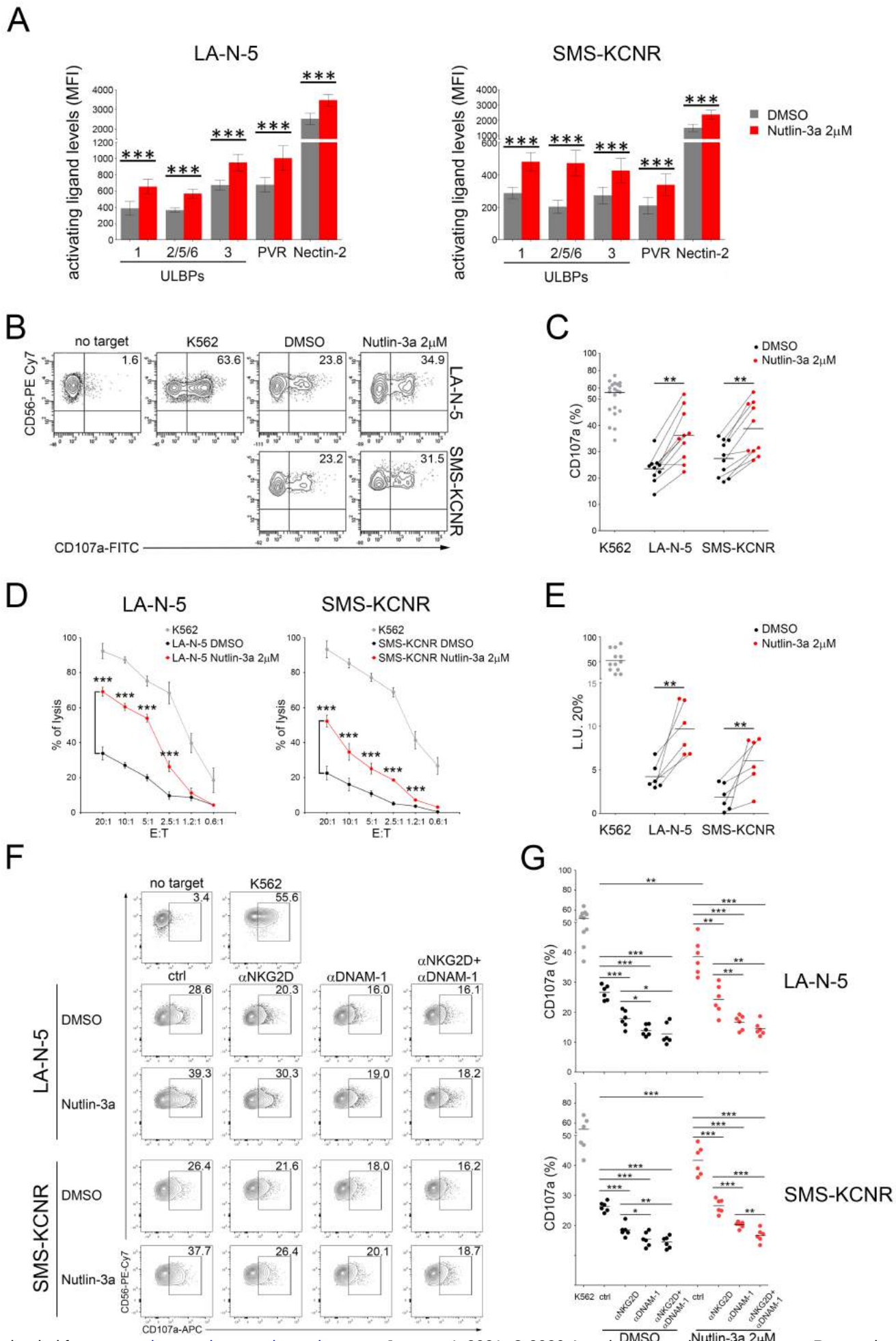


Figure 2

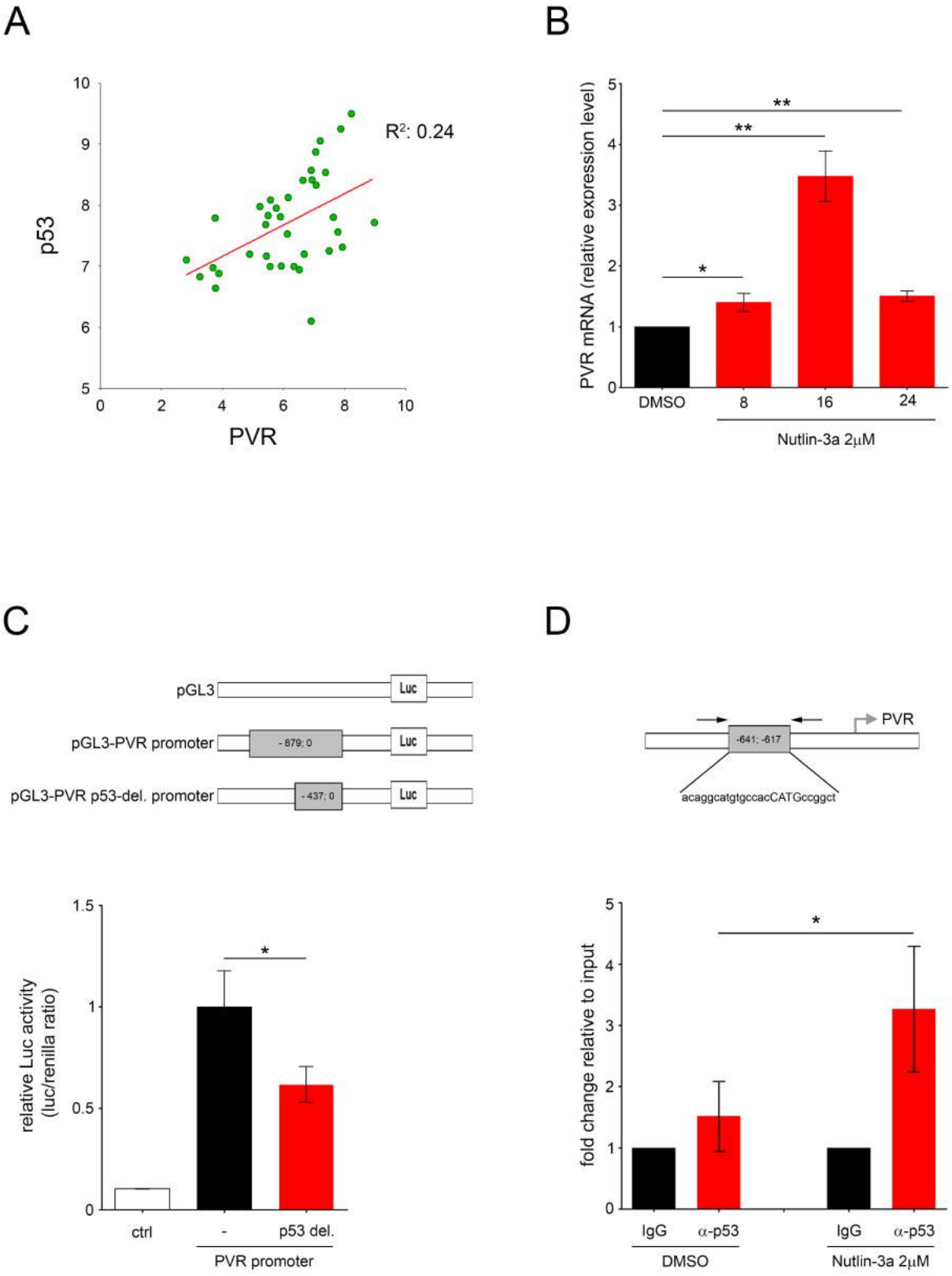
