SAPIENZA
UNIVERSITÀ DI ROMA

Molecular strategies aimed to boost Natural Killer cell-mediated immunotherapy of Neuroblastoma

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PhD school coordinator

Prof. Angela Santoni

Supervisor

Dott. Loredana Cifaldi

PhD student

Irene Veneziani

Matr: 1614946

Sommario

Neuroblastoma

Neuroblastoma (NB) is the most common extra-cranial solid tumour in childhood [1] and it may be considered a malignant manifestation of aberrant sympathetic nervous system development (Fig 1).

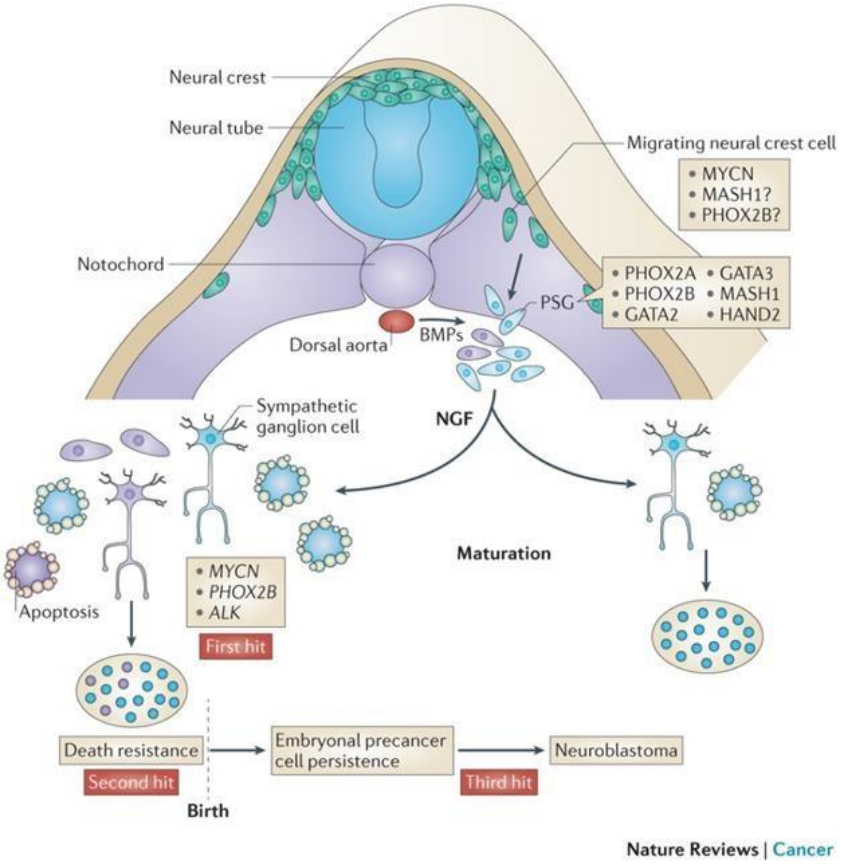

Fig 1. Development of the sympathoadrenal lineage of the neural crest ***(Cheung and Dyer. Nat Rev Cancer 2015)***

NB is the primary cause of death from paediatric cancer for children between the age of 1 and 5 years; roughly, it represents 6% of all paediatric tumours and accounts for approximately 15% of all paediatric cancer mortality. The median age at the time of diagnosis is between 18 and 22 mouths and 50% of the patients present a high-risk of metastatic tumour at the time of diagnosis [2].

There is a familial and a sporadic form of NB and both are defined by specific genetic mutations. The first is very rare (<2% of all NBs) and the major predisposition mutation identified in familial NB was in PHOX2B, a gene playing a critical role in the development of neural crest derived autonomic neurons [3]. Another common lesion associated with familial NB is in the ALK receptor tyrosine kinase gene [4]. ALK is expressed in the developing sympathoadrenal lineage of the neural crest, and it may regulate the balance between proliferation and differentiation [5]. While mutations of PHOX2B is relatively rare in sporadic NB, approximately 6–10% of NBs

carry somatic ALK activating mutations, and an additional 3–4% carry high level ALK gene amplifications [6]. However, the most common focal genetic lesion in sporadic NB is the amplification of MYCN, which occurs in approximately 22% of tumours and it is associated with poor outcome [7]. MYCN amplification or over-expression has been described in several other cancers that originate from tissues where MYCN is normally expressed and include retinoblastoma, Wilm's tumor, rhabdomyosarcoma, medulloblastoma, glioblastoma, and small cell lung cancer [8] (Fig 1).

Others important markers used to diagnose high-risk NB include ATRX mutations, but ATRX alterations alone are not sufficient to promote tumorigenesis, ploidy, chromosomes abnormalities and aberrant expression of tyrosine kinases receptors (Table 1) [2].

High-risk NB	
GENE	FUNCTION
ALK	ALK cooperates with MYCN to drive malignancy, as activation of ALK results in increased expression of MYCN by elevating activity of the MYCN promoter.
ATRX	ATRX encodes a SWI/SNF chromatin-remodelling ATP-dependent helicase, suggesting ATRX involvement in various developmental processes. However, ATRX mutations alone are not sufficient to promote tumorigenesis.
1p36.3	Deletion of 1p correlates with metastatic diseases.
17q	Gain of distal 17q is recurring feature of NB and primary tumours. 17q gain is itself a powerful independent predictor of poor survival
TrkB	Coexpressed at high levels with its ligand, BDNF, in unfavorable tumours, especially those with MYCN amplification. Activation of the TrkB–BDNF autocrine pathway can lead to invasion, metastasis, angiogenesis and drug resistance
MYCN	Mycn amplification occurs in 25%-33% of the cases and correlates with a greatly increased risk of fatal outcome.

Tab.1 Established characteristics for high-risk NB patients.

International Neuroblastoma Staging System (INSS), first published in 1988, used the extent of the initial surgical procedure to define the stage of the patient. Small localized tumours that were completely resected were considered Stage I lesions. Stage II tumours were small, may or may not have had lymphonode involvement, but could not be completely resected. Stage III lesions

were large tumours that crossed the anatomical midline of the patient and could not be completely resected. Lastly, Stage IV and IVS metastatic tumours were differentiated by the fact that IVS patients were <1 year of age with metastatic disease located in liver, skin and less than 10% of the bone marrow [9]. Most infants, with IVS metastatic disease, can be cured with moderate-intensity chemotherapy, and some patients with a special pattern of metastasis have a high likelihood of undergoing spontaneous regression without chemotherapy. Several possible mechanisms are proposed to support spontaneous regression in NBs: (i) neurotrophin deprivation, (ii) loss of telomerase activity, (iii) humoral or cellular immunity and (iv) alterations in epigenetic regulation and possibly other mechanisms, but the exact mechanisms responsible for spontaneous regression (and differentiation) are uncertain [10] [11].

Although the International NB Staging System (INSS) is currently used, a new International Neuroblastoma Risk Group (INRG) Staging System was recently proposed classifying NB patients into 16 risk groups on the assessment of 13 potential biological prognostic factors [12]. According to INRG, risk in NB is classified as low, intermediate, or high. Although low- and intermediate-risk patients generally have a favourable outcome (80%–95% event-free survival rate), high-risk patients have, 50% event-free survival rate, and there is also a subset of "ultra-high" risk patients who do not respond to therapy [13]. The biological hallmark of NB is the complexity of the genetic abnormalities acquired by the tumour cells, and some of these abnormalities are powerful prognostic high-risk NB markers.

MYCN and p53

MYCN belongs to a family that includes MYCL (L-Myc) and MYCN (N-Myc) [14] [15] [16]. While the role of L-Myc is less well understood, N-Myc expression is tissue-restricted, and N-Myc could substitute for c-Myc in murine development [17] [18]. It is crucial to understand that concentration of both c-MYC and MYCN proteins is finely adjusted allowing them to perform their normal functions [19]. While c-Myc is expressed in all proliferating tissues in the adult, MYCN expression in humans and mice is restricted to certain tissues in the developing embryo and is very low or absent in adult tissues [20] [21].

Structurally, c-Myc and MYCN proteins are very similar and both heterodimerize with MAX protein to mediates many of their functions. Myc–Max complexes bind gene promoters by recognizing a DNA sequence, called E-box; MYCN preferentially binds E-box CATGTG as well as the classic CACGTG. Moreover, under N-Myc–amplified conditions such as in NB, MYCN becomes less specific and can bind additional E-box motifs including CATTTG, CATCTG, and

CAACTG [22]. This in turn activates the transcription of downstream genes involved in diverse cellular functions, specifically in NB it can activate transcription of genes involved in metastasis, survival, proliferation, pluripotency, self-renewal, and angiogenesis and can suppress expression of genes that promote differentiation, cell cycle arrest, immune surveillance, and genes that antagonize metastasis and angiogenesis [23] (Fig. 2).

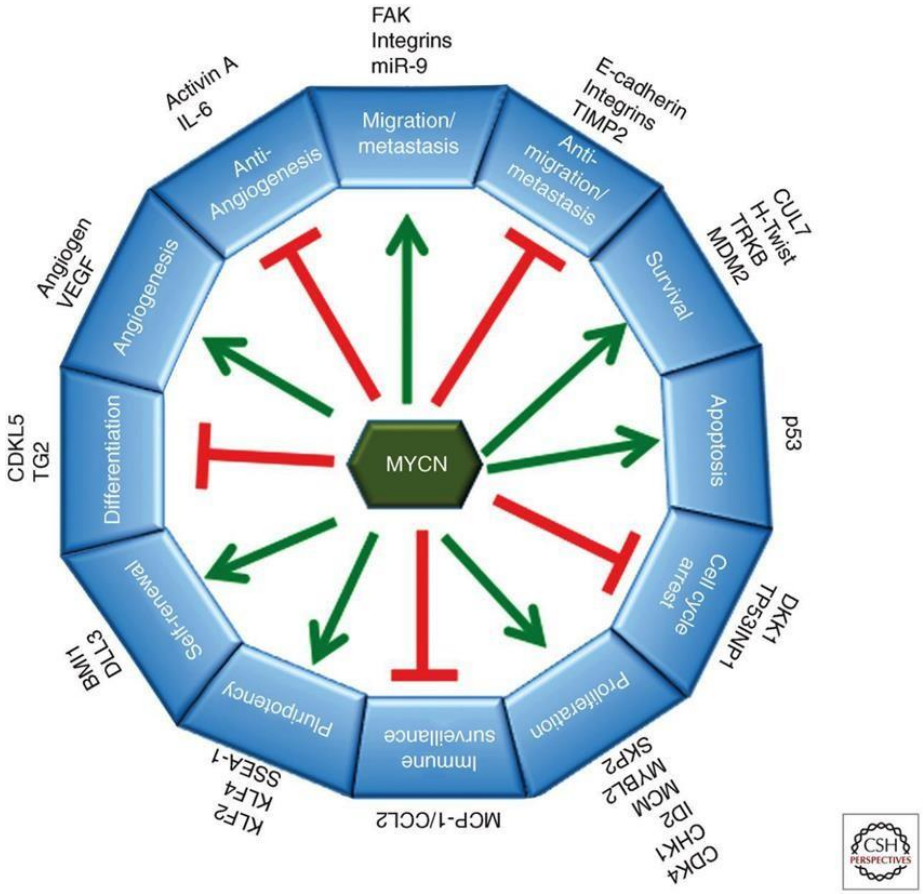

Fig 2. MYCN plays multiple roles in malignancy and maintenance of stem-like state ***(Huang M. et al. Cold Spring Harb Perspect Med 2013).***

Early studies found that several c-Myc target genes were expressed in some NB cell lines with MYCN amplification: significant overlap between c-Myc and MYCN-regulated gene sets had been reported [24] [25]. By contrast, MYCN expression inversely correlates with that of c-Myc indeed, in MYCN amplified NB cell lines, c-Myc mRNA transcription is repressed by the high levels of MYCN protein [24].

p53 is a tumour suppressor gene that is induced and activated by a variety of potentially tumorigenic stresses, including inappropriate oncogene signalling and DNA damage. p53 is a direct target gene of c-MYC because its promoter contains a non-canonical E-box (CATGTG)

located upstream of the transcription initiation site and it is recognized by MYC-MAX heterodimers [26]. Recently, it was demonstrated that p53 is also a direct transcriptional target of MYCN and that p53 accumulation in NB correlates with *MYCN* amplification [27]. p53, often referred to as "guardian of the genome", is mutated in up to 60% of many human malignancies, but in NB it is mutated only in 2% of the cases at the time of diagnosis [28]. Normally, p53 function is controlled by a complex network of regulators including $p14^{ARF}$, mouse double minute (MDM)2, and MDM4 [29]. In particular, MDM2 binds p53 and blocks its transcription and nuclear export, and mediates p53 degradation because MDM2 has an E3 ubiquitin ligase activity responsible for p53 ubiquitination [30].

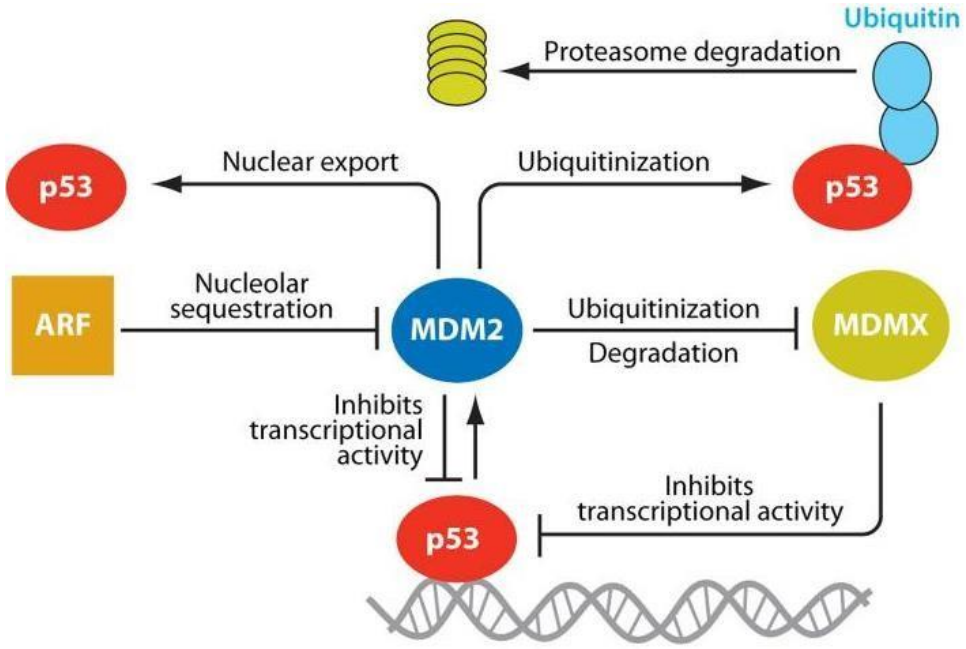

Fig.3 – Autoregulatory feedback loop of inhibition of p53 by MDM2. (*Annu Rev Pharmacol Toxicol, 2009)*

The activation of mammalian ATM promotes p53 responses by stimulating the rapid degradation of both MDM2 and MDM4 through C-terminal phosphorylation of both proteins [31]. The restoration of p53 function induces the expression of target genes such as p21 (CDKN1A), which leads to G1 cell-cycle arrest [32]. Moreover, DNA single strand breaks induce ATR activation which phosphorylates Chk1, leading to G2 cycle arrest [33]. It was demonstrated that both cell cycle arrest and senescence contribute to blocking tumorigenesis [34] and promote the expression of ligands for NK cell-activating receptors [35] [36].

The human MDM2 protein, the main negative controller of p53 function (Fig. 3), is another oncogene that is amplified in a variety of human cancers, including NB. Interestingly, both MDM2 and p53 are regulated by MYCN. Moreover, MDM2 also plays p53-independent roles in oncogenesis regulating MYCN mRNA stabilization [37] [38].

Neuroblastoma Immune Escape

Tumour growth is facilitated by cancer cell-driven immunosuppressive mechanisms. In the last 10 years many studies have contributed to elucidate the immune evasion mechanisms adopted by NB cells to escape the control of the host immune system. These studies have highlighted:

- the release of soluble molecules suppressing anti-tumour immune reactivity, such as the stress inducible NKG2D ligand MICA and the HLA class Ib molecule HLA-G [39];
- the decreased or absent expression of surface ligands for various natural killer (NK) cell-activating receptors, including NCRs, NKG2D and DNAM-1. In particular, defects in the expression of few components of the HLA class I related antigen processing machinery (APM) [40];
- the production of suppressive factors e.g. TGF-beta and IL-10, which may prevent expansion and activation of tumour-infiltrating lymphocytes reducing the expression of NKp30 and NKG2D receptor [41];
- the decreased expression of costimulatory molecules such as CD40, CD80, CD86, OX40L and 4-1BB-L, but not PD-L1and B7H2 in primary NB tumours [42];
- 4Ig-B7-H3 molecules expressed at the NB cell surface can exert a protective role from NK-mediated lysis by interacting with a still undefined inhibitory receptor expressed on NK cells [43].

The main effector cells of the immune system involved in tumour cell recognition are: (i) $CD8^+$ T cells, which specifically recognize through their T cell-receptor (TCR) peptides presented by antigen presenting cells (APC) in the context of MHC class I molecules and mediate cytotoxic functions; (ii) NK cells, innate effectors, which exert their cytotoxic activity through a diverse repertoire of activating and inhibiting receptors that recognize specific ligands on the surface of the target cells; (iii) $CD4^+$ T lymphocytes and macrophages are other important players of anti-tumour immune responses, mainly through the production of cytokines and inflammatory mediators. Defects in HLA class I and APM component expression may render NB cells an excellent target for NK cell-mediated cytotoxicity [44].

Neuroblastoma Treatment

A localized NB lesion is generally curable. However, long-term survival for children with advanced disease older than 18 months of age remains poor despite aggressive multimodal therapy. The therapies for these risk categories are very different.

- Low-risk disease can frequently be cured with surgery alone.
- Intermediate-risk disease is treated with surgery and chemotherapy.
- High-risk NB is treated with intensive chemotherapy, surgery, radiation therapy, bone marrow / hematopoietic stem cell transplantation [45], biological-based therapy with 13-cis-retinoic acid (isotretinoin or Accutane) [46] and immunotherapy (for example anti GD2-monoclonal antibody usually administered with the cytokines GM-CSF and IL-2) [47].

With the current treatments, patients with low and intermediate risk disease have an excellent prognosis with cure rates above 90% for low risk and 70–90% for intermediate risk. In contrast, therapy for high-risk NB in the last 20 years resulted to be effective only for about 30% of patients.

Chemotherapy agents used in combination have been found to be effective against NB. Agents commonly used for stem cell transplant conditioning are platinum compounds (cisplatin, carboplatin), alkylating agents (cyclophosphamide, ifosfamide, melphalan), topoisomerase II inhibitor (etoposide), anthracycline antibiotics (doxorubicin) and vinca alkaloids (vincristine) and some newer regimens include topoisomerase I inhibitors (topotecan and irinotecan) that have been found to be effective against recurrent disease. Many of this drugs cause a genotoxic stress that lead to DNA damage response (DDR) pathway activation with a consequent induction of ligands for NK-cell activating receptors [48] [49] [50].

Among the new strategies proposed to treat high-risk NB patients, the use of small molecules inhibitors is very promising. There are different compounds targeting proteins associated to NB. The most used inhibitors directly or indirectly target MYCN protein but can also affect downstream targets.

BET (bromodomain and extra-terminal domain) are a family of proteins that regulate the transcription of many genes, including MYCN. These proteins contain 2 bromodomain motifs in the amino terminus and an extraterminal protein–protein interaction domain in the carboxy terminus, constituting an additional class of proteins that regulate chromatin structure [51] [52]. These “chromatin readers” bind to acetylated lysines in chromatin, recruiting additional chromatin, modifying proteins and leading to cell context–dependent gene activation or repression [53]. The BET family consists of BRD2, BRD3, BRD4, and BRDT.

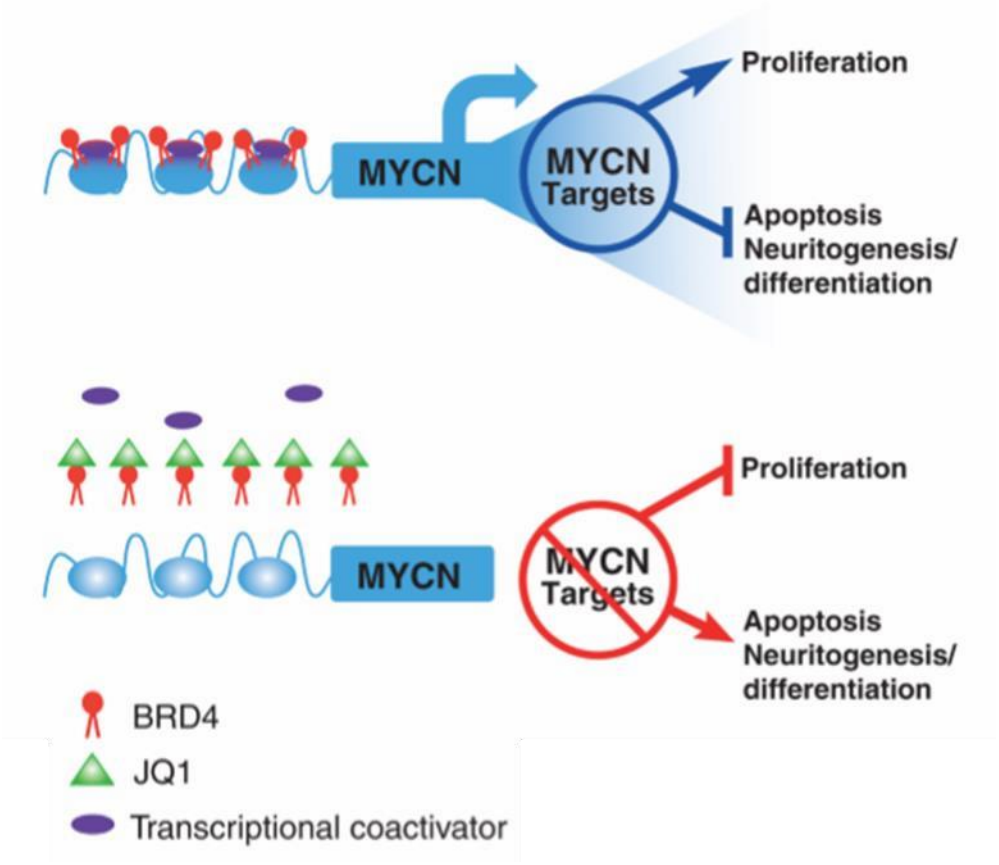

Fig.4. JQ1 mechanism of action ***(Schnepp and Maris. Cancer Discov. 2013)***

JQ1 is one of the BET bromodomain inhibitor and it can represent a promising strategy to block the growth of MYCN-dependent NB cells [54] [55]. Indeed, it competitively binds to bromodomain, displacing BRD4 fusion oncoprotein from MYCN promoter (Fig. 4).

Among MYCN downstream targets, p53 represents a good candidate because NB is rarely associated with p53 mutations [27]. However, the p53 pathway is often impaired in childhood cancers because of upstream p53/MDM2/$p14^{ARF}$ network aberrations [56]. One of the strategies to affect this pathway is by perturbing the p53/MDM2 interaction. Small molecules as Nutlin-3, a cis-imidazoline compound, can interact with MDM2 by mimicking the p53 N-terminal region where MDM2 binds and can induce p53 activation allowing its post-transcriptional acetylation [57]. Together with wt p53, expression level of MYCN and $p14^{ARF}$ may determine the outcome of the response to MDM2 inhibition [58].

The main prerequisite for p53 activation after Nutlin-3 treatment, however, is an intact p53 protein, as p53 loss-of-function mutations inevitably prevent MDM2 inhibitors from reinstating p53 function [59] (Fig. 5).