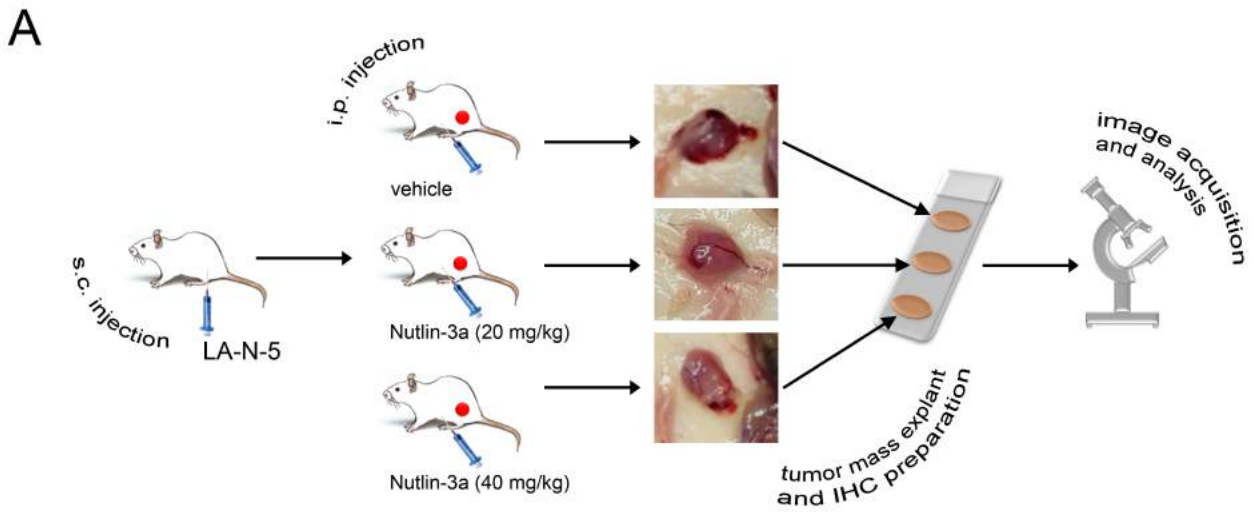
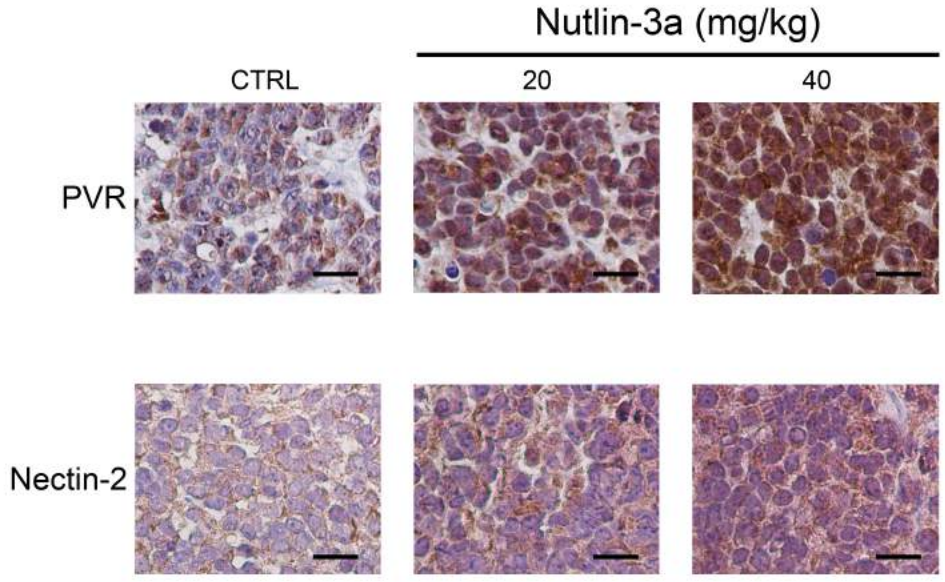


Figure 3





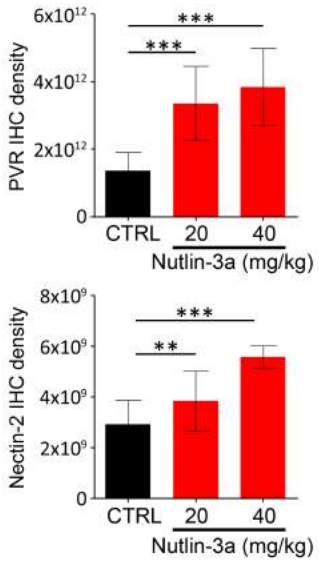


Figure 4

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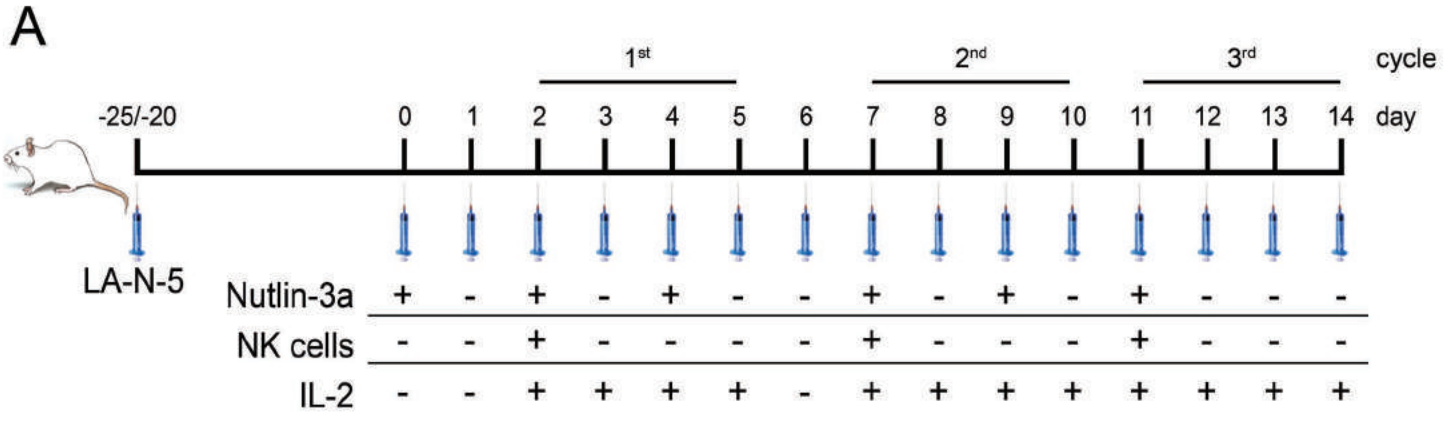
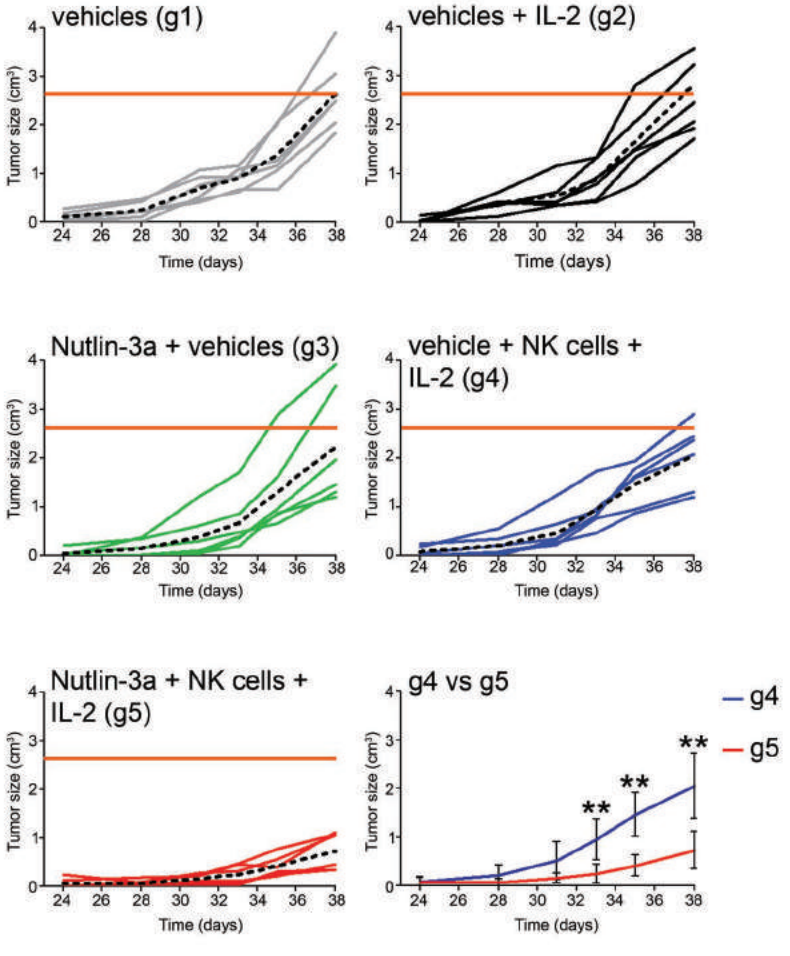