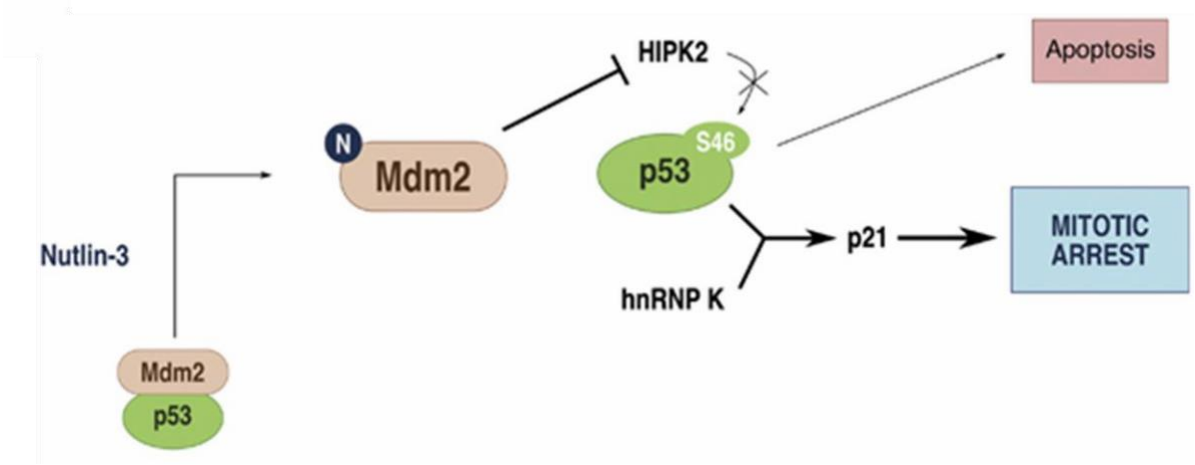

Fig. 5. Nutlin-3a mechanism of action

NK cells have an important anti-tumor activity because they can limit tumour growth. Moreover, especially NK cells are much less suppressed by tumor microenvironment then T cells and unlike B and T cells of adaptive immunity, they are capable of spontaneously destroying transformed cells without prior sensitization in an MHC-restricted manner [60]. The pre-clinical studies in mice in which NK cells are infused after allogeneic haematopoietic stem cell transplantation (HSCT), are very promising because suppress graft-versus-host-diseases (GVHD) and enhance graft-versus-tumor (GVT) against solid tumors and leukemias [61] [62]. The concurrent administration of NK cell stimulating cytokines, such as IL-2 or IL-15, can promote this effect [63].

Tumor-specific mAbs that recognize tumour antigens expressed on the surface of tumor cells are being used as cancer therapy. GD2 is a promising target for antibody therapy because it is abundant in most NB cells and its expression is restricted to nervous system cells among normal cells [64]. For this reason, an immunotherapy based on the use of anti-GD2 antibody for NB was investigated [47]. Anti-GD2 antibody induces ADCC via NK cells. NK cell-mediated surveillance mechanisms [65] and NK cell stimulation with interleukin IL-2 can also contribute to the antitumor effect [66]. Two intravenous (i.v.) injection of anti-GD2 IgG antibodies, chimeric 14.18 (ch14.18) and mouse 3F8 combined with i.v. injection of interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), have been tested in the clinic [47]. Despite the recent success of immunotherapy with the anti-GD2 antibody ch14.18 and cytokines, treatment of high-risk NB remains challenging.

Another strategy to enhance an NK cell-mediated immunotherapy is based on genetic modification of cells. Such as T cells, also NK cells may be genetically engineered to display a

CAR enables the specific targeting or augmented cytotoxicity of NK cells. It was demonstrated that NK-92-scFv(ch14.18)-zeta, a GD2-specific chimeric antigen receptor expressing cell line, successfully lyses partially or multidrug-resistant $GD2^+$ NB cells lines [67]. The reported side effects of a CAR-T cell based immunotherapy include neurologic toxicity, cytokine release syndrome, tumor lysis syndrome, immunogenicity [68]. For this reason, the long-lasting and production of pro-inflammatory cytokines from CAR-T cells *in vivo* is the cause of severe side effects [69]. NK cells have been considered better candidates for CARs because their short lifespans last for nearly 2 week *in vivo* and they mainly produce interferon IFN-γ. NK cells have been used for treating both hematopoietic malignancies (e.g., leukemia, lymphoma, and multiple myeloma) and solid tumors (e.g., melanoma, ovarian cancer, lung cancer, colorectal cancer, and glioblastoma) [70] [71]. NK cells have innate advantages in the treatment of certain solid tumors: IFN- γ has been shown to induce the permanent arrest of the growth of melanoma cells [72]; others solid tumors, such as the ovarian cancer, have high expression of MICA/B and ULBPs, which can promote NK cells function through the activating receptor NKG2D [73]. Moreover, recent studies have shown that IFN-γ can drive T-regulatory cells to promote antitumor immunity, suggesting that NK cells may contribute for the immunotherapy of solid tumors [74]. However, NK cell-immunotherapies for solid tumors, can control disease progression but scarcely abate disease [75] [76]. The possible reasons include (i) low infiltration rate of NK cells to the solid tumor; (ii) inhibitory factors in the tumor microenvironment; and (iii) inhibition from tumor cells, such as residual HLA expression. These crucial issues may be solved enhancing the migration of NK cells to tumor sites, altering the tumor microenvironment and combining with the targeting of checkpoint inhibitors aimed to activate NK cells [77].

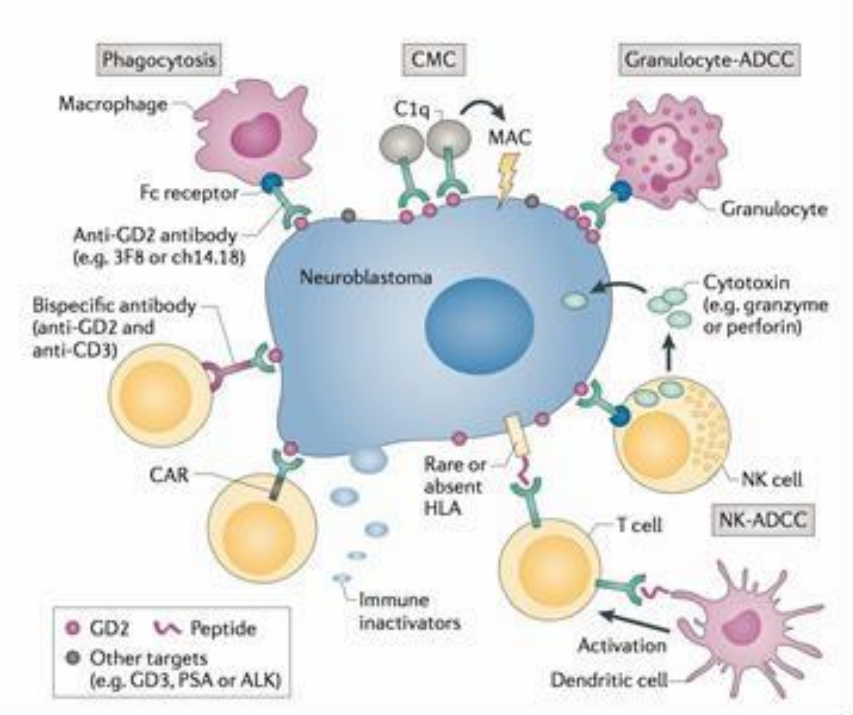

Fig. 6 Immunotherapy of Neuroblastoma ***(Nature Reviews, 2013)***

Natural Killer Cells

NK cells (around 5-20% of all circulating lymphocytes) are large granular lymphocytes that belong to the innate immune system. Unlike T or B cells of the adaptive immune system, NK cells do not rearrange T cell-receptor or immunoglobulin genes from their germline configuration [78]. The traditional cell surface phenotype defining human NK cells within the lymphocyte gate on the flow cytometric analyser shows an absence of CD3 and expression of CD56, the 140-kDa isoform of neural cell adhesion molecule (NCAM) found on NK cells and a minority of T cells [79]. Murine NK cells do not express CD56 therefore NKp46, a member of the highly conserved natural cytotoxicity receptor (NCR) family of NK cell-activating receptors, is used to defineNK cells across species [80] [81].

NK cells are part of hematopoietic system and are derived from CD34+ hematopoietic progenitor cells (HPCs) (Fig.7) [82], but they don't wholly develop in the bone marrow. As a matter of fact, a subset of NK cells, termed CD56bright for their high-density surface expression of CD56, are relatively dominant in secondary lymphoid tissue (SLT) compared with their more abundant CD56dim NK cell counterpart found in bone marrow, blood, and spleen [83]. CD56bright NK cell population are less mature than the CD56dim subset, that it is characterized by an increased expression of NK cells [84]. In SLT, dendritic cells (DCs) and other antigen presenting cells

(APCs) express membrane bound IL-15, which is required for NK cell maturation, suggesting that SLT may be a site for NK cell development *in vivo* [85]

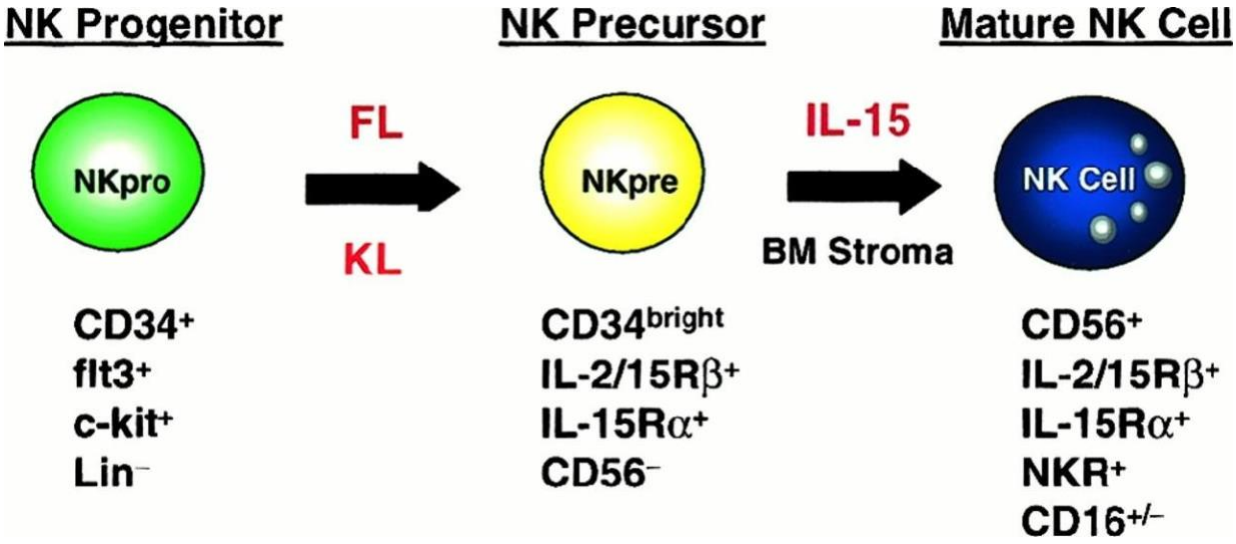

Fig.7 Schematic diagram of human NK cell development. ***(Blood 2001)***

Conversely to T cells, NK cells can recognize viral-infected or malignant-transformed cells without a prior exposure and their functions are based on a balance between activator and inhibitor signals [86]. They are a major source of cytokines that influence the host's immune response. TNF-α initiates cytokines and chemokines release that influence pro-inflammatory cytokine cascades, while interferon gamma (IFN-γ) production by NK cells is known to shape the Th1 immune response, activate APCs to further up-regulate MHC class I expression and improve anti-proliferative effects on viral- and malignant-transformed cells [87].

The majority of circulating NK $CD56^{dim}$ cells share a common killing mechanism with $CD8^{+}$ cytotoxic T lymphocytes (CTL) display against tumour target cells. NK cell killing of tumour cells proceeds through distinct mechanisms: (i) exocytosis of secretory lysosomes that contain lytic proteins such as perforin, granzymes, and (ii) NK surface expression of ligands that engage death receptors of the tumour necrosis factor (TNF) superfamily on tumour cells (eg, Fas, TRAIL) [88], thereby triggering apoptosis. In addition to antibody–independent natural cytotoxicity, expression of CD16 (FcγRIIIA, the low affinity receptor for IgG) on NK $CD56^{dim}$ cells mediates an antibody-dependent cellular cytotoxicity (ADCC) against IgG–coated cells [89]. Furthermore, NK cells can kill allogeneic cells in hematopoietic transplantation enhancing a graft-versus leukaemia activity [90]. Moreover, elimination of NK cells in mice results in a

higher incidence of spontaneous tumours, impaired clearance of inoculated tumour cells, and an increased rate of tumour metastasis [91].

NK cell education and function

NK cell education plays an important role in steady state conditions, where potentially auto-reactive NK cells must maintain tolerance for self, yet recognize infected or transformed cells after proper stimuli [92]. NK cell education is based on the signaling via the inhibitory receptors that recognize self-MHC class I ligand. The activation of NK cells is regulated by the integration of signals deriving from activating and inhibitory receptors expressed on their surface.

Inhibitory receptors and their ligands.

In MHC class I-sufficient humans, the NK cell's subset lacking inhibitory receptors for self-MHC class I is hypo-responsive to *in vitro* stimulation through several activating receptors and fail to reject MHC class I-deficient bone marrow *in vivo* [93]. Thus, expression of self-MHC class I-reactive inhibitory receptors improve the responsive potential of NK cells [94]. Inhibitory NK cell receptors recognizing self-MHC class I are considered the predominant mechanism responsible for NK cell tolerance to self. In human, MHC class I recognition is performed by a distinct set of receptors called killer cell immunoglobulin-like receptors (KIRs) which are specific for different polymorphic MHC class I molecules. Different KIR genes have varied effects on NK cell activity: those KIR genes with immune-receptor tyrosine-based inhibitory motif in the cytoplasmic tails (indicated by L=long in the nomenclature), transmit inhibitory signals after binding their cognate HLA ligand; those with short cytoplasmic tails (S) including immune-receptor tyrosine-based activator motif transmit activating signals [95]. There are two main KIR haplotypes, A and B, which depend upon the genes expressed. The A haplotype contains up to 7 expressed genes (KIR3DL3, KIR2DL3, KIR2DL1, KIR2DL4, KIR3DL1, +/-KIR2DS4, KIR3DL2). KIR B haplotypes contain up to 14 genes, (KIR2DS2, KIR2DL2, KIR2DL5B, KIR2DS3, KIR3DS1, KIR2DL5A, KIR2DS5, KIR2DS1) and the majority of them is stimulatory [96]. Other inhibitory NK cell receptors for human leukocyte antigens (HLA, or human MHC class I) with cytoplasmic ITIMs, such as NKG2A (CD159a) and LIR-1 (ILT2, CD85j), also display variegated expression patterns. The ligand of NKG2A is the non-classical MHC class I molecule HLA-E (Fig. 8 and Fig. 9).

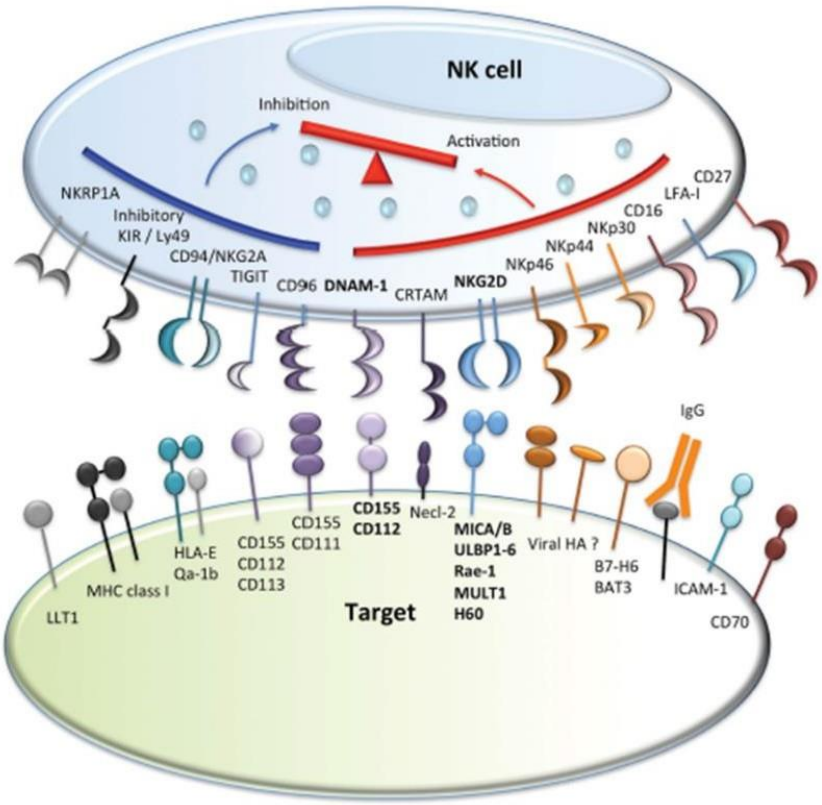

Fig.8 NK-cell receptors and their cognate ligands ***(CJ Chan et al. Cell Death and Differentiation 2014)***

Activator receptors and their ligands.

In contrast to inhibitory receptors, most activating receptors are expressed by all NK cells. Furthermore, activating receptors use diverse signaling cascades, whereas inhibitory receptors appear to use a common mechanism for inhibition [97] [98]. Activating receptors associated with ITAM, can be subdivided into two groups, the first includes rapidly evolving receptors expressed on NK cells, such as KIR2DS, KIR3DS, and NKG2C (CD159c); some activating KIRs bind classical MHC class I [99], whereas NKG2C binds HLA-E [100]. Generally, the binding of activating receptors to MHC class I is less strong than that of their related inhibitory receptor counterparts (Fig. 9).

The second group of ITAM-associated receptors, including CD16, NKp30 (CD337), and NKp46 (CD335), are expressed on most resting NK cells. Natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46 were identified for their role in natural cytotoxicity towards tumour cells [101]. Recently, it was discovered that B7H6 is a functional ligand for the NK cell-activating receptor NKp30 [102]. Information about others NCR receptor's ligands are not well defined.

NK receptor for the Fc fragment of IgG (FcγR) targets immune complexes to effector cells. Multipe FcγRs exist, which differ in ligand affinity, cellular distribution, and effector function.

In human, NK cells, macrophages, and polymorphonucler cells (PMN) express FcγRIII (CD16) [103]. Moreover, CD16 represents the only FcγR receptor on NK cells and especially it is expressed on $CD56^{dim}$ NK cells subset [78]. Similar to both NK cell cytokine release and NK cell-mediated killing, CD16-mediated ADCC is tempered by inhibitory Fc receptors expressed on APCs, as the absence of these inhibitory Fc receptors improves the efficacy of ADCC *in vivo* [104]. In general, FcγR receptors are protein that contains two canonical Ig-like extracellular domains, a weakly hydrophobic transmembrane domain and a short cytoplasmic domain, (four amino acid) [105]. CD16 often associates with FcϵRI-γ chains or CD3-ζ chains within the cell membrane: both chains have an ITAM in their cytoplasmic tails [106]. Upon FcγR binding, these ITAMs are phosphorylated, and through the activation of PI3K, NF-κb and ERK pathways, NK cell degranulation, cytokine secretion, and finally tumour cell lysis occurs [107]. ADCC stimulates IFNγ release by NK cells activating nearby immune cells to promote antigen presentation and adaptive immune responses.
CD16 is probably the best example of an NK cell receptor coupling the innate and the adaptive immune responses. In fact, the existence of this receptor on NK cells underlines their ability not only to recognize early infected and transformed target cells but also to bind the antibodies that are generated late during the immune response, when the adaptive immunity is activated [108].

NKG2D and DNAM-1 receptors and their ligands

A second category of activating receptors do not contain ITAMs or associate with ITAM–carrying adaptors. They include NKG2D (CD314) and DNAM-1 (CD226) (Fig 9).
NKG2D is a lectin-like type 2 transmembrane receptor expressed as a homodimer in humans by all NK cells [109]. It associates with the adaptor protein DAP10, which carries a phosphatidylinositol-3 kinase (PI3K) binding motif; the phosphorylated form of this tyrosine motif can bind the p85 subunit of PI3K and Grb2 [110]. In tumour microenvironment (TME), many cytokines regulate NKG2D expression on NK cells and its signalling. IL-15 enhances the surface expression of this receptor and increases also the expression of DAP10; on the other hands, cytokines such as IL-4 and TGFβ down regulates NKG2D and other NK cell markers expression *in vitro* and *in vivo* as a result to decrease NKG2D dependent cell killing [111] [112].

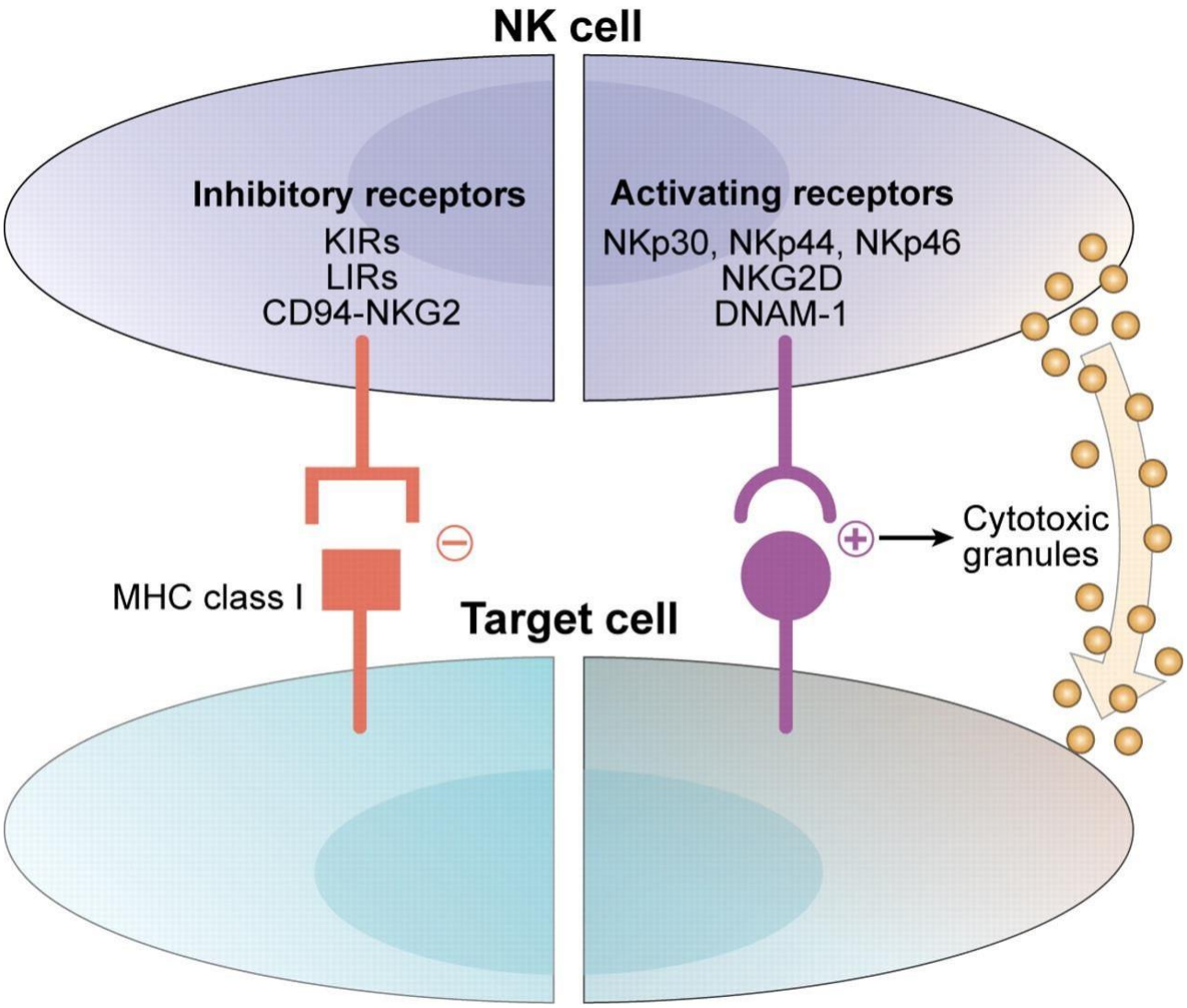

Fig.9 NK-cell reactivity is determined by the balance between activating and inhibitory signals.
(Nina Shah et al. Blood 2009)

The ligands for NKG2D are self-proteins related to MHC class I molecules. In humans, these ligands consist of the MHC class I chain-related protein (MIC) family (e.g., MICA and MICB) and the UL16-binding protein (ULBP1-6) family [113].