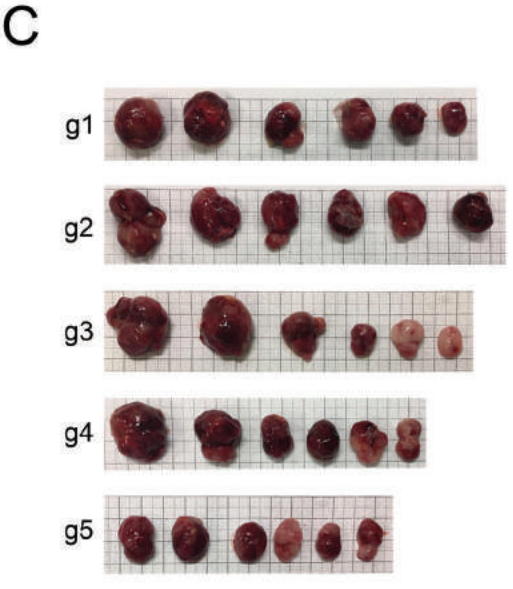
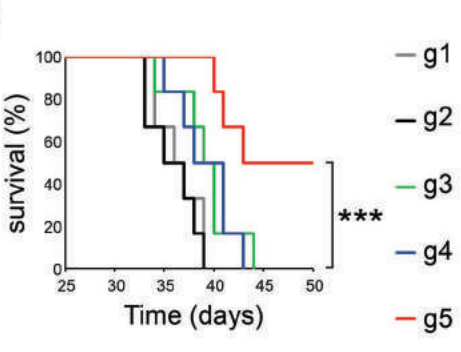
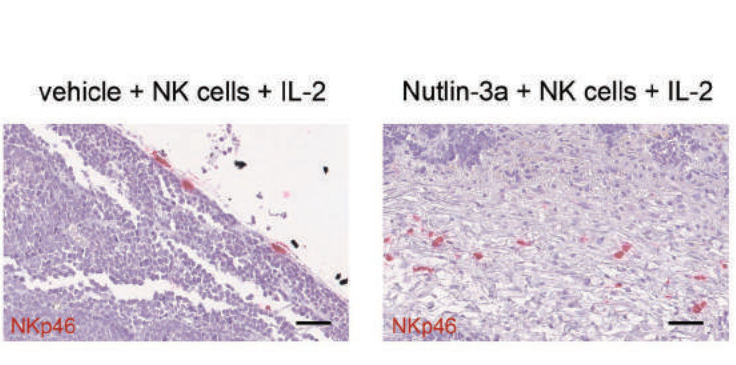
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.