Generally, NKG2D ligands are absent on the cell surface of healthy cells but are frequently upregulated upon cellular stress associated with viral infection and malignant transformation [114] and NKG2D-dependent elimination of tumour cells expressing NKG2D ligands has been well documented *in vitro* and in tumour transplant experiments [115]. In humans, NKG2D ligands have been described on different primary tumours. MIC and ULBP families of ligands are found prevalently expressed by carcinomas of the breast, lung, colon, ovary, kidney, and prostate, melanomas, gliomas, and leukemia. Regulation of NKG2D ligands exerts at different levels including transcription, mRNA stability, translation, protein stabilization, and excretion/shedding of ligands from cells [116] [117] [118]. DNA damage response mediated by

ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related), induces the transcription of an array of NKG2D ligands, such as ULBP1 and ULBP2 and MIC family molecules [50]. In particular, ATM is recruited and activated by DNA double-strand breaks and is involved in several pathways, including the activation of p53 [119] which also induces expression of ligands for NK cell activating receptors such as ULBP1 and ULBP2 [120]. At the post-translational level, NKG2D ligands such MICA, MICB, and ULBPs were found to be cleaved from the cell surface by proteases ADAM10/17 and MMP14 and in some cases by tumour derived exosomes [121] [122].

Another NK activating receptor is DNAM-1 (CD266) that is a 65-kDa surface glycoprotein expressed by NK cells. DNAM-1 triggers NK cell–mediated cytolysis by promoting actin and granule polarization by using immunoreceptor tyrosine tail (ITT)-like motif, Grb2, and other downstream molecules such as Vav-1, PI3K, and phospholipase C-γ1 to activate ERK and Akt [123]. Moreover, in the last few years, it was underlying the recruitment and phosphorylation of Tyr322 and Ser329 in the DNAM-1 cytoplasmic tail for DNAM-1 adhesion and signalling [124]. DNAM-1 has been found to interact with the poliovirus receptor PVR (CD155) and with Nectin-2 (also known as CD112 or PVRL2), CD96, and TIGIT inhibitory immune receptors [125]. The genes for the DNAM-1 ligands, CD155 and CD112, belong to the nectins, a large family of Ig-like molecules involved in cell-cell adhesion [126] [127]. Additionally, the interaction of NK cell receptors with members of the nectin-like molecules underlines the importance of adhesion in the triggering of NK cell functions; it also shows that β2-integrins, LFA-1, may participate in triggering NK cell-mediated cytotoxicity by interacting with DNAM-1 [128] [129]. Generally, ligands for DNAM-1 activating receptor are not expressed on cell surface but recent studies have been shown that also CD155 and CD112, such as ligands for NKG2D receptor, may be up modulated by activating ATM/ATR pathway on tumour cells of epithelial or neuronal origin. On the other hand, the hypermethylation of PVR promoter-associated CpG islands induces PVR down-modulation in many tumours [130].

Nutlin

Several classes of low molecular weight inhibitors of the p53-MDM2 interaction have been reported to be able to disrupt the binding between these proteins, then restoring p53 functions.

Nutlins are the first selective class of *cis*-imidazoline small-molecules antagonist of the p53-MDM2 interaction. Through extensive chemical modifications of the lead compound, three inhibitors were ultimately obtained: Nutlin-1 and Nutlin-2 are racemic mixtures and Nutlin-3a is

an active enantiomer isolated from racemic Nutlin-3. Nutlin-1, Nutlin-2, and Nutlin-3 disrupted the MDM2-p53 interaction with IC^{50} values of 260 nM, 140 nM, and 90 nM, respectively, with approximately 150- to 200-fold difference in affinity between enantiomers [57]. Among nutlins compounds, Nutlin-3a has been largely studied for its therapeutic potential and mechanism of action in human cancer. Three amino acid residues from the p53 peptide (Phe^{19}, Trp^{23}, and Leu^{26}) are essential for the p53 binding to the MDM2 hydrophobic pocket [131]. Nutlins bind, in an enantiomer specific manner, to the p53 pocket on the surface of MDM2 by mimicking the interaction of the three critical p53 amino acid residues with the hydrophobic cavity of MDM2. *In vitro* Nutlin-3a show excellent cell permeability to cell membranes, stabilize and accumulate p53 in cell nuclei leading to activation of p53 pathway and the transcription of p53 target genes containing the p53 recognition sequence in their promoter regions (e.g., $p21^{Waf1}$, MDM2). Although many of the p53 signal transduction components have been identified, the mechanisms that control the choice of p53 response and its execution are still poorly understood. Activation of the p53 pathway by nutlins may induces p21-dependent cell cycle arrest and/or apoptosis, but in normal cells, p53 activation by MDM2 inhibitors leads to cell cycle arrest and not cell death [132] [133] (Fig. 10). Induction of cell cycle arrest by p53 is characterized by depletion of S-phase cells and accumulation at G1/S (74) and/or G2/M (75) [134]. Moreover, several studies have shown that normal cells treated with Nutlin-3a are more resistant to apoptosis then transformed cells because Nutlin-3a is less toxic to normal cells than to neoplastic cells [135] [136]. These results suggest that p53 activation by Nutlin-3a is nontoxic to normal cells and are thus encouraging from a therapeutic perspective.

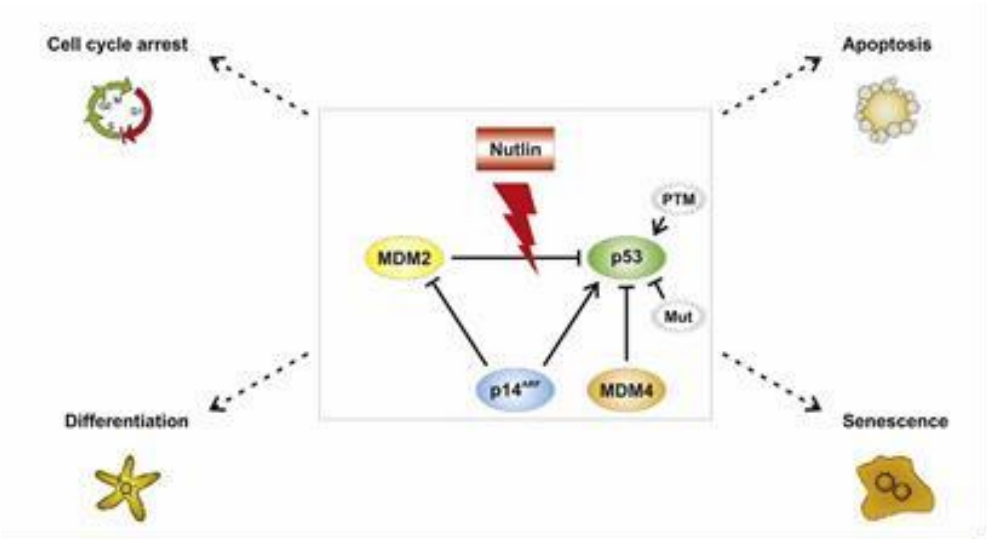

Fig.10 Nutlin activated p53-induced pathway ***(Van Maerken et al. Cancer letter, 2014)***

Another modality of action of Nutlin-3a is represented by the possibility to induce a cellular senescence, a characteristic irreversible cell cycle arrest. The first evidence that Nutlin-3a may induce cellular senescence was obtained in a model of oncogenically transformed fibroblasts [136]. Interestingly, a cellular senescence-like phenotype was achieved in neoplastic cells faster than in their corresponding primary cells, suggesting that Nutlin-3a and oncogenic signalling cooperate in mediating cellular senescence. A number of subsequent studies have underlined that senescence-like status induced by Nutlin-3a was strictly dependent on the presence of functional p53 [137] [138].

Generally, in response to genotoxic stress, p53 undergoes post-translational modifications (e.g. phosphorylation), but recently, various studies demonstrated that Nutlin-activated p53 is free of phosphorylation on six key serine residues but functions well or better as a transcription factor than p53 induced by two genotoxic drugs such as doxorubicin and etoposide [139]. This study revealed that p53 phosphorylation is not necessary for p53 activation, and that p53 transcriptional activity depends on its nuclear level rather than phosphorylation status. Moreover, overexpression of MDM2 as a result of amplification of the *mdm2* gene locus has been shown to correlate well with wild type (wt) p53 status.

Based on this observation, osteosarcoma cancer cell lines with the amplification of *mdm2* gene have responded very well to nutlin treatment both *in vitro* and *in vivo* [57].

Potential therapeutic use of Nutlin-3a in tumours

During the past two years, more than 160 studies have been published using Nutlin-3a to study potential therapeutic applications of this compound. All these studies performed *in vitro* and in animal models confirmed the anti-tumoural potential of the p53/MDM2 inhibitory approach. In addition, most studies demonstrated that patients with functional p53 pathway could take advantage of Nutlin-3a treatment.

With respect to hematological malignancies, acute myeloid leukemia (AML), B-chronic lymphocytic leukemia (B-CLL), and multiple myeloma are potentially attractive tumour types for Nutlin-3a-based therapy, because they show a relatively low percentage of p53 deletion and/or mutation at diagnosis (<15%) [140]. *Ex vivo* experiments using AML [141], B-CLL [142], and multiple myeloma [143] patient specimens have indeed shown that inhibition of MDM2 by Nutlin-3a effectively triggers apoptosis. Moreover, Nutlin-3a synergizes with doxorubicin and cytosine arabinoside in killing myeloblasts in AML and with doxorubicin, chlorambucil, and fludarabine in killing leukemic cells in B-CLL patient specimens [144]. Importantly, the

combination effect of this drugs with Nutlin-3a is specific for cancer versus normal cells, as revealed by the lack of toxicity to peripheral blood mononuclear cells or bone marrow-derived hematopoietic progenitors and bone marrow stromal epithelium cells [142] [143]. In addition, it was demonstrated that Nutlin-3a might retain its activity in cancers missing upstream signals that regulate p53, such as ATM [141].

On the other hand, all paediatric tumours are very attractive therapeutic targets for Nutlin-3a treatment because most of them are p53 wt at diagnosis. A preclinical study in Rhabdomyosarcoma (RMS) showed that exposure to MDM2-p53 interaction inhibitor, decreased the viability of embryonal rhabdomyosarcoma (eRMS) and alveolar rhabdomyosarcoma (aRMS) cells with wt p53 compared to p53 mutated cell lines where there were only minimal changes. In RMS, MDM2 inhibition induced increased p53 levels and apoptosis markers (Bax, cleaved caspase-3 and cleaved PARP) as well as indicators of cell cycle arrest [145]. No changes were recorded in the mutated p53 RMS cell lines. Moreover, Miyachi et al. found Nutlin-3a induced increases of p53 protein through a post-transcriptional mechanism in RMS cell lines with wt p53 as well as a dose dependent increase in the mRNA levels and protein levels of p21 and MDM2; by contrast, p53 mutated RMS cell lines had little effect on the mRNA and protein levels of p53, p21, and MDM2 [146].

A good cytotoxic activity of Nutlin-3a has been demonstrated also in a variety of *in vitro* studies carried out on both $p53^{wild\text{-}type}$ and $p53^{null}$ NB cell lines. Nevertheless, treatment of a subcutaneous neuroblastoma xenograft model with twice-daily oral Nutlin-3a (200 mg/kg) only partially inhibited tumour growth, despite good sensitivity (IC^{50} of 5.56 µM) of these cells *in vitro* [147] [148] [149]. NB cell line with wt p53 treated with Nutlin-3a show a rapid accumulation of p53 protein, within 1–2h of treatment, and in a robust induction of p53 target gene expression, regardless of their differentiation status [150]. The expression levels of MYCN and $p14^{ARF}$ may determine the outcome of the response to MDM2 inhibition: knockdown and overexpression experiments consistently showed in these studies that both MYCN and $p14^{ARF}$ sensitize NB cells p53 wt to Nutlin-3a treatment. Gamble et al. showed that Nutlin-3a is more active in MYCN amplified compared with non-amplified NB cell lines, by an increased p53 transcriptional response, increased apoptosis and enhanced growth inhibition in MYCN amplified NB cell lines [58].

Of note, p53 is a direct transcriptional target of MYCN and its signalling pathway is potentially functional but impaired by MDM2 binding in MYCN amplified NB cells [27]. It was hypothesised that p53 antagonists such as MDM2 should be more active in MYCN amplified NB cells. Several studies have shown, by pre-clinical studies, the activity of the MDM2/p53 antagonist Nutlin-3a

in NB cell lines alone and in combination with conventional chemotherapy (such as cisplatin, etoposide and camptothecin) [151]. A synergistic antitumor effect in NB has also been documented when Nutlin-3a is combined with the CDK inhibitor (R)-roscovitine [152] or with the anti-VEGF monoclonal antibody bevacizumab [153]. An interesting observation is that Nutlin-3 does not only induce G1 cell cycle arrest and apoptosis in NB cells p53 wt, but also premature cellular senescence and neuronal differentiation depending on the cellular background [154]. These observations indicate that Nutlin-3 has pleiotropic activities to counteract the malignant phenotype of NB cells and suggest that selective MDM2/p53 inhibitors are well suited for treating tumours that are arrested in their differentiation, such as NB. Nutlin-3a was also able to induce anti-tumour activity against metastatic NB lesions, as MYCN DNA content, MYCN mRNA levels and the decreased amount of metastatic foci in liver and lungs Nutlin-3a treated mice [148].

Aim of the project

During my PhD I studied molecular mechanisms that induce immune-evasion response in NB and I explored new strategies to render NB cells more susceptible to NK cell-mediated recognition and lysis. The results showed here may represent the base for a new NK cell-mediated immunotherapy of NB.

As reported above, the expression of ligands for NK cell-activating receptors is necessary for a proper anti-tumor NK cell activation and function. In particular, MICA, MICB, ULBP1, ULBP2/5/6 and ULBP3 ligands, are specific for NKG2D receptor and PVR and Nectin-2 ligands are specific for DNAM-1 receptor. In the last years, many studies showed ULBP1, ULBP2/5/6 and ULBP3 gene expression is regulated by c-Myc and p53 transcription factors [120]. p53 and MDM2 are two direct target of MYCN that is the well-established marker of poor prognosis in NB cells and it plays a key role in several pathways involved in metastasis (angiogenesis, motility, and maintenance of pluripotency).

First, based on this consideration, we asked if MYCN was involved, directly or not, in the expression of activating ligands. Then we tried to modulate MYCN expression by using the BET-bromodomain inhibitor JQ1 that, presently, is a good candidate for MYCN targeting. We explored also new molecular strategies to subvert MYCN function, because direct MYCN modulation is still very challenging. Of note, several chemotherapeutic agents have been reported to function as potent immune adjuvants and to be able to induce the expression of ligands for NK cell- activating receptors in tumour cells of different origin. Since in NB p53 gene is mutated only in 2% of the cases, especially in relapse, we also investigated whether the activation of DDR pathway caused by chemotherapeutic agents currently used in the treatment of NB, could positively affect the expression of ligands for NK cell-activating receptors leading to a better recognition and lysis by NK cells.

Despite an initial response to intensive chemotherapy, relapse with chemoresistant disease is common and can rarely be solved. Moreover, the overall long-term survival of high-risk patients currently remains less than 50%, with survivors often having long-term toxicities as a consequence of the intensive chemotherapy. Thus, there is a continuing need to identify novel and less toxic therapies to improve survival of this group of patients.

As reported above, Nutlin-3a is a small molecule that enhances p53 activity by disrupting the interaction of p53 with its inhibitor MDM2; moreover, it was studied that Nutlin-3a is less toxic to normal cells then to transformed cells by restoring p53 function. Of note, p53 is a direct transcription factor of ligands for NK cell-activating receptors [120], and promotes cellular

senescence, a characteristic irreversible cell cycle arrest. Interestingly, it was demonstrated that a cellular senescence status is necessary to induce the expression of some ligands for DNAM-1 and NKG2D NK cell-activating receptors [49]. Senescence-like status induced by Nutlin-3a was strictly dependent on the presence of functional p53. For all these reasons, by treating NB cell lines and primary cells with Nutlin-3a and by restoring p53 function, we aim to potentiate the expression of activating ligands on cancer cell surface. The effect of the small molecule Nutlin-3a on NB cells can be promising for a new NK cell-based immunotherapy.

Materials and methods

Cell lines and reagents

Human NB cell lines were obtained as follow: GICAN and ACN from Interlab Cell Line Collection, Banca Biologica and Cell Factory (www.iclc.it), SK-N-AS, SH-SY5Y, SH-EP, SK-NSH, SK-N-BE(2)c, IMR-32 from the American Type Culture Collection (ATCC), LA-N-1 from Creative Bioarray, LA-N-5 from the Leibniz-Institut DMSZ, SMS-KCNR from Children's Oncology Group Cell Culture, while the Tet-21/N cell line was kindly provided by Dr M. Schwab (University of Heidelberg, Heidelberg, Germany). The human non-small-cell lung cancer cell line A549 was purchased from Sigma-Aldrich. All NB cell lines were characterized by (i) HLA class I typing by PCR-SSP sets (Genovision) according to the manufacturer's instructions, and (ii) array CGH (see below). The human erythro-leukemia cell line K562 was purchased from ATCC and used as control target for NK cell functional assays. Cells were grown in RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific), 2 mM glutamine, 100 mg/mL penicillin and 50 mg/mL streptomycin (Euro Clone S.p.a.). Doxycycline (Sigma Aldrich) was used at 10 ng/mL. Lipofectamine 2000 was used, according to manufacturer's instructions (Invitrogen), to transfect SK-N-SH cells with pIRVneoSV empty vector or pIRVneoSV-MYCN, both kindly provided by G. Giannini ("La Sapienza" University of Rome, Italy). Cell Tracker Deep Red (Thermo Fisher Scientific) was used at the final concentration of 1µM to mark $5x10^5$ LA-N-5 NB cell line. Cisplatin (Accord Healthcare Limited), etoposide (Teva Italia), irinotecan (Campo, Pfizer), and topotecan (GlaxoSmithKline) were kindly provided by the pharmacy of our institution. JQ1 (Selleckchem) was dissolved in DMSO and used at concentrations of 0,5 µM/L for LAN-5 and SH-SY5Y.

Nutlin-3a (Cayman Chemical) was dissolved in DMSO and used at different doses at on both NB cell lines and NB primary cells, or at 20 and 40mg/kg (Nutlin-3a mg/mouse body weight) for *in vivo* studies. Animal experiments were conducted under the auspices of protocols approved by the Animal Care and Ethics Committee of the University of New South Wales (New South Wales, Australia).

Antibodies, western blotting, flow cytometry and ROS Production.

The following antibodies were used: anti-MYCN, anti-p53, anti-Actin (B8.4.B, FL-393 and I-19, respectively, Santa Cruz Biotechnology), anti-MYC (Y69, OriGene), and anti-MDM2 (2A10, Calbiochem-Millipore) for western blotting; antiCD107a-FITC (H4A3), anti-CD3-Alexa-700 (UCHT), antiCD56-PE-Cy7 (B159), anti-CD45 (HI30), FITC-conjugated rat anti-mouse IgG1

(A85–1) and PE-conjugated rat anti-mouse IgM (R6–60.2) purchased from BD Biosciences; anti-ULBP1-PE (170818), anti-ULBP2/5/6-PE (165903), anti-ULBP3-PE (166510), anti-MICA (159227), anti-MICB (236511), antiTRAIL/R2-APC (17908), anti-CD155/PVR-PE (300907), antiNectin-2/CD112-APC (610603) purchased from R&D Systems; W6/32 which recognizes human fully-assembled MHC class I heavy chains and goat F(ab')2 Fragment anti-mouse IgG FITC (IM1619, Dako) for flow cytometry; anti-MICA, antiNectin-2 (62540 and 154895, respectively, Abcam) and anti-PVR (Novus Biological) for immunohistochemistry assay. Apoptosis of tumour cells was evaluated with APC-conjugated AnnexinV (BD-Pharmingen) and propidium iodide (PI) (Sigma-Aldrich). ROS production was evaluated in drug-treated NB cell lines by using CellROX Deep Red Reagent (C10422, Invitrogen) and measured by flow cytometry. Flow cytometry was performed on FACSCantoII (BD Bioscences) and analyzed by FACSDiva Software (BD Biosciences). Whole-cell extracts were quantified by the bicinchoninic acid assay (Thermo Fisher Scientific), resolved on 8–10% SDS-PAGE and electroblotted. Filters were probed with primary antibodies followed by goat anti-mouse IgG HRP conjugated (Jackson).

Patient samples

Tumour samples from 12 NB patients and sections of normal intestinal mucosa and colon carcinoma, diagnosed at the Bambino Gesu Children's Hospital, were used. For each patient, written informed parental consent and approval by the Ethical Committee of the Institution were obtained.

Array CGH

DNA from NB cell lines was tested by high-resolution array comparative genomic hybridization (CGH) SNP arrays. The test involved the use of a 180 K platform with a mean resolution of approximately 40 kb (4 x 180 platform, Agilent Technologies). A copy number variant was defined as a displacement of the normal value of at least three consecutive probes, and the mapping positions refer to the Genome Assembly hg19 (build 37). SNP-array and oligoarray data were analysed with Genomic Workbench 7.0.40 software (Agilent). The quality of the test was assessed on the strength of the QCmetrics values. Polymorphisms (http://projects.trag.ca/variation/) were not included because considered normal variants.

Senescence-associated β-galactosidase (SA β-Gal) activity

Subconfluent Tet-21/N left untreated or treated with doxycycline, cultured in six-well plates, were fixed using 4% formaldehyde for 10 min at room temperature, washed twice with PBS and then stained for β-gal activity at pH 6.0, according to manufacturer's instructions (Promega). Images were acquired on an Olympus IX51 inverted microscope, and SA β-Gal positive (blue) cells were counted for five fields of view (x 20 magnification) per well.

ChIP analysis

Cells were treated with formaldehyde (1% final concentration), added 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 min at RT. Cells were washed with cold phosphate buffered saline, scraped and lysed in L1 buffer (2mM EDTA, 50mM Tris-HCl [pH8.1], 0.1% NP40, 10% Glicerol and protease and phosphatase inhibitors) for 20 min at 4°C in rotation. The lysates were homogenized by 15 dounce strokes and then centrifuged at 5000 rpm for 5 min at 4°C. Nuclear pellets were resuspended in L2 buffer (5mM EDTA, 50mM Tris-HCl (pH8.0), SDS 1% and protease and phosphatase inhibitors) and kept on ice for 10 min. Nuclear lysates were sonicated to obtain chromatin fragments of an average length of 200 to 500 bp and centrifuged at 10000 rpm for 10 min at 4°C. After determining the DNA concentrations, each chromatin sample was divided into aliquots of 150 µg. The sonicated supernatant fractions were diluted 10 fold with dilution buffer (5mM EDTA, 50mM Tris-HCl (pH8.0), NP40 0.5%, NaCl 200 mM and protease and phosphatase inhibitors). For each condition one aliquot for the specific antibody (anti p53) and one aliquot for the IgG control were incubated with Protein A Sepharose, saturated with BSA and salmon sperm, for 3 hours at 4°C on a rotating platform. The pre-cleared chromatin samples were centrifuged at 14000 rpm for 5 min and incubated with the respective antibody or IgG overnight with gently rotation at 4°C. Immuno-precipitated samples were recovered by incubation with saturated protein A sepharose on a rotating platform for 3 hours at 4°C. Before washing, the supernatant of the IgG control was taken as input sample. After extensive washing (5 min at 4°C in rotation and subsequent centrifugation at 3000rpm for 2 min) with wash buffers (2 washes with 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150 mM NaCl; 2 washes with 0.1% SDS, 1% triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl; 1wash with 0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1; 1 wash with 10mM HCl, 1mM EDTA, pH 8.0) samples were eluted in elution buffer (100 mM NaHCO3, SDS 1%) at room temperature while vortexing for 30 min. After the elution, samples were centrifuged at 13000 RPM for 5 minutes and the eluate was collected. The samples

treated with RNAse A for 10 min at RT were incubated at 67°C over night to reverse the protein-DNA cross-linking. Then in each sample the $NaHCO_3$ was neutralized with 6 µl Tris-HCl 1M (pH 6-7.5). After treatment with proteinase K, the DNA was extracted with phenol-chloroform, precipitated with ethanol and re-suspended in 50 µl of distilled water. 5 ng of the immunoprecipitated, IgG and input samples were used for PCR with the specific oligonucleotides spanning 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 termocycler "CFX Connect Real time PCR detection system" (Bio-Rad). The primer pair efficiency, the relative quantity of each immunoprecipitated and IgG ($\Delta C(t)$) respect to Input sample and the Standard Error of the mean of the relative quantity were determined with CFX Manager™ software (Bio-Rad). The percentage of the relative quantity of each immunoprecipitated sample was normalized respect to IgG and expressed as percentage of input chromatin (% input).

Quantitative mRNA expression

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific). First-strand cDNA was synthesized 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 from Applied Biosystems, Thermo Fisher Scientific (Hs00792195_m1 for MICA, Hs00360941_m1 for ULBP1, Hs00607609_m1 for ULBP2, Hs00225909_m1 for ULBP3, Hs00197846_m1 for PVR, Hs01071562_m1 for Nectin-2). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method and $2^{-\Delta Ct}$ considered as expression level, with GAPDH (Hs02758991_g1) as endogenous control.

NK cell isolation

Human NK cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors with the RosetteSep NK-cell enrichment mixture method (StemCell Technologies) and Ficoll-Paque Plus (Lympholyte Cedarlane) centrifugation. NK cells were routinely checked for the $CD3^-CD56^+$ immunophenotype by flow cytometry and those with purity greater than 90% were cultured with 600 IU/mL of recombinant human IL-2 (PeproTech) at 37°C and used up to 5 d after isolation for *in vitro* functional assays. NK cells isolated for in mice injections were expanded in NK MACS medium (Miltenyi Biotec).

Cytotoxicity and degranulation assay

NK cell cytotoxic activity was tested by a standard 4-h ^{51}Cr release assay. Degranulation assay was performed by co-culturing NK cells with target cells at 1:1 ratio for 3 h, in complete medium in presence of anti-CD107a and in the last 2 h of GolgiStop (BD Bioscence). Then, cells were stained with anti-CD56 and anti-CD45 and expression of CD107a was evaluated by flow cytometry in the $CD56^+CD45^+$ subset. Specific lysis was converted to lytic units (L.U.) calculated from the curve of the percentage lysis. One lytic unit is defined as the number of NK cells required to produce 20% lysis of 10^6 target cells during the 4 h of incubation.

Immunohistochemistry assay

LA-N-5 bearing NSG mice were treated intraperitoneally with DMSO or Nutlin-3a at 20mg/kg (drug dose/mouse body weight) and at 40mg/kg every two days for two weeks. Tumour masses were harvested, formaldehyde-fixed, thus paraffin-embedded blocks were cut into 3-mm sections and baked for 60 min at 56°C in a dehydration oven. Tissues slides from DMSO, Nutlin-3a at 20mg/kg and at 40mg/kg masses were mounted on the same slide and parallel stained for the same antibody. Antigen retrieval and deparaffinization were performed on a PT-Link (Agilent Technologies) using the EnVision FLEX Target Retrieval Solution kits at high pH (Agilent Technologies) for PVR and Nectin-2, as per manufacturer's instruction. Slides were then blocked for endogenous peroxidase for 10 min with a peroxidase blocking solution (Agilent Technologies), rinsed in the appropriate wash buffer (Agilent Technologies), and incubated for 30 min with 5% PBS/BSA. Slides were then incubated overnight at 4°C with primary antibodies PVR (1:50) and Nectin-2 (1:300). Twenty minutes incubation with secondary antibody coupled with peroxidase (Agilent Technologies) has been subsequently performed. Bound peroxidase was detected with diaminobenzidine (DAB) solution and EnVision FLEX Substrate buffer containing peroxide (Agilent Technologies). Tissue sections were counterstained with EnVision FLEX Haematoxylin (Agilent Technologies). Formaldehyde-fixed paraffin-embedded blocks from NB patients were prepared as described above and the expression of MICA (1:200) and Nectin-2 (1:300) was evaluated. Sections of normal intestinal mucosa, colon carcinoma and kidney were used as positive controls for MICA, Nectin-2 and PVR respectively. Isotype-matched mouse mAbs were used as negative controls.

Xenograft neuroblastoma model and treatment of NSG mice

All in vivo experiments utilized 4- to 6-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (Charles River Laboratories Italia srl), Briefly, 5 x 10^6 cells of LA-N-5 NB cells resuspended in 100-mL PBS were injected subcutaneously (sc) into flank of the mice [155]. Mice were randomly divided into 4 groups (5 mice for each group), and treated with 1) control solvent, 2) Nutlin-3a, 3) IL-2 4) NK cells plus IL-2 or 5) NK cells plus IL-2 plus Nutlin-3a. 40mg/kg of Nutlin-3a was intraperitoneally (i.p.) injected every two days for three cycles. NK cells were peritumorally injected 48 hours after the first Nutlin-3a injection, every 5 days, for two cycles. IL-2 ($10x10^3$ U/mouse) was injected i.p. every day, starting from the first NK cell injection and for the entire NK cell treatment. Tumour size was assessed every day by caliper measurement, until 45 days after LA-N-5 injection. All animal experiments were performed in accordance with a protocol approved by the Italian Ministry of Health and Usage Committee of Children's Hospital Bambino Gesù.

Statistical analysis

Digital images of western blotting were analysed by Image J (http:// rsbweb.nih.gov/ij/index.html) and statistical significance of densitometric values was evaluated by the two-tailed unpaired Student's *t*-test. Normalized values were analysed for correlation by the regression analysis using GraphPad software. p values lower than 0.05 were considered to be statistically significant.

Results

Expression of MYCN inversely correlates with that of ligands for NK cell-activating receptors in NB cell lines

NK-cell cytotoxicity is triggered by the interaction of NK-cell activating receptors with their specific ligands. In particular, NKG2D receptor recognizes MICA, MICB, ULBP1, ULBP2/5/6 and ULBP3 ligands [156], while DNAM1 receptor interacts with PVR and Nectin-2 ligands [125]. We asked whether the expression levels of the MYCN oncogene correlated with a mechanism of immune escape involving down-regulation of NK-cell-activating receptor ligands on NB cells. To investigate this possible correlation, we assessed the protein levels of MYCN by western blotting (Fig.12) and the surface expression of activating ligands by flow cytometry in a panel of 12 NB cell lines (Fig.11), including 6 MYCN non-amplified (non-MNA) and 6 MYCN amplified (MNA).

Regression analysis of normalized densitometric values of western blotting and mean fluorescence intensity values of flow cytometry analysis revealed a significant inverse correlation between the expression of MYCN and that of the activating ligands MICA, MICB, ULBP1, ULBP2/5/6, ULBP3 and PVR. A trend toward an inverse correlation was also found between expression of MYCN and that of MHC class I and of Nectin-2. On the other hand, MYCN expression did not correlate with that of TRAIL-R2 (Fig.11).

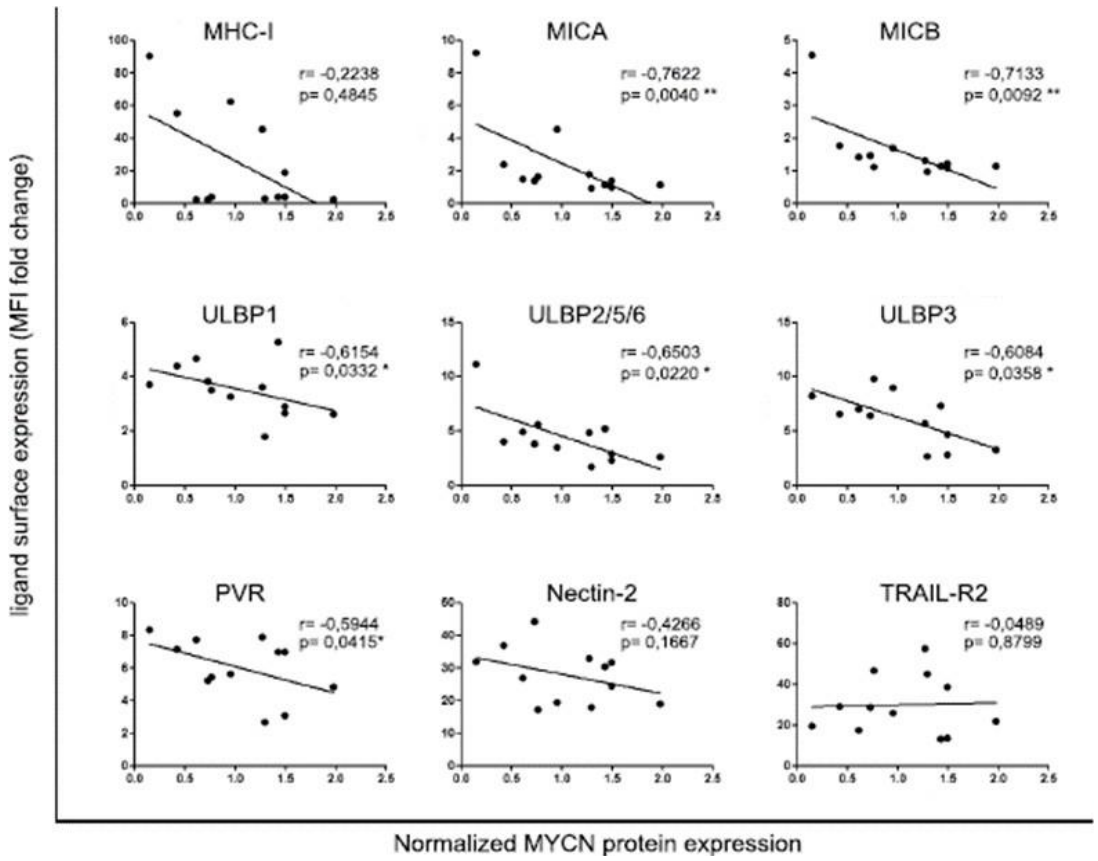

Fig.11 : Scatter plots showing the correlation between MYCN and the surface expression of activating ligands for NK-cell receptors in twelve NB cell lines. The means of four independent immunoblot analyses of MYCN expression in NB cell lines are plotted against the means of fifteen independent flow cytometric analyses of surface expression of the indicated activating ligands; Spearman correlation r and *p* value are shown for each plot.* $p<0.05$, **$p<0.01$.

c-MYC and p53 are two transcription factors involved in the induction of ULBP ligands [120]. Of note, *p53* gene is a direct transcriptional targets of MYCN in NB [157].

To assess whether p53 and c-MYC could be involved in the expression of activating ligands in NB, the status of MYCN, c-MYC, p53 and MDM2 was evaluated in NB cell lines in terms of amplification, gain, deletion and protein expression by high-resolution array CGH analysis and western blotting (Fig.12), respectively. *p53* gene was lost in four NB cell lines tested [SK-N-AS, ACN, SK-N-BE(2)c and LA-N-1 [158]], three of them [SK-N-AS, ACN and SK-N-BE(2)c] also lost *MDM2* gene. *c-MYC* gene gain was detected in two NB cells lines (SK-N-AS and ACN), whereas *MYCN* gene amplification or gain were found in five [SK-N-BE(2)c, LA-N-1,LA-N-5, SMS-KCNR, IMR-32] and two (GICAN, SK-N-SH) NB cell lines, respectively (Table 2).

NB cell lines	*MYCN*	Chr. coordinates of *MYCN* gain/amp	*c-MYC*	Chr. coordinates of *c-MYC* gain	*p53*	Chr. coordinates of *p53* loss	*MDM2*	Chr. coordinates of *MDM2* loss
SK-N-AS	single copy	—	gain	Chr8: 46924418–129841304 Cytoband: 8q11.1-q24.21 Size: 82.9 Mb	loss	Chr17: 51885–22242373 Cytoband: 17p13.3-p11.1 Size: 22.1 Mb	loss	Chr12: 37944373–112034509 Cytoband: 12q11-q24.12 Size: 74 Mb
GICAN	gain	Chr2: 16066442–18776030 Cytoband: 2p24.3-p24.2 Size: 2.7 Mb	single copy	—	single copy	—	single copy	
ACN	single copy	—	gain	Chr8: 59043056–146280020 Cytoband: 8q12.1-q24.3 Size: 87.2 Mb	loss	Chr17: 84287–22080868 Cytoband: 17p13.3-p11.2 Size: 21.9 Mb	loss	Chr12: 38766104–133767986 Cytoband: 12q12-q24.33 Size: 95 Mb
SH-SY5Y	single copy	—	single copy	—	single copy	—	single copy	—
SH-EP	single copy	—	single copy	—	single copy	—	single copy	—
SK-N-SH	gain	Chr2: 17019–48571447 Cytoband: 2p25.3-p16.3 Size: 48.5 Mb	single copy	—	single copy	—	single copy	—
SK-N-BE(2)c	amp	Chr2: 16082217–16469668 Cytoband: 2p24.3 Size: 387 Kb	single copy	—	loss	Chr17: 29169–22242373 Cytoband: 17p13.3-p11.1 Size: 22.2 Mb	loss	Chr12: 37873948–131685128 Cytoband: 12q11-q24.33 Size: 93.8 Mb
LA-N-1	amp	Chr2: 16066442–16487029 Cytoband: 2p24.3	single copy	—	loss	Chr17: 183662–22205821 Cytoband: 17p13.3-p11.1	single copy	—

Chr: chromosome; amp: amplification.

Tab 2. Array CGH analysis about the status of MYCN, c-MYC, p53 and MDM2

All these genetic aberrations were confirmed at the protein level, except for SK-N-BE(2)c, which expressed high levels of mutant p53, due to a previously reported missense mutation. Consistently with previously published studies, MYCN expression was inversely correlated with c-MYC expression. In contrast, MYCN expression directly correlated with both p53 and MDM2 expression, as evaluated in p53 wt NB cell lines (SH-EP, GICAN, SH-SY-5Y, SK-N-SH, LA-

N-5, SMS-KCNR, IMR-32 and Tet-21/N). This indicates that p53 is more functional in non-MNA NB cells, even if expressed at low levels, than in MNA NB cells in which it is inhibited by MDM2.

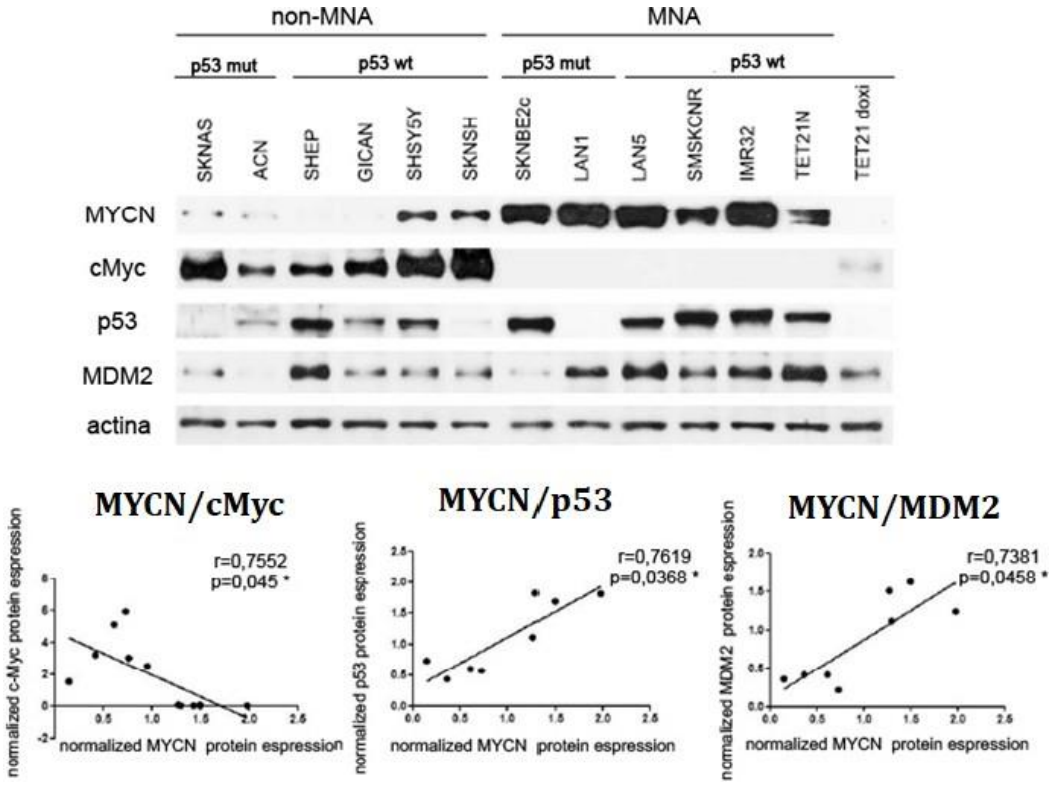

Fig.12 MYCN is inversely correlated with c-MYC and directly correlated with p53 and MDM2 in NB cell lines Immunoblot analysis of MYCN, c-MYC, p53 and MDM2 in the NB cell lines indicated. An anti-Actin Ab was used for normalization. One representative experiment out of the five performed is shown. Scatter plots showing the correlation between MYCN and c-MYC (B), p53 (C) or MDM2 (D) in twelve NB cell lines. The average of densitometric values of 5 independent experiments for each marker is plotted; Spearman correlation r and *p* values value are show for each plot.

The modulation of MYCN expression affects that of ligands for NK cell-activating-receptors and the susceptibility of Neuroblastoma cells to NK cell-mediated lysis

To investigate whether modulation of MYCN expression could affect that of activating ligands, we used the conditionally MYCN-expressing Tet-21/N cell line that loses MYCN expression following treatment with doxycycline (Doxy). Doxycycline treatment caused a drastic down-regulation of MYCN expression, early induction of c-MYC expression (at 8 h) and delayed reduction of p53 and MDM2 expression (at 24 h) (Fig.13). Following 16 h treatment with doxycycline, the expression of MICA, ULBP2/5/6, ULBP3 and PVR was significantly higher in

Doxy-treated Tet-21/N than in untreated Tet-21/N cells. Up-regulation of NK cell-activating-receptor ligands was detected up to 24 h after doxycycline treatment, with a peak at 16 h (Fig.14).

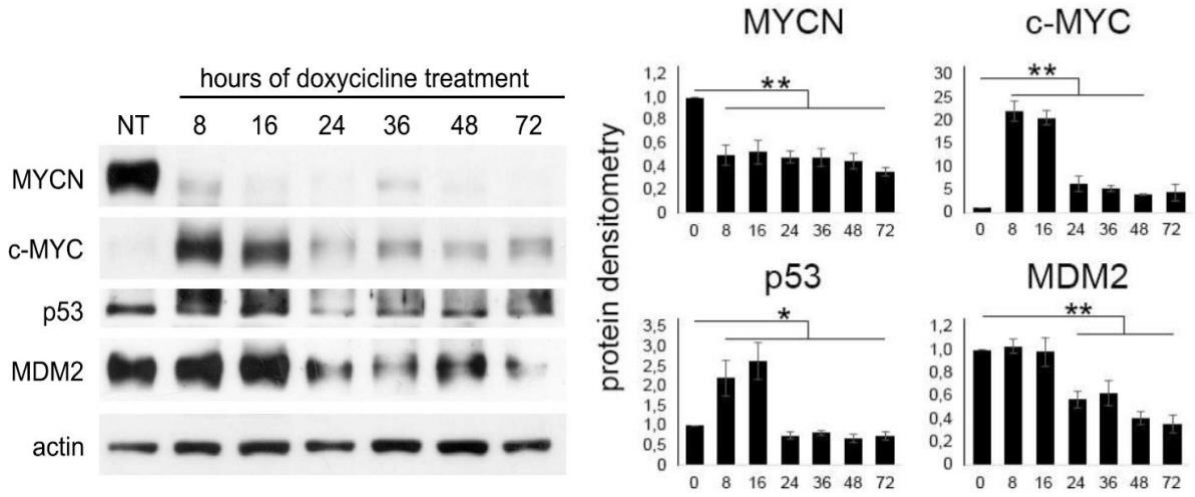

Fig 13. Modulation of MYCN affects the expression of ligands for NK-cell activating receptors. Representative exemple of immunoblot analysis of MYCN, c-Myc, p53 and MDM2 in Tet-21/N either left untreated (0) or treated with doxycycline for the indicated time. An anti-actin Ab was used for normalization. Densitometry analysis of Actin-normalized proteins values of three independent experiments are shown. Mean ± SD; * $p<0.05$, ** $p<0.01$.

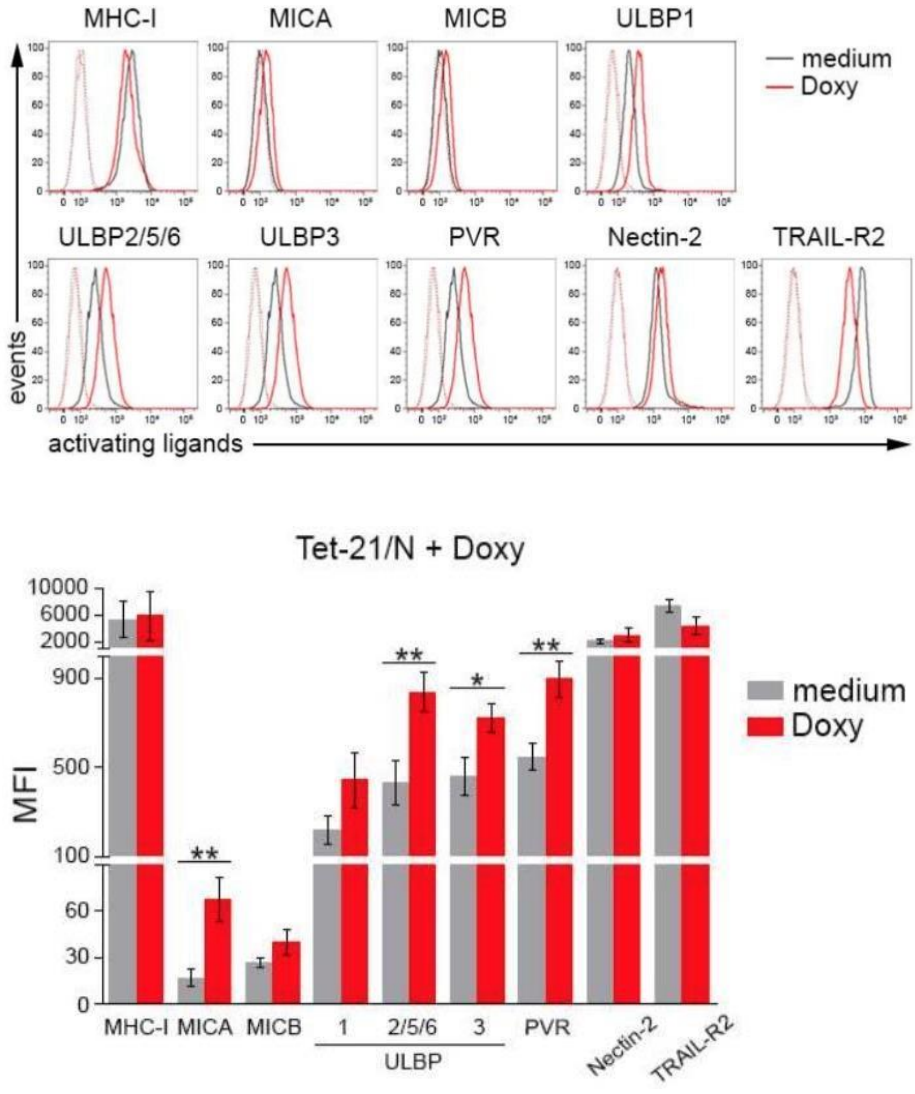

Fig.14 Representative flow cytometric analysis of surface expression of activating ligands for NK-cell receptors in Tet-21/N either left untreated (medium, gray line) or treated with doxycycline for 16 h (Doxy, red line); dotted lines, isotype-matched negative controls (left panel). Summary of five independentflow cytometric analyses (right panel). p values, compared with untreated and Doxy-treated Tet-21/N cells (two-tailed paired Student's t-test); *$p < 0.05$, **$p < 0.01$.

In Tet-21/N cell line a state of cell senescence was detected 16 h after doxycycline treatment, as evaluated by senescence associated-β-galactosidase (SA-β-Gal) staining (Fig.15). Three days after doxycycline treatment, the occurrence of cell differentiation, revealed by changes of cell morphology, became evident (Fig.15). This finding is consistent with results reported by other authors [49] on the expression of activating ligands in senescent cells, but not in mature and differentiated cells.