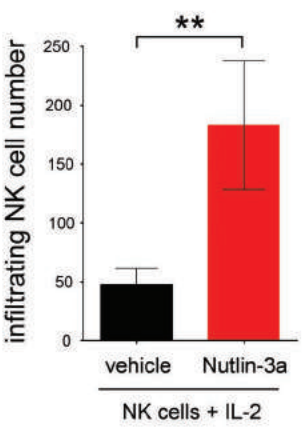
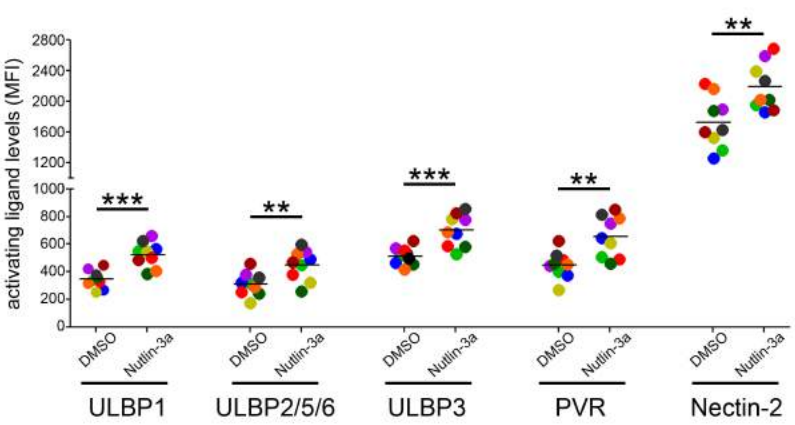