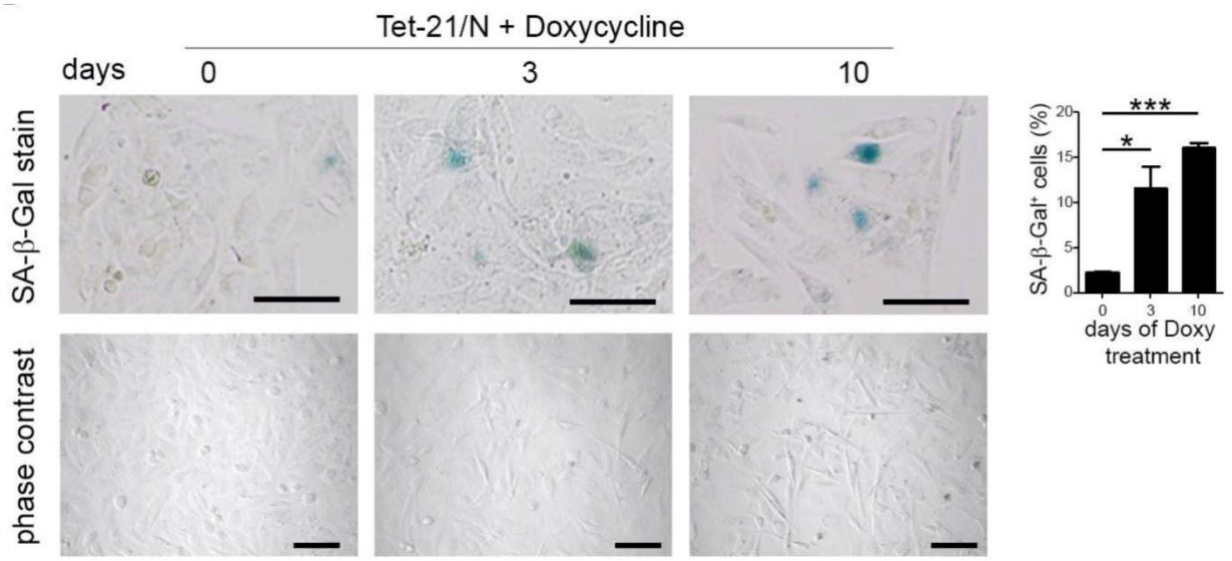

Fig.15 SA-β-Gal staining and morphology of Tet-21/N cells left untreated or treated with doxycycline for 3 or 10 days, bar 50 m. The percentage of SA-β-Gal positive cells, evaluated from 5 different fields for each condition, by counting at least 200 cells per field, is reported, mean ± sd (right panel), (two-tailed paired Student t test) *p<0.05, ***p<0.001.

To further confirm that MYCN expression affects that of ligands for NK cell-activating-receptors, we transiently transfected a non-MNA NB cell line as SK-N-SH with piRV-neoSV vector bearing MYCN cDNA (SK-N-SH-MYCN) or the empty vector (SK-N-SH-ctrl) as control. The overexpression of MYCN, peaking at 16 h and gradually decreasing until 72 h, induced downregulation of c-MYC (from 16 to 72 h) and upregulation of both p53 (from 16 to 48 h) and MDM2 (from 16 to 24 h), as revealed by western blotting (Fig.16). SK-N-SH-MYCN cells showed decreased surface expression of activating ligands as compared with SK-N-SH-ctrl cells, significantly evident 48 h after transfection.

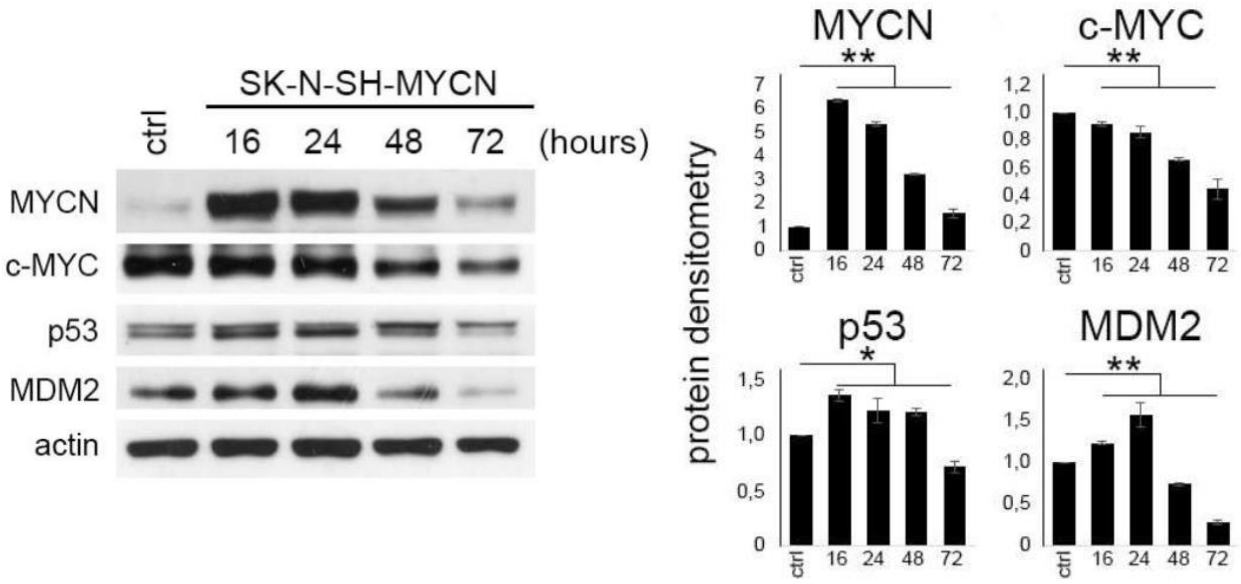

Fig.16 Representative example of immunoblot analysis of SK-N-SH either left transfected with empty vector (ctrl) or with piRVneoSV-MYCN (MYCN) for the indicated time (left panel). Densitometry analysis of Actin-normalized proteins values of three independent experiments are shown (right panel).

SK-N-SH-MYCN cells showed decreased surface expression of activating ligands as compared with SK-N-SH-ctrl cells, significantly evident 48 h after transfection (Fig.17).

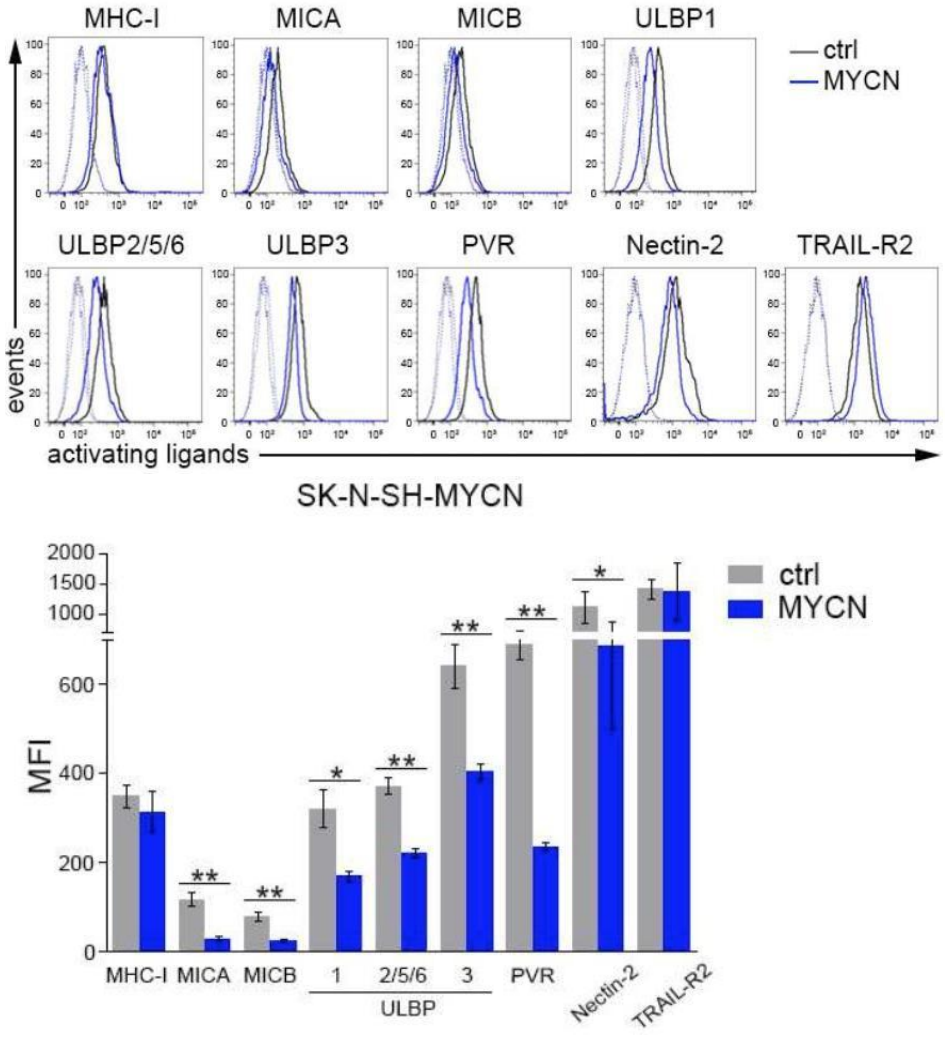

Fig.17. Representative flow cytometric analysis of surface expression of activating ligands for NK-cell receptors in SK-N-SH transfected for 48 h with control vector (ctrl, gray line) or with MYCN cDNA vector (MYCN, blue line); dotted lines, isotype-matched negative controls (left panel).
Summary of five independent flow cytometric analyses (right panel). p values, compared with ctrl and MYCN cDNA vector transfected cells (two-tailed paired Student's t-test); *p < 0.05,**p < 0.01.

Later, to test whether the increased expression of NK cell-activating receptor ligands induced by MYCN modulation could affect NK cell-mediated recognition of NB cells, we performed degranulation and cytotoxicity assays using untreated and Doxy-treated Tet-21/N cells, as well as SK-N-SH-ctrl and SK-N-SH-MYCN cell lines as targets. After 16 h treatment with doxycycline, Tet-21/N cells were significantly more susceptible to NK cell-mediated lysis than untreated cells in both degranulation and cytotoxicity assays. Conversely, SK-N-SH-MYCN cell lines were less susceptible to NK cell recognition and lysis, compared with control cells, as evaluated in both degranulation and cytotoxicity assays (Fig. 18).

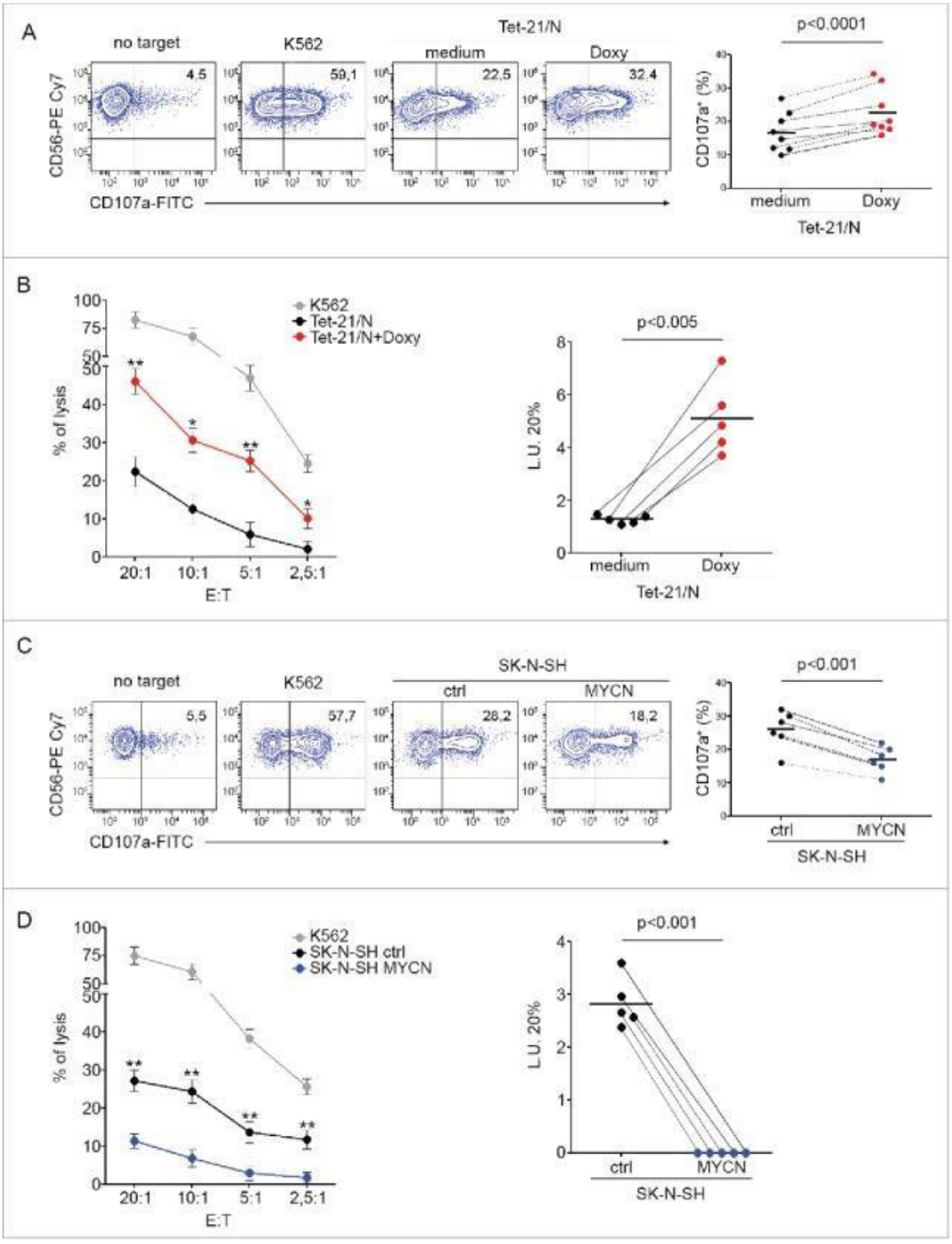

Fig. 18. Modulation of MYCN renders NB cells differently susceptible to NK-cell-mediated lysis. (A) Degranulation of human $CD3^{-}CD56^{+}$ $CD45^{+}$ NK cells from healthy donors, measured as CD107a cell-surface expression following stimulation with Tet-21/N cells, either left untreated (medium) or treated with doxycycline for 16 h (Doxy). K562 cells were used as positive control. The percentage of $CD107a^{+}$ NK cells is indicated. A representative experiment out of the eight performed is shown (left panel). Summary of NK-cell degranulation of cells isolated from eight healthy donors is shown (right panel). Dots, percentage of $CD107a^{+}$ NK cells; horizontal bars, average values. Dashed lines connect percentage of $CD107a^{+}$ NK cells from each donor cells. *p* values, compared with untreated and doxycycline-treated Tet-21/N cells (two-tailed paired Student's *t*-test). (B) Tet-21/N untreated or treated as in (A) were tested as targets for NK cells at the indicated effector:target (E:T) ratios in a standard ^{51}Cr-release assay. One representative experiment out of the five performed is shown (left panel). *p* values, compared with untreated and doxycycline-treated Tet-21/N cells (two-tailed paired Student's *t*-test); $*p < 0.05$, $**p < 0.01$. Summary of cytotoxic assay of Tet-21/N cells treated as in A and tested as targets of NK cells isolated from five healthy donors in a standard ^{51}Cr-release assay (right panel). Specific lysis was converted to L.U. 20%. Dots, L.U. 20% of the effector/target pairs tested; horizontal bars, average values. Dashed lines connect L.U. 20% from each donor cells obtained with the indicated target. *p* values, compared with untreated and doxycycline-treated Tet-21/N cells (two-tailed paired Student's *t*-test). (C) Degranulation assay of human NK cells, as in (A), following stimulation with SK-N-SH cells transfected for 48 h with piRVneoSV vector (ctrl) or with piRVneoSV- MYCN (MYCN). One representative experiment out of the six performed (left panel) and the summary of six experiments (right panel) are shown. (D) Cytotoxic activity of healthy donor NK cells, as in (B), against SK-N-SH cells transfected for 48 h with ctrl or MYCN cDNA vector. One representative experiment out of the five performed (left panel) and the summary of five experiments (right panel) are shown.

The expression of MYCN in primary Neuroblastoma samples is inversely correlated with that of ligands for NK cell-activating receptors

Next, the expression of MYCN and that of MICA, ULBP1, ULBP2, ULBP3, PVR andNectin-2 was investigated in 12 different primary neuroblastoma samples by qPCR. Similarly to NB cell lines, MYCN expression was inversely correlated with that of activating ligands also in NB patient samples (Fig.19). A significant inverse correlation between the expression of MYCN and that of MICA and Nectin-2 was detected.

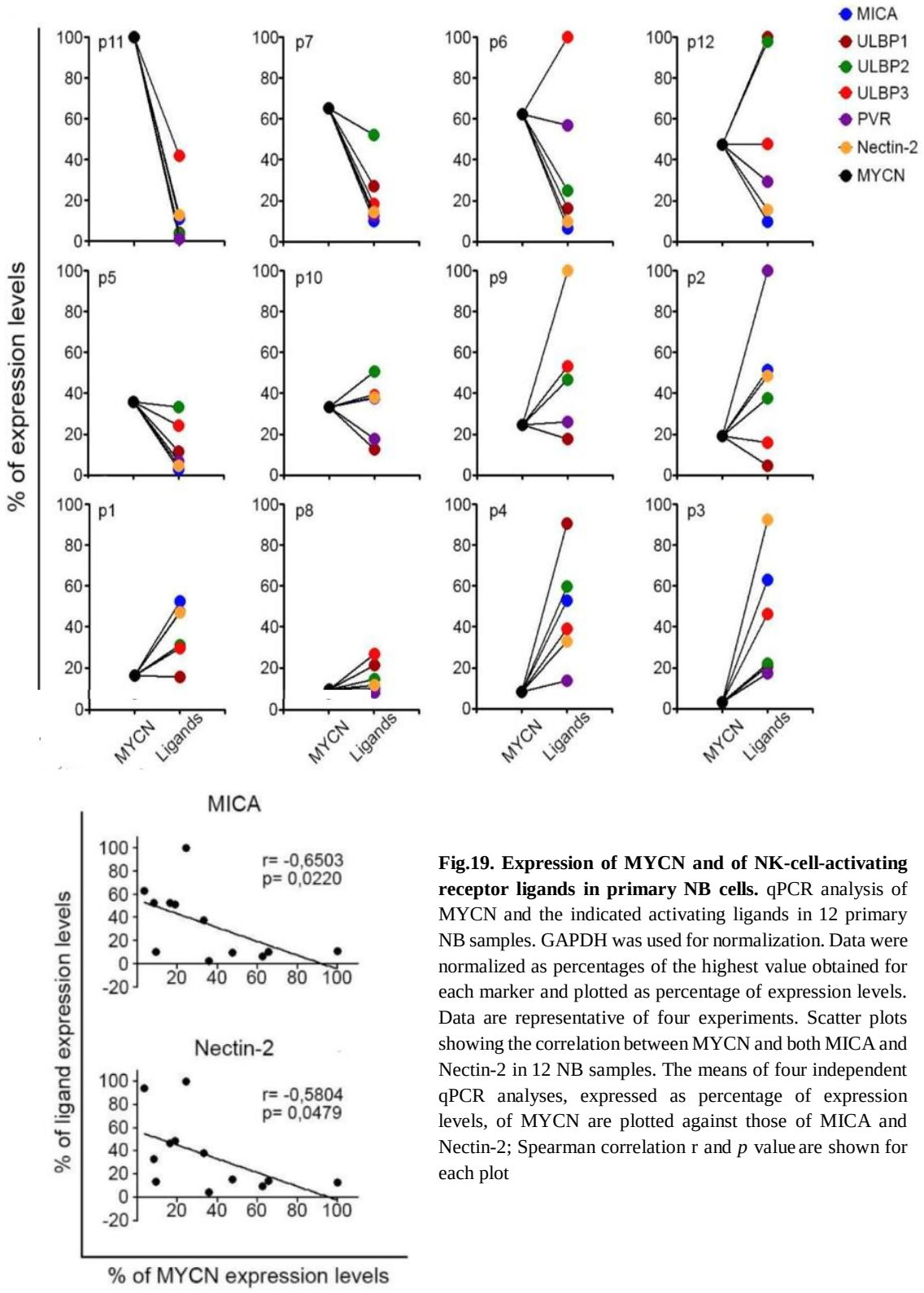

Fig.19. Expression of MYCN and of NK-cell-activating receptor ligands in primary NB cells. qPCR analysis of MYCN and the indicated activating ligands in 12 primary NB samples. GAPDH was used for normalization. Data were normalized as percentages of the highest value obtained for each marker and plotted as percentage of expression levels. Data are representative of four experiments. Scatter plots showing the correlation between MYCN and both MICA and Nectin-2 in 12 NB samples. The means of four independent qPCR analyses, expressed as percentage of expression levels, of MYCN are plotted against those of MICA and Nectin-2; Spearman correlation r and *p* value are shown for each plot

Thus, high levels of MYCN expression corresponded to low levels of expression of activating ligands and *vice-versa*, with the exception for some activating ligands in patients 6, 12 and 8. Interestingly, primary sample from patient 12 expressed moderate levels of MYCN and high

levels of ULBP1 and ULBP2. Notably, this patient had 4S NB, a tumour characterized by high rate of spontaneous regression [159].

Finally, the expression of MICA and that of Nectin-2 protein was evaluated by immunohistochemical assay in primary NB samples from patient 11 and 3 that displayed markedly different expression of MYCN mRNA and of activating ligands (Fig.20). The stroma-poor tumour from patient 11, characterized by *MYCN* gain and low differentiation grade, showed low expression of both MICA and Nectin-2 (Fig 20 upper panels). By contrast, the stroma-rich tumour from patient 3, characterized by undetectable MYCN expression (lower panels), with mature differentiation grade, showed high expression levels of both MICA and Nectin-2 in ganglion cells. Thus, the inverse correlation between the expression of MYCN and MICA and Nectin-2 ligands, detected at the mRNA level, was confirmed at the protein level in primary NB samples.

These data has been published in the journal OncoImmunology [160].

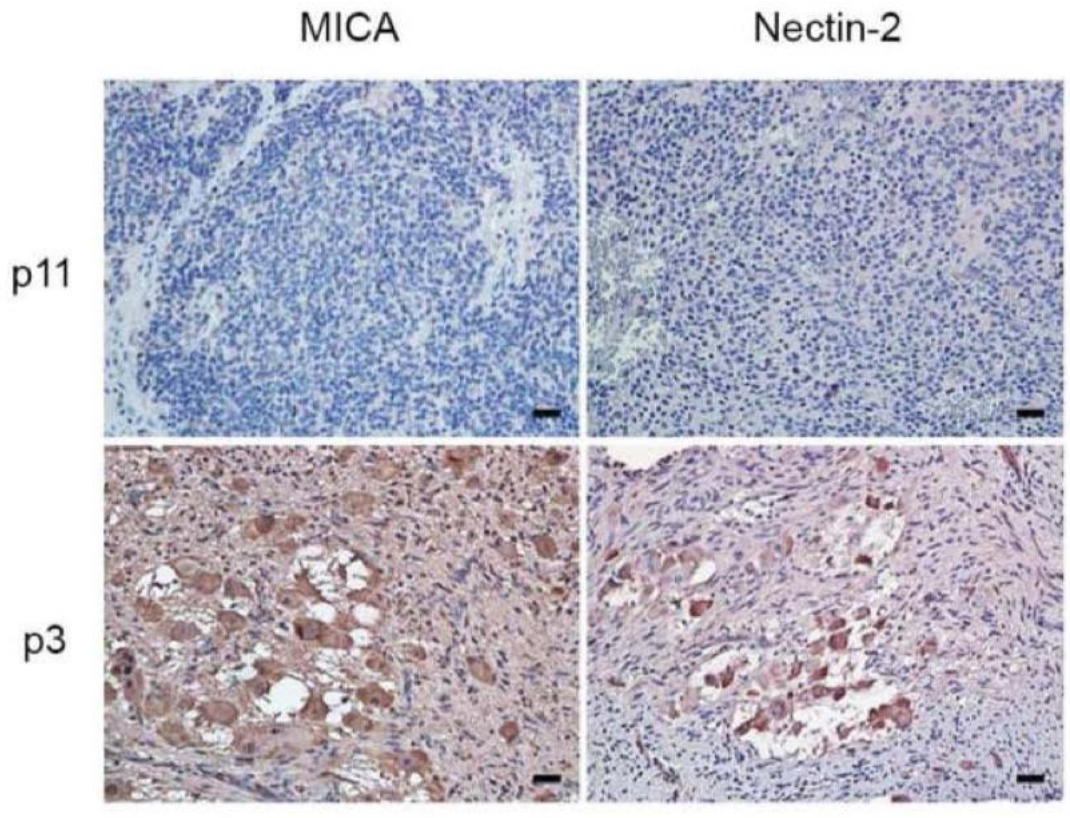

Fig.20. Expression of MICA and Nectin-2 in primary NB samples by immunohistochemistry assay. MICA and Nectin-2 expressing cells are shown in brown. Nuclei are counterstained with hematoxylin (blue). Original magnification, ×20. Scale bars 30 µm.

JQ1 negatively affects the activating ligands expression for NK cell-activating-receptors in NB

In view of these data, we explored molecular strategies aimed to inhibit MYCN functions. We used JQ1 (BET bromodomain inhibitors) to investigate its effect on the expression of activating ligands.

The *MYCN* non-amplified (non-MNA) SH-SY5Y and both *MYCN*-amplified (MNA) LA-N-5 and IMR-32 NB cell lines were cultured in the presence of JQ1 at different doses for 24, 48 and 72 hours in order to identify optimal conditions enabling cell viability and preventing apoptosis which could compromise the surface expression of ligands. Cell viability and apoptosis were assessed by cell count and AnnexinV/PI staining, respectively. The dose of 0,5 μM did not affect cell growth (Fig. 21A) or apoptotic state (Fig. 21B) compared with higher drug doses at all three time points tested. At 72 hours IMR-32 cell line was more sensible to JQ1 treatment compared with the others NB cell lines, as evaluated by important cell arrest and apoptosis induction even at low JQ1 doses.

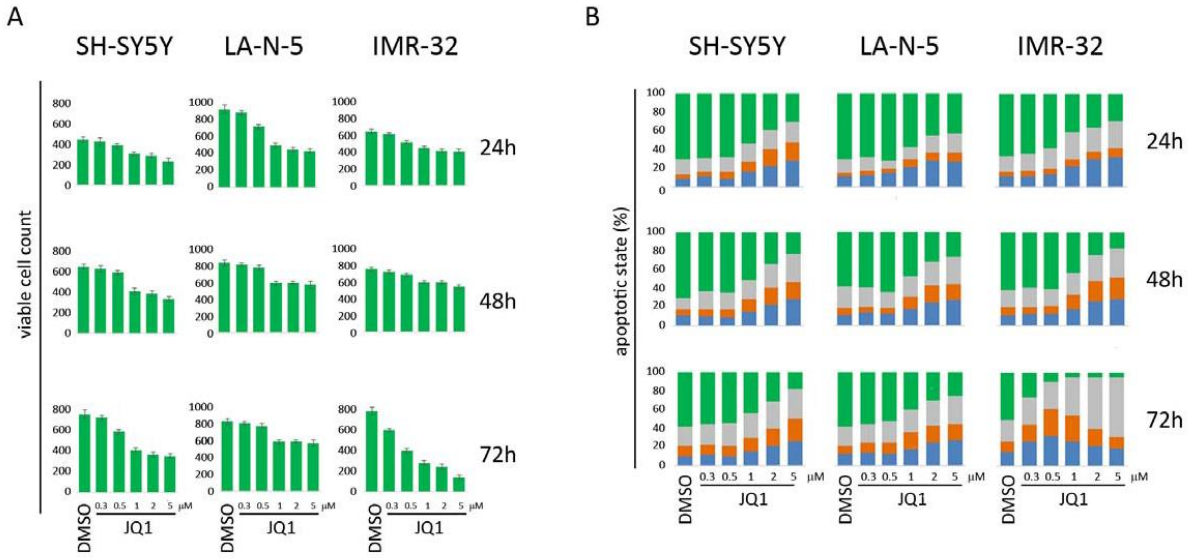

Fig.21 Cell count and apoptotic state of NB cell lines upon JQ1 treatment. NB cell lies SH-SY5Y, LAN-5 and IMR-32 were untreated (DMSO) or treated with JQ1 at the indicated concentrations for 24, 48 and 72h. (A) Cell count of NB cell lines at each condition was measured by trypan blue exlusion-based method. (B) Apoptosis state was evaluated by AnnexinV/PI staining and flow cytometry analysis. The percentage of cells in the early phase of Apoptosis (AnnexinV$^+$PI$^-$, blu bar), in the late phase of apoptosis (AnnexinV$^+$PI$^+$, orange bar) and dead cells (AnnexinV$^-$PI$^+$, gray bar) are indicated in stacked histogram. A representative experiment out of three performed is shown.

Then, we performed western blotting analysis to evaluate the effect of JQ1 on the expression level of MYCN, together with that of c-MYC, p53 and the two main p53 functional readouts, MDM2 and p21. As shown in Fig. 22A, JQ1 treatment (0,5 μM for 24h, 48 or 72 hours for both

SH- SY5Y and LA-N-5, and for 24 or 48 hours for IMR-32) efficiently downregulated MYCN in NB cell lines, with more evident effects on the MNA LA-N-5 cell line and on IMR-32 already at 24h. JQ1 downregulated also c-MYC as evident in SH-SY5Y, consistent with the fact that the promoter of *c-MYC* shares BET-bromodomains with that of *MYCN* oncogene [161]. These data indicate that in NB cell lines JQ1 impaired the expression of c-MYC, one of the two transcription factors known to regulate the expression of ligands for NK cell-activating receptors [162]. Moreover, p53 protein levels were very weakly increased upon JQ1 treatment with a significant decrease of MDM2 and no effect on p21 (Fig. 22A). We supposed that the weak increase of p53 levels results from the balance of a double mechanism i) the p53 molecular stabilization mediated by the reduced levels of MDM2 and ii) the p53 down-regulation mediated by the reduced levels of MYCN. Thus, consequently, the weak increase of p53 operated by pre-apoptotic dose of JQ1 is not functional enough as evaluated by the unmodified p21 level. These data suggest that the only down-regulation of MYCN mediated by JQ1 is not sufficient to restore p53 function.

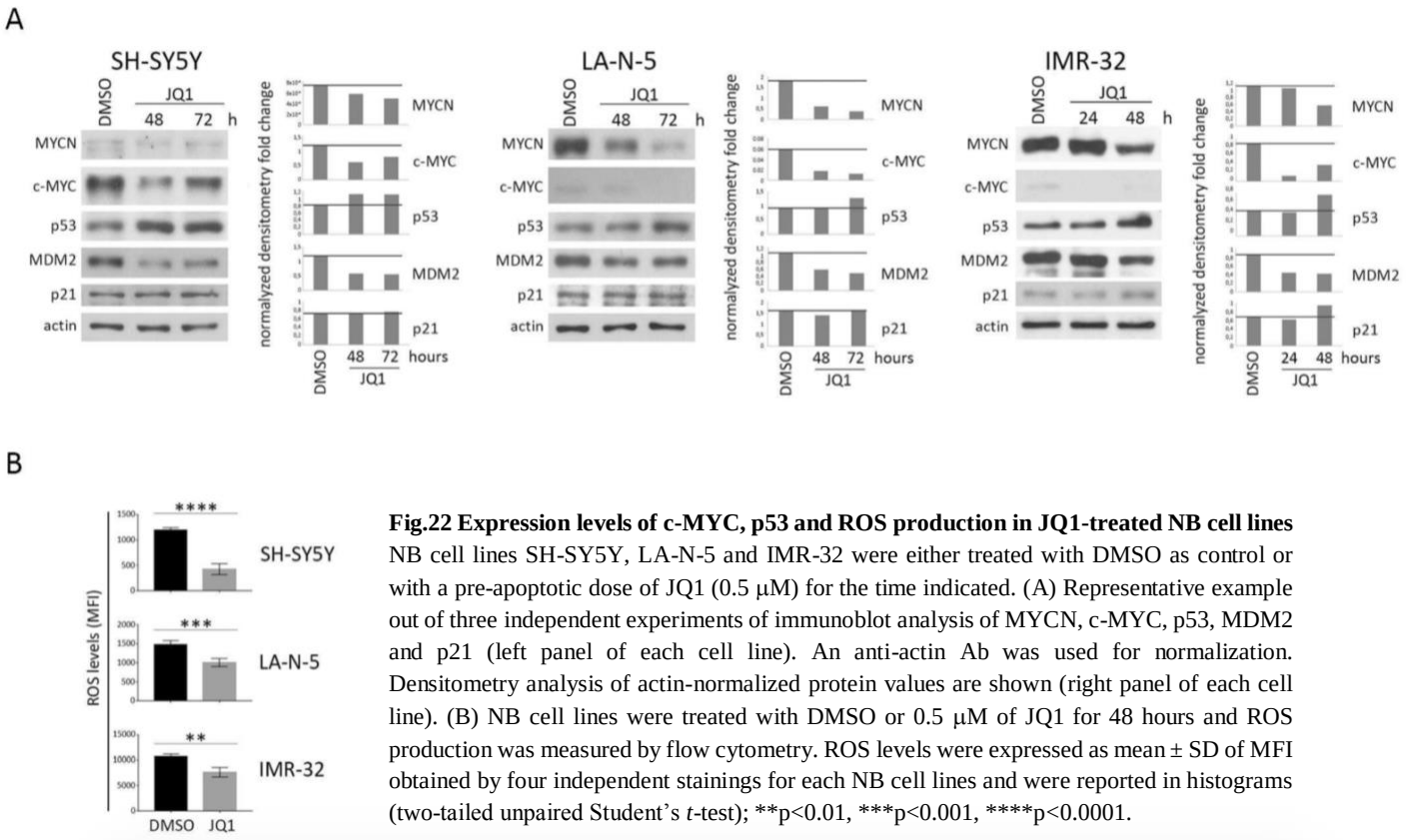

Fig.22 Expression levels of c-MYC, p53 and ROS production in JQ1-treated NB cell lines NB cell lines SH-SY5Y, LA-N-5 and IMR-32 were either treated with DMSO as control or with a pre-apoptotic dose of JQ1 (0.5 μM) for the time indicated. (A) Representative example out of three independent experiments of immunoblot analysis of MYCN, c-MYC, p53, MDM2 and p21 (left panel of each cell line). An anti-actin Ab was used for normalization. Densitometry analysis of actin-normalized protein values are shown (right panel of each cell line). (B) NB cell lines were treated with DMSO or 0.5 μM of JQ1 for 48 hours and ROS production was measured by flow cytometry. ROS levels were expressed as mean ± SD of MFI obtained by four independent stainings for each NB cell lines and were reported in histograms (two-tailed unpaired Student's *t*-test); **p<0.01, ***p<0.001, ****p<0.0001.

Moreover, we evaluated the levels of ROS, extensively reported to be correlated with the expression of ligands for NK cell-receptors [164, 178, 179], in JQ1-treated NB cell lines. As shown in Fig 2B, 0.5 μM of JQ1 at 48 hours strongly down-regulated the levels of ROS in all three NB cell lines (Fig.22B). These data were in line with other evidences in tumours showing the down-regulation of ROS levels operated by JQ1 treatment, by affecting several BRD4 genes involved in ROS production [180, 181]. Overall, these data indicate that pre-apoptotic dose of JQ1 in NB cell lines, not only impaired c-MYC expression and do not restore the impaired p53 function, but also affect a stress-induced event as ROS production, all these crucial factors involved in the induction of activating ligands. The effect of JQ1-mediated MYCN downregulation was evaluated on surface expression of activating ligands by flow cytometry analysis. The expression levels of ULBP1, ULBP3, PVR and Nectin-2 were significantly downregulated in both SH-SY5Y, LA-N-5 and IMR-32 cell lines upon JQ1 treatment (Fig. 23). These data indicate that in spite of an efficient MYCN downregulation mediated by JQ1, the impaired function of both c-MYC and p53 led to a compromised expression of their target genes for activating ligands.

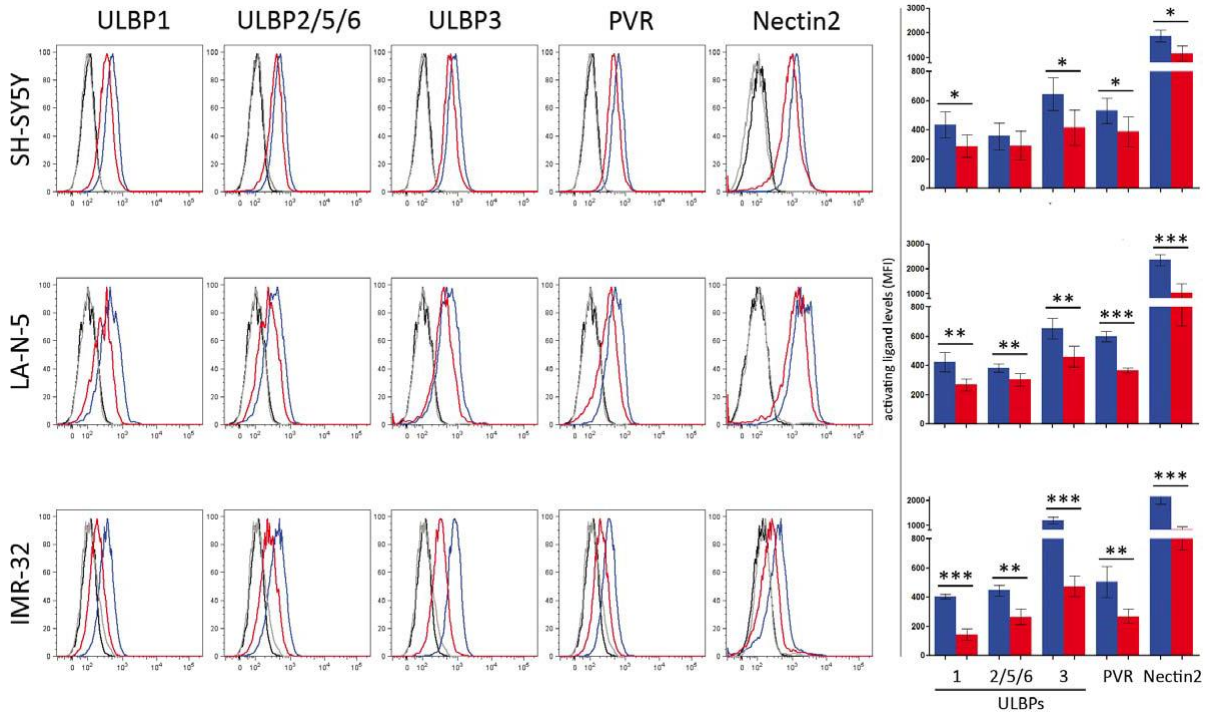

Fig.23 JQ1 downregulates the expression of ligands for NK cell-activating receptors

NB cell lines SH-SY5Y, LA-N-5 and IMR-32 were treated with DMSO (blue bar) or JQ1 (0.5 μM, red bar) for 48 hours and surface expression of ligands for NK cell-activating receptors was evaluated by flow cytometry analysis. Representative flow cytometry analysis of surface expression of each ligands for NK cell-activating receptors (right panel) and the summary of five independent experiments (left panel) were reported. *p* values, compared DMSO- and JQ1-treated NB cell lines (two-tailed unpared Student's *t*-test); *p<0.05, **p<0.01, ***p<0.001.

These results were consistent with our previous data regarding the modulation of MYCN through a Tet-off cellular model in which we appreciated the increase of activating ligands only in conditions of up-modulation of c-MYC and p53 [160]. Here, these evidences confirmed that the MYCN downregulation molecular strategies aimed to cure NB should be accomplished by a sustained activation of p53 and all the events, excluding those inducing side effects (such as c-MYC up-modulation), involved in the induction of ligands for NK cell-activating receptors.

JQ1-treated NB cell lines are less susceptible to NK cell-mediated recognition and killing

To evaluate if the decreased expression levels of activating ligands could affect NK cell-mediated recognition and killing of NB cells, we performed both NK cell degranulation and ^{51}chromium release assay using DMSO- and JQ1-treated NB cell lines as targets. Consistently with the decreased levels of activating ligands, JQ1-treated NB cell lines were significantly more resistant to NK cell-mediated degranulation and cytotoxicity (Fig. 24A-B).

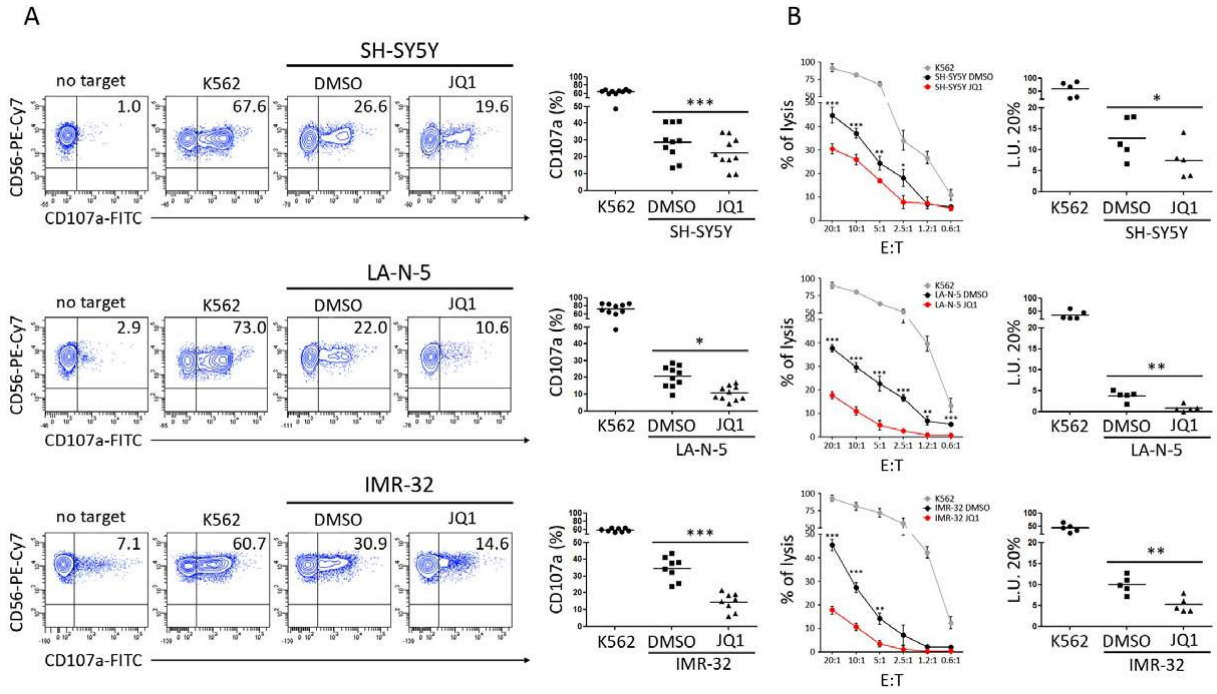

Fig. 24 JQ1 renders NB cell lines more resistant to NK cell-mediated recognition and lysis.
DMSO and JQ1-treated SH-SY5Y, LA-N-5 and IMR-32 cell lines were used as target cells in NK-cell functional assays. (A) Degranulation of human $CD3^{-}CD56^{+}CD45^{+}$ NK cells isolated from healthy donors, measured as cell surface expression of CD107a marker, following stimulation with SH-SY5Y, LA-N-5 and IMR-32, either treated with DMSO or JQ1 (0.5 □M) for 48 hours. K562 cell line was used as positive control. The percentage of $CD107a^{+}$ NK cells is indicated. A representative experiment out of ten performed is shown (left panel). Summary of NK cell-degranulation of cells isolated from ten healthy donors is shown (right panel). Dots, percentage of $CD107a^{+}$ NK cells; horizontal bars, average values. (B) DMSO and JQ1-treated SH-SY5Y, LA-N-5 and IMR-32 cell lines were used as targets cytotoxic assay of NK cells isolated from five healthy donors by standard ^{51}Cr-release assay at the indicated effector:target (E:T) ratios in a standard ^{51}Cr-release assay. One representative experiment out of the five performed is shown (right panel). Specific lysis was converted to L.U. 20%. Summary of cytotoxic assays of NK cells

isolated from five healthy donors are reported (left panel). Dots, L.U. 20% of the effector/target pairs tested; horizontal bars, average values. In A and B, *p* value, compared with DMSO- and JQ1-treated NB cell lines (two-tailed unpaired Student's *t*-test); *p<0.05, **p<0.01, p<0.001***.

These data suggested that JQ1, the most efficient drug inhibiting *MYCN* oncogene functions, does not show immunomodulatory capacity in terms of induction of ligands for NK cell-activating receptors and NK cell anti-tumour activity in NB cell lines.

All these data regarding JQ1 treatment of NB cells have been submitted to an indexed journal and presently are under review.

Drugs Used for NB Treatment Did Not Induce the Expression of Ligands for NKG2D- and DNAM1-Activating Receptors on NB Cell Lines

It was demonstrated that p53 is a transcriptional factor for ULBP1 and ULBP3 and since it is mutated only in 2% of NB cases [13], we investigated whether drugs commonly used in the treatment of NB could induce the activation of DDR pathway that, of note, is involved in p53 function. The genotoxic drugs cisplatin (DNA binder), etoposide (topoisomerase II inhibitor), irinotecan, and topotecan (topoisomerase I inhibitors) were used to treat *in vitro* the following NB cell lines: SK-N-AS, SH-SY5Y, SH-EP, SK-N-SH, SK-N-BE(2)c, LA-N-5, and IMR-32. These cell lines were cultured with cisplatin at 2 μM, etoposide at 0,1 μM, irinotecan at 1M and topotecan at 10 nM for 24h without inducing apoptosis. For this reason, 24h drug treatment was the best time point to evaluate the levels of activating ligands. Under these conditions, cisplatin, etoposide, irinotecan, and topotecan did not induce the expression of MHC class I molecule ligands for activating killer-immunoglobulin-like receptors (KIRs), MICA, MICB, ULBP1, ULBP2/5/6, ULBP3 ligands for NKG2D, PVR, and Nectin2 ligands for DNAM1 and TRAIL-R2 interacting with TRAIL. Some exceptions were found for the induction of ULBP2/5/6 on SK-N-AS and IMR-32 by etoposide and of Nectin2 on SK-N-AS and SH-EP by topotecan and irinotecan, respectively (Fig. 25).