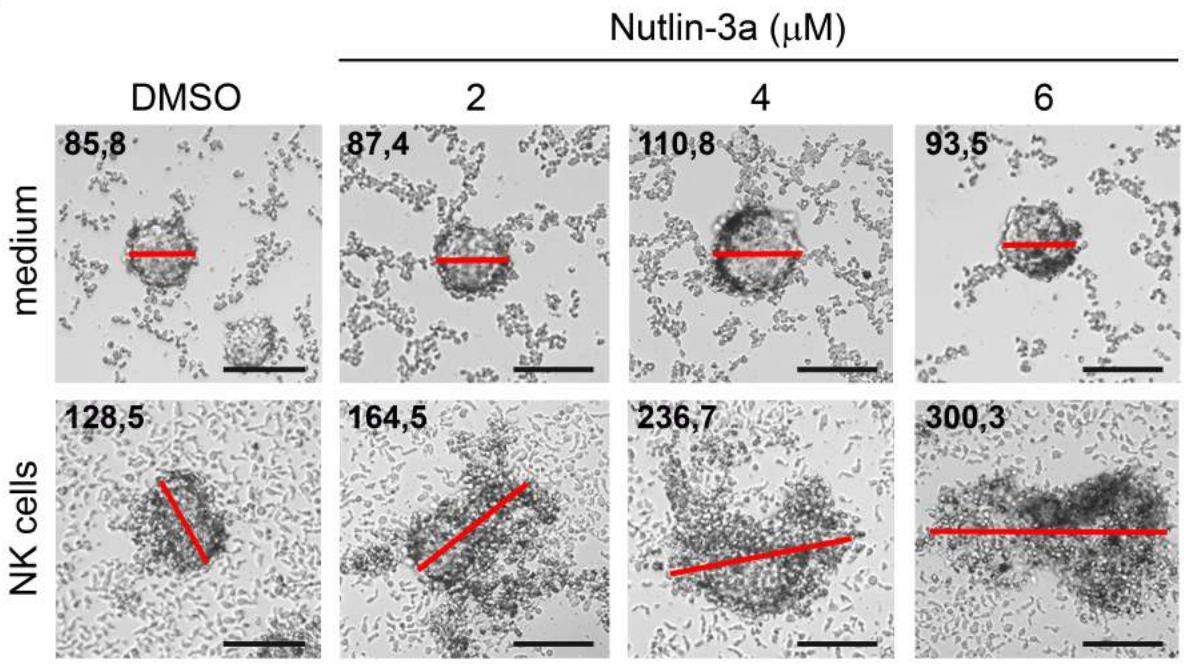
Figure 5

A



patient n.	age	stage	MYCN	subtype
1	2y 5m	4	amp	NB
2	3y	4	amp	NB
3	3y	4	amp	NB
4	3y 9m	4	gain	NB
5	2y 8m	4	gain	NB
8	3y 7m	4	single copy	NB
14	3m	4s	gain	NB
20	2y 10m	2B	amp	GNBL
25	4y 1m	1	nd	GNBL

B



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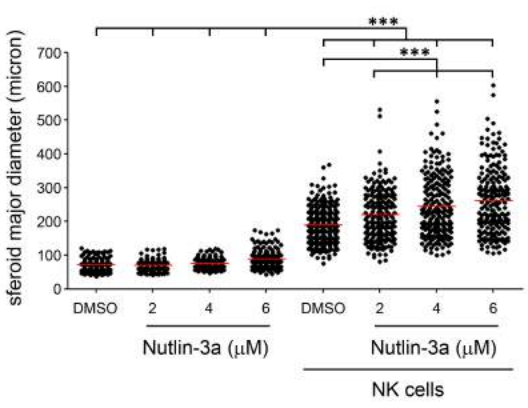

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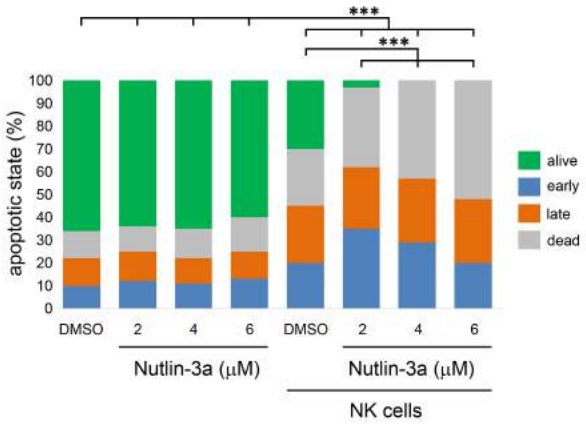

AACR American Association for Cancer Research

Cancer Immunology Research

Nutlin-3a enhances natural killer cell-mediated killing of neuroblastoma by restoring p53-dependent expression of ligands for NKG2D and DNAM-1 receptors

Irene Veneziani, Paola Infante, Elisa Ferretti, et al.

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