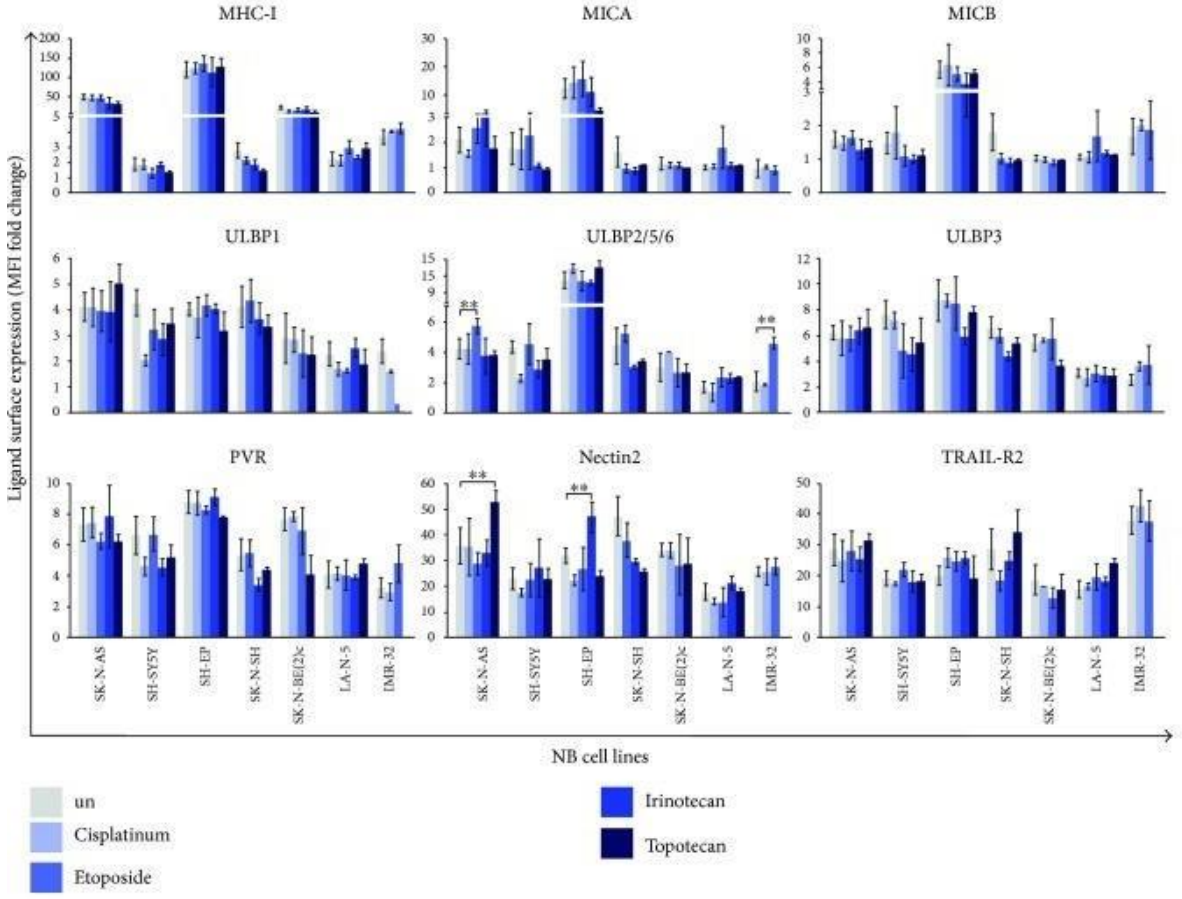

Fig.25. Anticancer drugs do not affect the expression of ligands for NK cell-activating receptors in NB cell lines. The NB cell lines SK-N-AS, SH-SY5Y, SH-EP, SK-N-SH, SK-N-BE(2)c, LA-N-5, and IMR-32 were untreated (un) or treated with cisplatin (at 2 μM), etoposide (at 0,1 μM), irinotecan (at 1nM), and topotecan (at 10nM) for 24 hours, and the surface expression of each indicated ligands for NK cell-activating receptors was analysed by flow cytometry. The surface expression of activating ligands was expressed as mean fluorescence intensity (MFI) that was normalized to MFI of IgG isotype Ab used as control (MFI fold change). The mean ± SD of MFI fold changes obtained by 5 independent stainings of each drug-treated NB cell line are reported in histograms (two-tailed paired Student's t-test) (** $p < 0.01$).

These data suggest that the drugs commonly used for chemotherapy of NB patients, induce apoptosis of p53 wild-type NB cell lines and, at pre-apoptotic concentrations, do not induce the expression of activating ligands. Thus, these drugs do not exert immune adjuvant effects on antitumor NK cell-mediated functions.

ATM, ATR, CHEK1, and CHEK2 Status on NB Cell Lines

To assess if the impaired modulation of activating ligands by the four drugs tested could be related to altered signalling upon drug-mediated genotoxic DNA damage, the status of genes encoding ATM (*ATM*), ATR (*ATR*), Chk1 (*CHEK1*), and Chk2 (*CHEK2*), the main molecules involved in DDR, was analysed in our panel of NB cell lines (Table 3). a-CGH revealed that the *ATM* gene was normal in SH-SY5Y, SK-N-SH, and LA-N-5 but was lost in SK-N-AS, SH- EP, and IMR-32. By contrast, the status of the *ATR* gene was normal in all NB cell lines with the exception of SK-N-AS and SH-EP in which *ATR* gain was detected. In addition, the status of genes for checkpoint kinases was shown to be partially altered: *CHEK1*was lost in both SK-N-AS and IMR-32, *CHEK2* was lost in both SK-N-AS and SK-N-BE(2)c, and both were normal in all other cell lines. Moreover, *ATM* and *CHEK1* were subjected to copy-neutral loss of heterozygosity (cnLOH) in SK-N-BE(2)c. cnLOH represents one example of genomic abnormality in which no change in chromosomal copy number occurs and is often associated with resistance to standard therapeutic modalities and poor survival in tumours [163]. Interestingly, SK-N-BE(2)c showed a large region of cnLOH (79.7 Mb) that included *ATM* and *CHEK1* genes. Thus, cnLOH can lead to an effective knockout of gene expression.

These data suggest that the impaired induction of the expression of activating ligands upon DDR was due at least in part to the loss of *ATM*, *CHEK1*, and *CHEK2* and the gain of *ATR* in NB cell lines tested. Additional molecules involved in the DDR pathway triggered by genotoxic drugs and leading to defective modulation of activating ligand expression in NB cells should be investigated.

NB cell lines	*ATM* gene	Chromosomal coordinates of *ATM* loss/cnLOH	*ATR* gene	Chromosomal coordinates of *ATR* gain	*CHEK1* gene	Chromosomal coordinates of *CHEK1* loss/cnLOH	*CHEK2* gene	Chromosomal coordinates of *CHEK2* loss
SK-N-AS	Loss	Chr11: 72159638–134720344 Cytoband: 11q13.4–q25 Size: 62.5 Mb	Gain	Chr3: 94300781–197840323 Cytoband: 3q11.2–q29 Size: 103.5 Mb	Loss	Chr11: 72159638–134720344 Cytoband: 11q13.4–q25 Size: 62.5 Mb	Loss	Chr22: 23283056–31436822 Cytoband: 22q11.22–q12.2 Size: 8.1 Mb
SH-SY5Y	Single copy	—	Single copy	—	Single copy	—	Single copy	—
SH-EP	Loss	Chr11: 93455106–119038765 Cytoband: 11q21–q23.3 Size: 25.6 Mb	Gain	Chr3: 133671502–197801441 Cytoband: 3q22.1–q29 Size: 64.1 Mb	Single copy	—	Single copy	—
SK-N-SH	Single copy	—	Single copy	—	Single copy	—	Single copy	—
SK-N-BE(2)c	Copy-neutral LOH	Chr11: 55196818–134924542 Cytoband: 11q13.2–q24.3 Size: 79.7 Mb	Single copy	—	Copy neutral LOH	Chr11: 55196818–134924542 Cytoband: 11q13.2–q24.3 Size: 79.7 Mb	Loss	Chr22: 16153099–51224252 Cytoband: 22q11.1–q13.33 Size: 35.0 Mb
LA-N-5	Single copy	—	Single copy	—	Single copy	—	Single copy	—
IMR-32	Loss	Chr11: 85989063–134446160 Cytoband: 11q14.2–q25 Size: 48.4 Mb	Single copy	—	Loss	Chr11: 85989063–134446160 Cytoband: 11q14.2–q25 Size: 48.4 Mb	Single copy	—

Tab.3 ATM, ATR, CHEK1, and CHEK2 Status on NB Cell Lines

ROS Production and p53 Stabilization in NB Cell Lines upon Cisplatin, Etoposide, Irinotecan, and Topotecan Treatment

The production of ROS in genotoxic drug-treated tumour cells has been associated with the induction of NKG2D and DNAM1 ligands [164]. Then, wt p53 NB cell lines SH-SY5Y, SH-EP, SK-N-SH, LA-N-5, and IMR-32 were treated for 24h with cisplatin, etoposide, irinotecan, and topotecan at the above-mentioned concentrations, and ROS levels were measured by flow cytometry. Both LA-N-5 and IMR-32 produced ROS upon incubation with the four drugs, with the exception of LA-N-5 after etoposide treatment. SK-N-SH produced ROS after cisplatin and etoposide but not irinotecan and topotecan treatment. SH-SY5Y produced ROS only after topotecan treatment, whilst SH-EP was completely refractory to generate ROS upon the treatment of all drugs used. Then, to assess if our genotoxic drugs at the doses reported above induced the stabilization of p53 in NB cell lines, the wt p53 ATM single copy LA-N-5 NB cell line was treated with cisplatin, etoposide, irinotecan, or topotecan for 2, 5, and 8h, and the level of p53 was evaluated by Western blotting. LA-N-5 cells showed higher levels of p53 2 hours after drug treatment, particularly evident after cisplatin and etoposide treatment that returned to normal levels at latter time points.

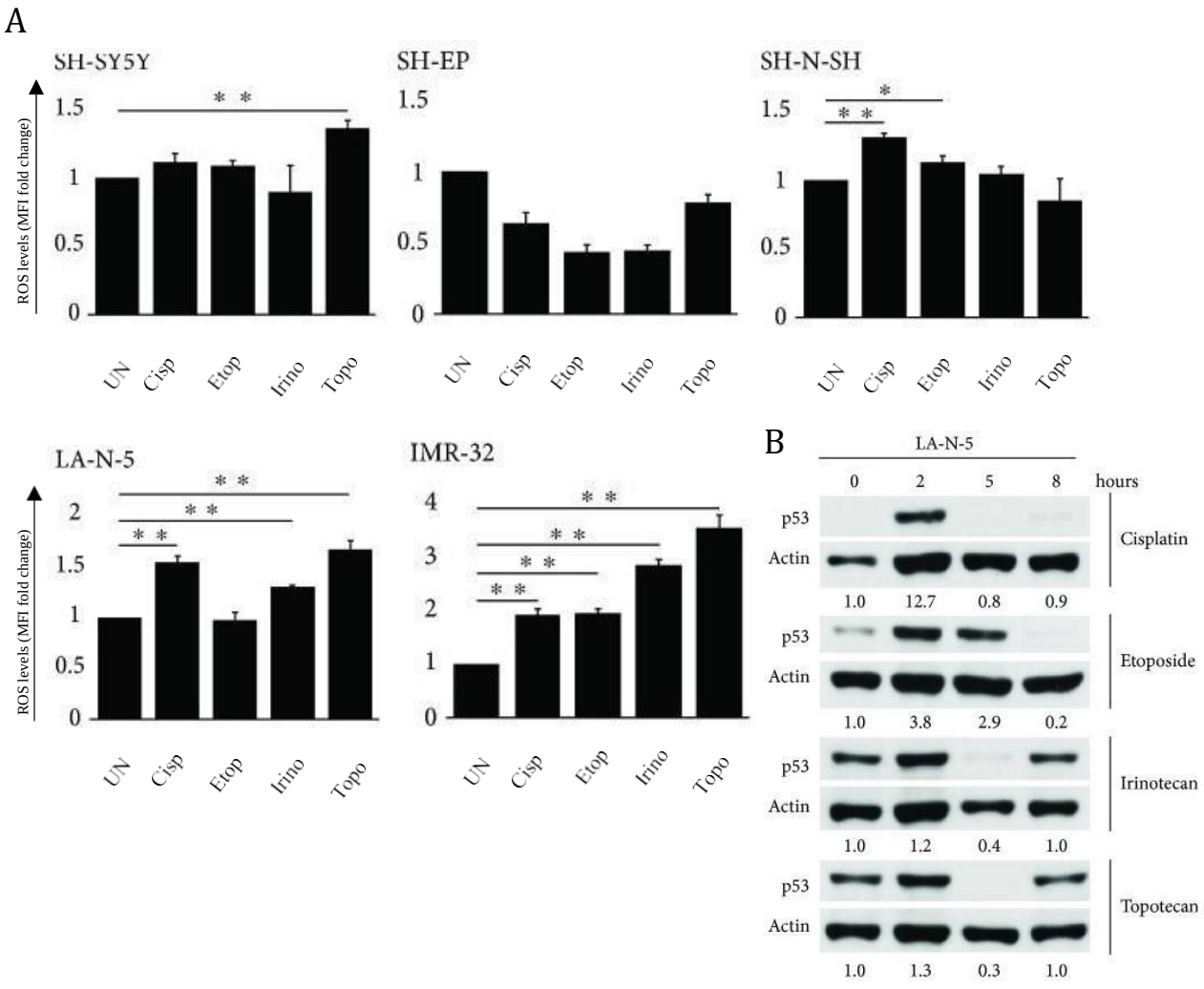

Fig.26 ROS production and p53 stabilization in drug-treated NB cell lines. (A) NB cell lines were untreated (UN) or treated for 24 hours with cisplatin, etoposide, irinotecan, and topotecan, at the same concentrations described in Fig.14, and ROS production was measured by flow cytometry. Drug-induced ROS levels were expressed as MFI normalized to MFI of untreated cells used as control (MFI fold change). The mean ± SD of MFI fold changes obtained by 4 independent stainings of each drug-treated NB cell line are reported in histograms (two-tailed paired Student's t-test) (∗p < 0 05, ∗∗p < 0 01). (B) LA-N-5 was untreated (0) or treated with the same drugs at the same doses described above for 2, 5, and 8 hours, and levels of p53 were measured by immunoblot analysis. An anti-actin Ab was used for normalization. Densitometric analysis of p53 normalized to actin and relative to untreated cells (point 0) are reported below each immunoblot. A representative out of two independent experiments performed is shown.

These data indicate that the impaired induction of ligands for NK cell-activating receptors in NB cell lines upon pre-apoptotic doses of genotoxic drugs could depend also on the abnormal production of ROS and on the transient rather than persistent p53 stabilization.

Cisplatin Does Not Render NB Cell Lines More Susceptible to NK Cell-Mediated Recognition

To test whether the impaired, genotoxic drug-dependent modulation of ligands for NK cell-activating receptors in NB cell lines affected NK-cell-mediated recognition, we performed an NK cell degranulation assay by using drug-treated NB cell lines as target cells. Cisplatin was previously reported to induce the MICA and MICB expression in non-small-cell lung cancer A549 cell line, through DNA stress-induced ATM-ATR signalling, resulting in enhanced sensitivity to NK cell-mediated cytotoxicity [165]. Therefore, we tested the A549 cell line as a positive control to compare the susceptibility of cisplatin-treated SH-SY5Y and LA-N-5 NB cell lines to NK cell-mediated recognition. We performed an NK cell degranulation assay by using as target the A549 cell line treated with 10 μM of cisplatin for 48 hours and both SH-SY5Y and LA-N-5 cell lines treated with 2 μM of cisplatin for 24 hours. The cisplatin treated A549 cell line was significantly more susceptible to NK cell recognition than untreated cells, as previously reported. In contrast, cisplatin-treated SH-SY5Y and LA-N-5 NB cell lines induced NK cell degranulation comparably to untreated cells (Fig. 27). These data confirm that genotoxic drugs such as cisplatin do not render NB cell lines more susceptible to NK cell recognition.

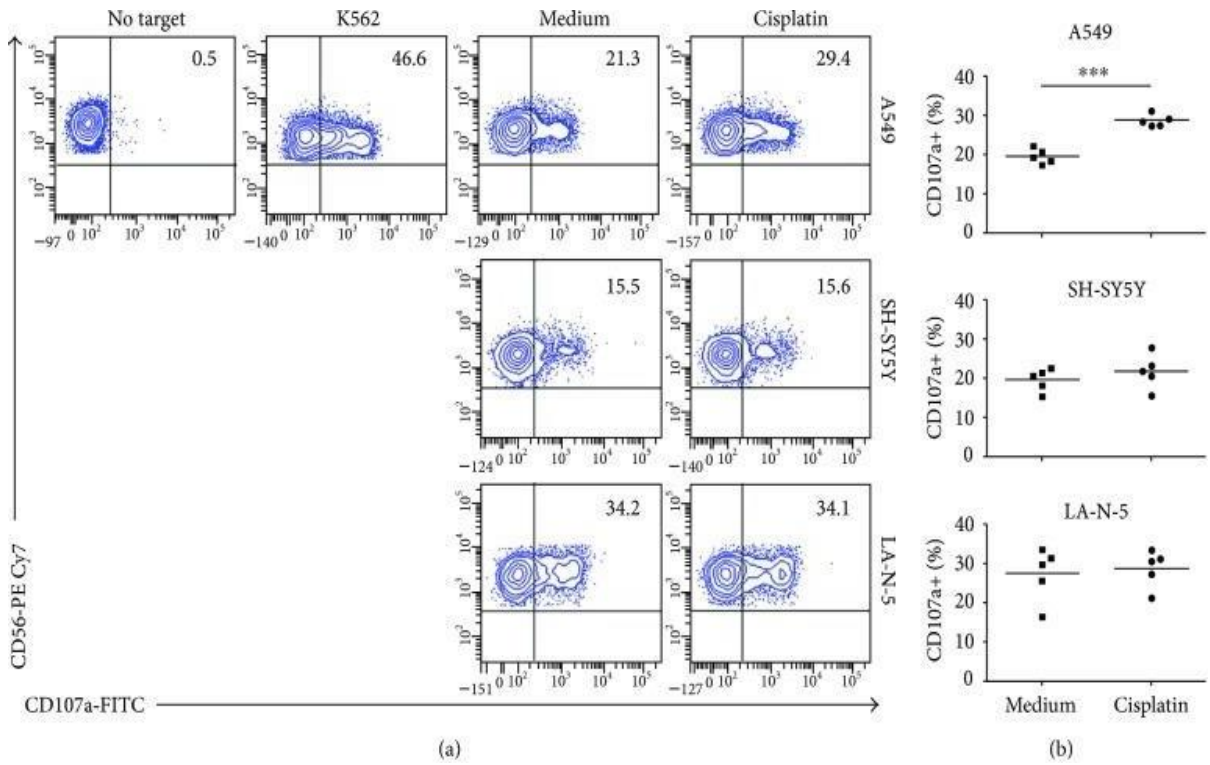

Fig.27 Cisplatin does not sensitize NB cell lines to NK cell recognition. The NB cell lines SH-SY5Y and LA-N-5 were untreated (medium) or treated with 2 μM of cisplatin for 24 hours and used as targets in an NK cell degranulation assay, measured as the CD107a cell-surface expression on human CD3−CD56+CD45+ NK cells from healthy donors. The K562 cell line was used as positive control for NK cell activation. The A549 cell line was untreated or treated with 10 μM of cisplatin for 48 hours and used as positive control for drug effect. (a) A representative experiment out of five performed is shown. The percentage of CD107a+ NK cells is indicated. (b) Summaries of NK cell degranulation of NK cells, isolated by five different healthy donors, following stimulation with untreated and cisplatin-treated cell lines are shown. Dots, percentage of CD107a+ NK cells; horizontal bars, average values. p value compared untreated with cisplatin-treated cell lines (two-tailed paired Student's t-test) (*** $p < 0.001$).

In conclusion, based on these data and concepts, in order to boost the NK cell-mediated immunotherapy of NB, the combined use of immune adjuvant agents could represent a winning approach. Thus, the effect of molecules efficiently inducing the expression of ligands for NK cell-activating receptors on NB cells remains to be evaluated.

All these data regarding the drug effects on NB cells have been published in the Journal of Immunology Research [166].

Nutlin-3a enhances the expression of ligands for NK cell-activating receptors and renders NB cell lines more susceptible to NK cell-mediated recognition and lysis.

Since we evaluated both NB refractoriness to genotoxic drugs and the inefficient JQ1-mediated targeting of MYCN in inducing the expression of ligands for NK cell-activating receptors in NB cells, we needed to explore other molecular strategies. Considering that the expression of such ligands depends on the function of the crucial transcription factor p53 [120], we evaluated the effect of the small molecule Nutlin-3a on NB cells that is known to be able to promote p53 function despite the upstream signals that regulate p53 are altered.

For this reasons, both non-MNA (SH-SY5Y p53 wt and SK-N-AS p53 mut), and MNA (SMS-KCNR, LA-N-5, both p53 wt and SK-N-BE2C p53 mut) cell lines undergone to Nutlin-3a treatment. A dose-response curve, at 24h and 48h, was performed to set the right dose to not induce an apoptotic effect. Analysis of normalized densitometric values of western blotting, analysed at 48h with progressive increase in the Nutlin-3a concentration (0,5-1-2-3-10 μM), show a significant increase in MDM2, p53 and p21 levels in SH-SY-5Y, SMS-KCNR and LA-N-5 but not in SK-N-AS and SK-N-BE2C cell lines. MYCN shows no expression changes in all cell lines (Fig.28). Mean fluorescence intensity values of flow cytometry analysis performed at 48h with a drug concentration of 2μM, revealed a strong and significant up-modulation of ULBP1, ULBP2/5/6, ULBP3 and PVR activating ligands in SMS-KCNR and LA-N-5 cell lines, both MNA and p53 wt. A less but still significant increase in ULBP1 and ULBP3 activating ligands is evident in non-MNA p53 wt SH-SY-5Y cell line. No result in terms of activating ligands up-regulation can be detected in SK-N-AS (non-MNA) and SKNBE2C (MNA) cell lines, both p53 mut (Fig.28).

Therefore, we can say Nutlin-3a is a drug able to induce the activating ligand expression on MYCN amplified NB cell lines.

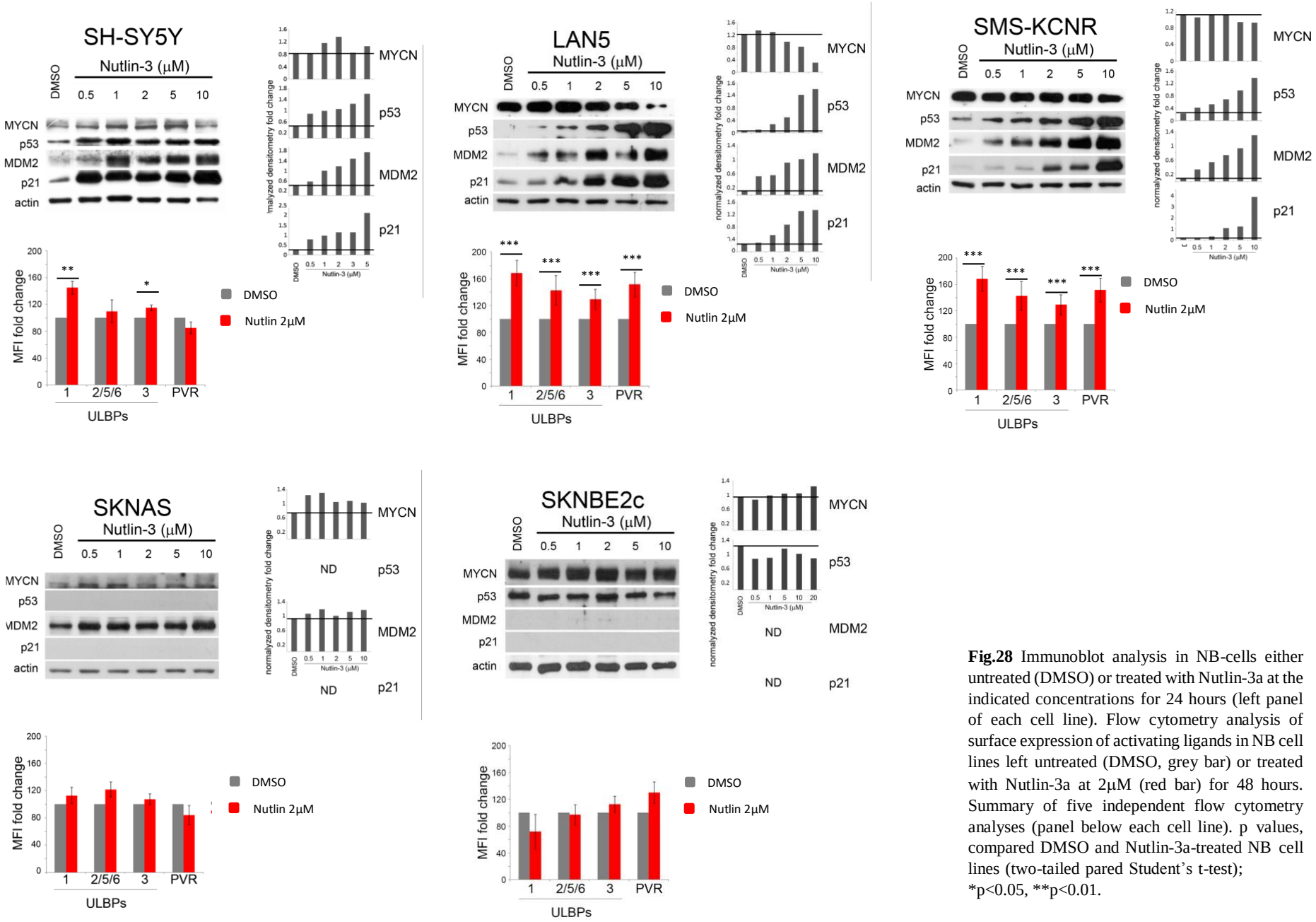

Fig.28 Immunoblot analysis in NB-cells either untreated (DMSO) or treated with Nutlin-3a at the indicated concentrations for 24 hours (left panel of each cell line). Flow cytometry analysis of surface expression of activating ligands in NB cell lines left untreated (DMSO, grey bar) or treated with Nutlin-3a at 2μM (red bar) for 48 hours. Summary of five independent flow cytometry analyses (panel below each cell line). p values, compared DMSO and Nutlin-3a-treated NB cell lines (two-tailed pared Student's t-test);
*p<0.05, **p<0.01.

To test if the induced expression of ligands for NK cell-activating receptor mediated by Nutlin-3a may affect NK cell-mediated recognition and lysis, we performed degranulation and cytotoxic assays after 48h in LA-N-5 and SMS-KCNR cell lines. We observed that cells treated with Nutlin-3a were significantly more susceptible to NK cell-mediated lysis than those with DMSO as control.

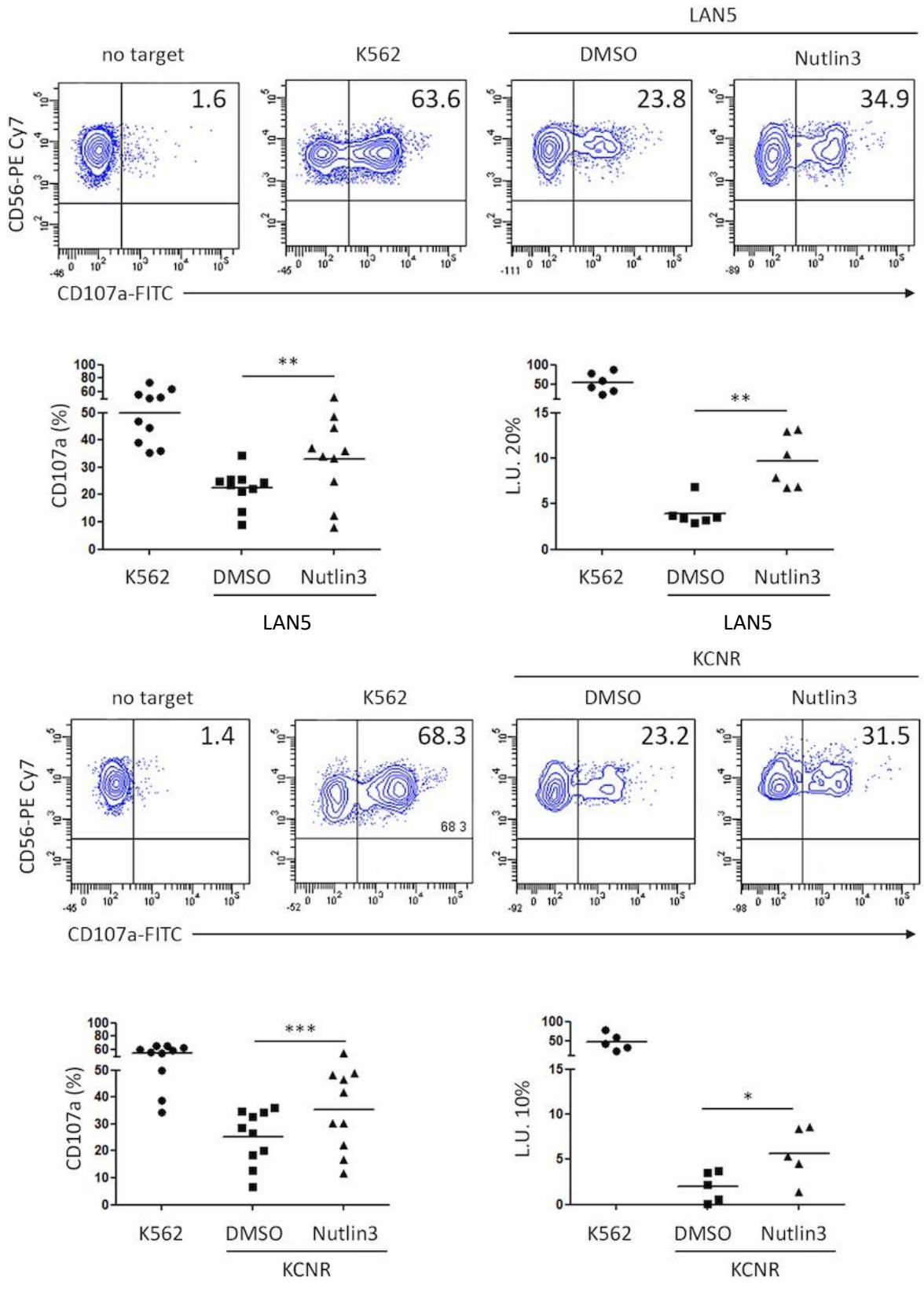

Fig. 29 Degranulation of human CD3-CD56+CD45+ NK cells from healthy donors, measured as CD107a cell-surface expression following stimulation with LA-N-5 and SMS-KCNR, either left untreated (DMSO) or treated with Nutlin-3a at 2μM for 48 hours. K562 cells were used as positive control. The percentage of CD107a+ NK cells is indicated. A representative experiment out of the ten performed is shown. Summary of NK cell-degranulation of cells isolated from ten healthy donors is shown. Dots, percentage of CD107a+ NK cells; horizontal bars, average values. DMSO or Nutlin-3a-treated NB cell lines as in A were used as targets for NK cells in a standard ^{51}Cr-release assay. Summary of cytotoxic assay of NK cells isolated from healthy donors, six for LA-N-5 and five for SMS-KCNR. Specific lysis was converted to L.U. 20%. Dots, L.U. 20% of the effector/target pairs tested; horizontal bars, average values. p value, compared with DMSO and Nutlin-3a-treated NB cell lines (two-tailed paired Student's t-test). *p<0.05, **p<0.01, ***p<0.001.

PVR is upregulated at the transcriptional level and it is direct p53 target gene.

Next, we analysed whether ligands for NK cell-activating receptors were upregulated at the transcriptional level by quantitative real-time PCR. Surprisingly, PVR mRNA (relative by 3-fold) was strongly upregulated upon induction of p53 after 16 h Nutlin-3a treatment in LA-N-5 p53 wt cell line (Fig. 30A).

In a next step, we investigated whether the transcriptional upregulation of PVR involved a direct p53 binding to PVR promoter. Bionformatic analisys by Trap and Genomatix Mathinspector software revealed three putative p53-binding sites on *PVR* promoter region. In addition, we performed the chromatin immunoprecipitation (ChIP) with a p53-specific mAb in LA-N-5 cells untreated or treated with Nutlin-3a for 16 hours that revealed a binding of p53 wt to a specific region on *PVR* promoter in LA-N-5 cells upon Nutlin-3a treatment (Fig. 30B). The binding of p53 to *PVR* promoter was quantified by qPCR indicating an increase of 2,7 fold compared with untreated cells. Of note, in the same experimental conditions, no binding using the p53-specific mAb on a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence was detected by ChIP ruling out nonspecific precipitation. Altogether, these data show that PVR mRNAs is upregulated by a direct binding of p53 to *PVR* promoter in LA-N-5 cell line after Nutlin-3a treatment.

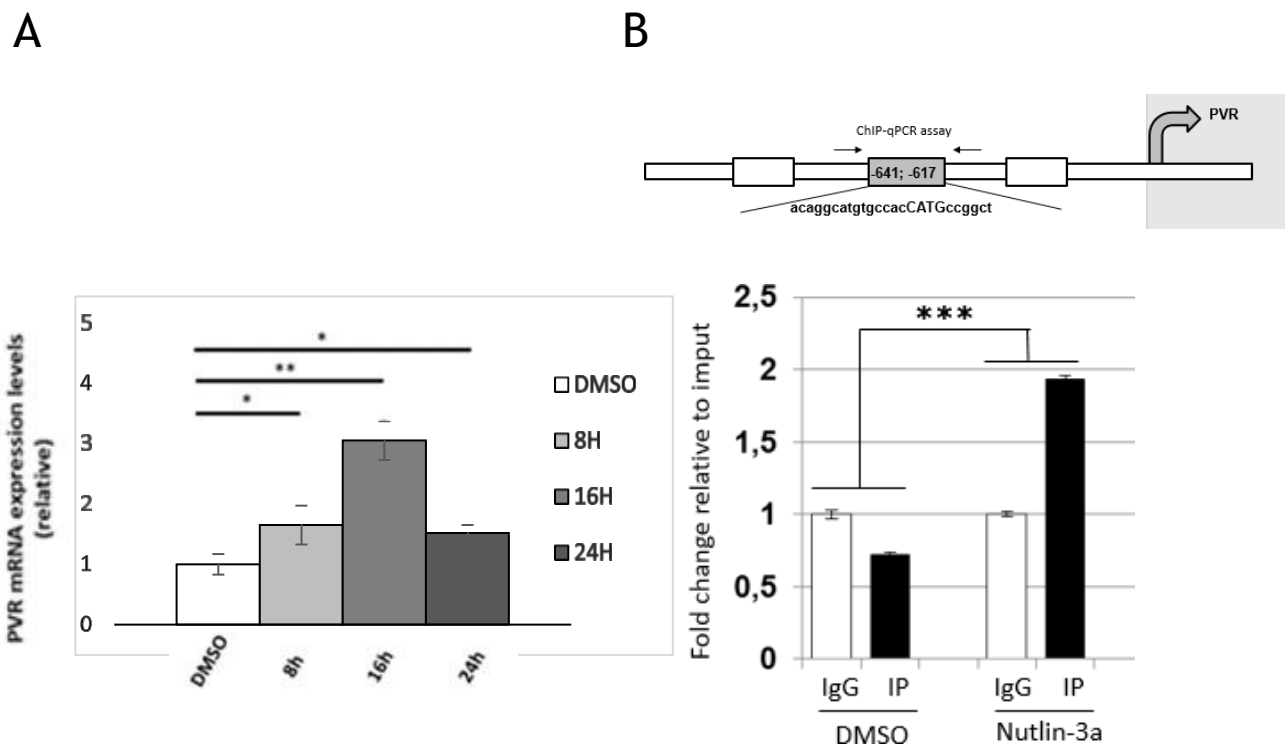

Fig. 30 p53-binding site in PVR gene. A qPCR analysis of PVR in LA-N-5 NB cell line. One representative experiment of 5 indipendent qPCR is shown. GAPDH was used for normalization **B** Binding of p53 to p53-binding site in the PVR gene promoter was analysed in LA-N-5, either untreated (DMSO) or treated with Nutlin-3a (2μM) for 16 hours, by ChIP assay. The amount of immunoprecipitated chromatin bound by either isotype control (IgG) or p53 (IP) was quantified by real-time PCR with specific primers. Specific signals were set relative to signals obtained for the imput chromatin. One representative experiment of 3 indipendent ChIP is shown. p value, compared with normalized (IP/IgG) DMSO and Nutlin-3a-treated LA-N-5 (two-tailed paired Student's t-test). *p<0.05.

All these data suggest that a Nutlin-3a pharmacological treatment aimed to improve the expression of ligands for NK cell-activating receptor, could represent a novel promising therapeutic strategy to restore the NK cell-mediated immune response against high-risk NB cells.

Nutlin-3a upregulates *in vivo* the expression of PVR and Nectin-2 in NB cell line grown in xenograft murine model.

Based on the *in vitro* studies, in order to evaluate the efficacy of Nutlin-3a also *in vivo*, we performed *in vivo* experiments in xenograft murine model. MYCN-amplified LA-N-5 cells were injected subcutaneously into NSG mice, and tumours thereby generated were treated intraperitonealy (i.p) with Nutlin-3 (or vehicle as control) at the doses of 20 mg/kg and 40mg/kg for two weeks. Mice were sacrificed at the end of treatment and tumour masses were harvested. Tissue sections of LA-N-5 xenografts from NSG mice treated with control DMSO or Nutlin-3a

were stained with PVR and Nectin-2 antibodies. Immunohistochemistry assay showed a significant increased expression of both PVR and Nectin2 ligands, in slides from treated compared with untreated mice (Fig. 31).

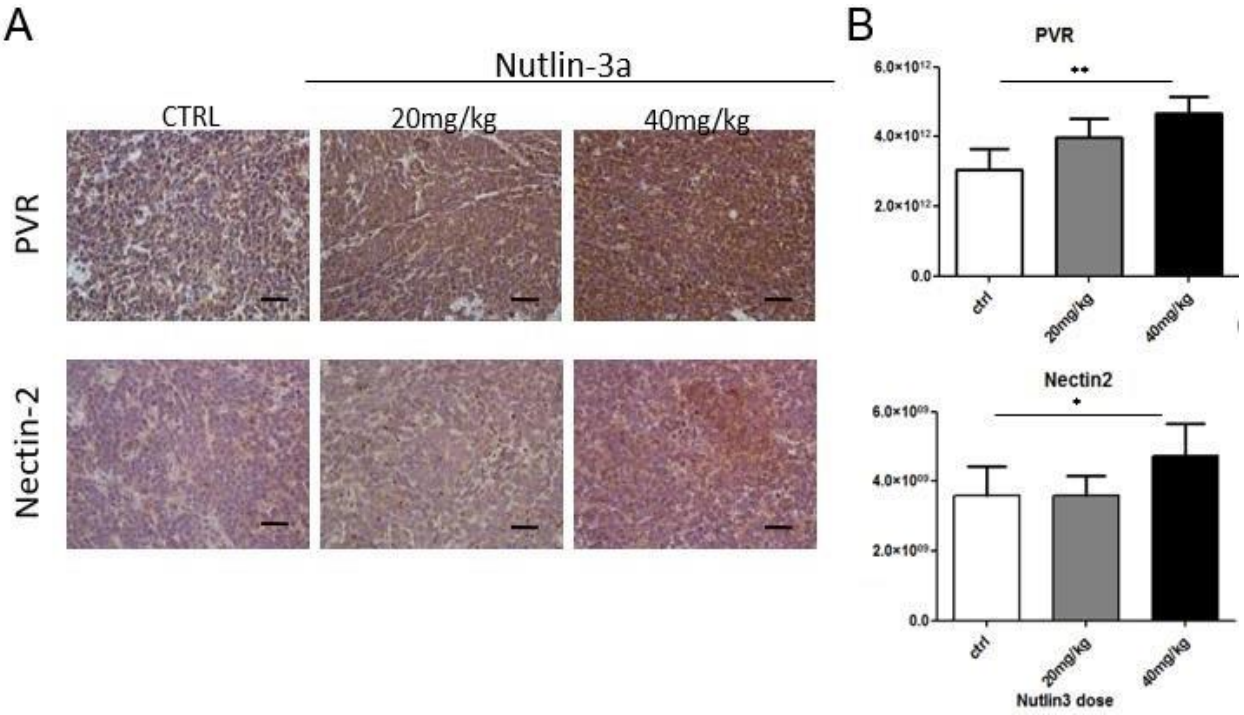

Fig.31 (A) Immunohistochemical staining of LA-N-5 xenograft using PVR and Nectin-2 antibodies. LA-N-5 xenografts were treated intraperitoneally with DMSO or Nutlin-3a at 20mg/kg (drug dose/mouse body weight) and at 40mg/kg indicated doses every two days for two weeks. Groups of five NSG mice for each condition were assessed. Mice were sacrificed at the end of Nutlin-3a treatment and tumour masses were harvested, formaldehyde-fixed, thus paraffin-embedded blocks were cut into 3-mm sections. Tissues slides from DMSO, Nutlin-3a at 20mg/kg and at 40mg/kg masses were mounted on the same slide and parallel stained for the same antibody. A representative out of six independent slides for each condition is shown. (B) Immunohistochemical staining was measured by ImageJ software and the mean+SD of integrated density of ten independent areas on four independent slides for each condition are reported in histogram. p value, compared with DMSO and Nutlin-3a-treated mice (two-tailed paired Student's t-test). *$p<0.05$, **$p<0.01$.

NK cells combined with Nutlin-3a treatment improve the overall survival of MYCN-amplified NB cell-bearing mice (ongoing assay).

Next, we asked if the increased expression of ligands for NK cell-activating receptors mediated by Nutlin-3a treatment, may improve NK cell activity in impairing tumour growth. NSG mice injected subcutaneously into the right flank with LA-N-5 NB cell line were divided into 4 groups: 1) injected with vehicles, 2) i.p treated with Nutlin-3a (40mg/kg), 3) intravenously (i.v.) treated with NK cells and 4) i.p. treated with Nutlin-3a plus i.v. treated with NK cells. We have preliminary results showing that the combined treatment of Nutlin-3a with NK cells could significantly improve mice overall survival (data not shown). Altogether, these results

demonstrate that *in vivo* treatment of MYCN-amplified NB with Nutlin- 3a and NK cells, affects tumour growth.

Nutlin-3a render NB primary cells more susceptible to NK cell-mediated recognition and lysis by improving the expression of some ligands for NK cell-activating receptors.

Since we demonstrated a significant up-modulation of ULBP1, ULBP2/5/6, ULBP3 and PVR ligands in NB cell line after Nutlin-3a treatment, we explored Nutlin-3a effect also on primary NB samples isolated by NB patients characterized by different stage of the disease. Therefore, NB primary cells treated with 2μM of Nutlin-3a for 48h showed an up-regulation of some ligands for NK cell-activating receptors with different results among NB primary cells (data not shown). Primary cells obtained by one patient with high-risk NB, characterized by MYCN-amplification, spontaneously developed in culture spheroids. To examine the NK cell ability to attack these spheroids, we performed a co-cultured of NK cells and Nutlin-3a pre-treated NB spheroids at the ratio E:T of 1:1. Then, we acquired images by optical microscopy and measured by Image J the major diameter of spheroids at 0 and 4h after NK cell adding. The diameter of spheroids increased under NK cell attack as the result of NK cell-mediated disaggregation. As shown in Fig. 32, Nutlin-3a was able to render significantly more susceptible spheroids to NK cells attack. These data were confirmed also by apoptotic analysis of spheroids upon NK cell exposition.

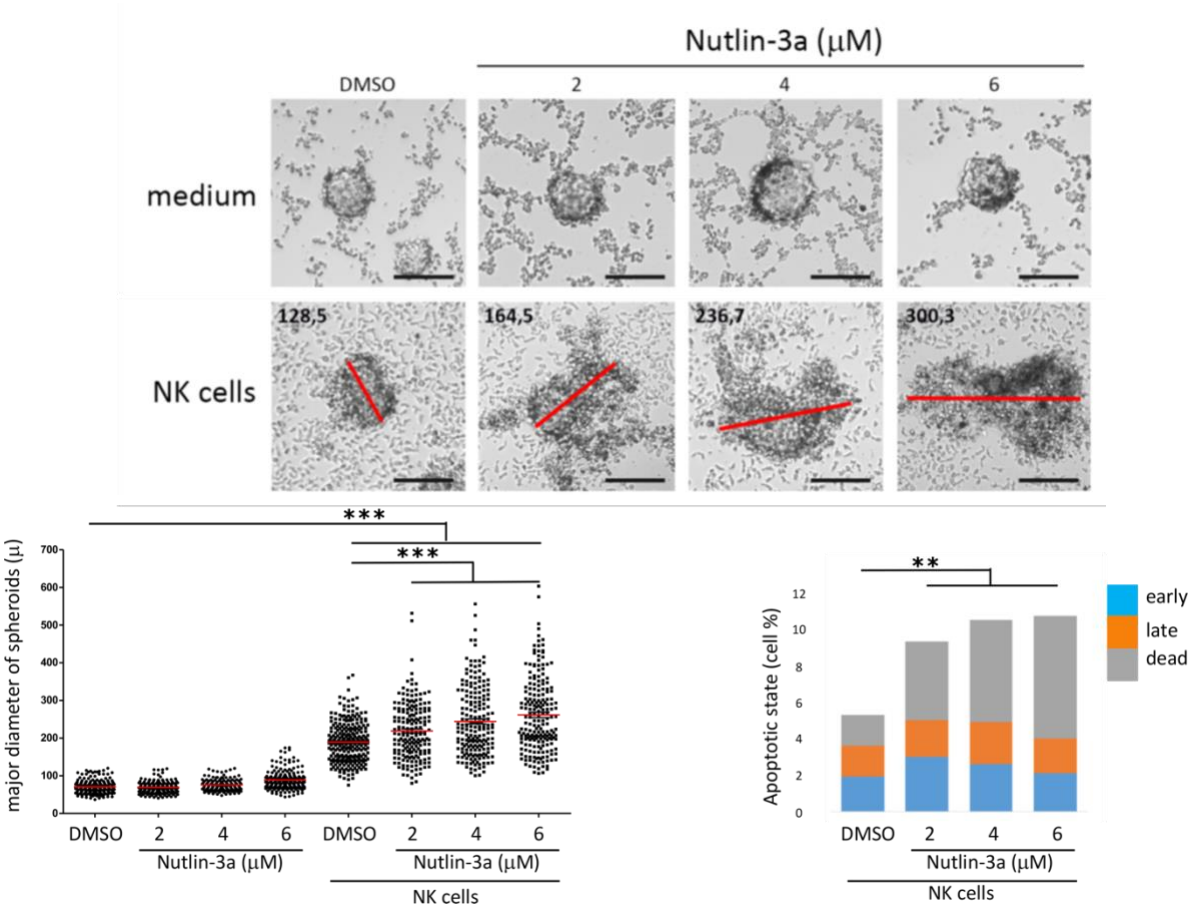

Fig. 32 Primary NB spheroids are recognized and destroyed by NK cells. Optical Microscopy acquisition and ImageJ analysis of the major diameter of spheroids of primary NB samples spontaneously developed *in vitro* isolated by a patient with high-risk NB upon *in vitro* NK cell attack. Apoptotic state was measured by Annexin V/PI staining.

Discussion

The antitumor activity of NK cells is strictly dependent on the expression of ligands for NK cell-activating receptors on tumour cell surfaces [156, 167]. The down-modulation of such ligands represent one of the mechanisms of immune-evasion for many tumours, including NB [168]. The up-regulation of activating ligands represent efficient strategies aimed to boost the NK cell-based anti-tumour immunotherapy.

MYCN oncogene represents the well-established predictor of poor prognosis in high-risk NB and it is known to be an important transcriptional factor able to confer metastatic and immune-evasion capabilities and to regulate several cellular processes such as survival, differentiation and apoptosis [8, 20]. MYCN expression in NB cells is positively correlated with that of p53 and inversely with that of c-MYC [27], in agreement with the finding that both transcription factors are involved in the induction of ULBPs ligands [120]. p53 is mutated in 2% of the cases of NB, especially in relapse [13]. The function of p53 protein is inhibited by the MDM2, which is overexpressed in many human cancers, including NB [157]. Of note, p53 and MDM2 genes are both direct transcriptional targets of MYCN in NB. Consequently, there are many experimental evidences suggesting a direct or indirect involvement of MYCN in the modulation of these surface activating ligands.

Based on these observations, we hypothesize that the inverse correlation between the expression of MYCN and that of c-MYC or the p53 functional status could explain differences in expression of ULBPs ligands in NB cell lines. Our data demonstrate that MYCN has an immunosuppressive role dampening the expression of ligands for NK cell-activating receptors and its overexpression contributes to protect NB cells from NK cell-mediated recognition and killing, thus delineating a novel mechanism of tumour escape from the control of the innate immune system. Our study shows that the expression of MYCN is inversely correlated with that of the activating ligands for NKG2D and DNAM-1 NK-cell receptors in NB cells. Moreover, since higher levels of soluble MICA have been reported to be found in sera of NB patients compared with healthy donors [168], the putative role of MYCN in regulating cell surface expression of activating ligands by non-transcriptional mechanisms cannot be excluded and needs further investigation. As a consequence, MYCN expression could represent a biomarker allowing to predict the susceptibility of NB cells to NK cell-mediated immunotherapy.

MYCN targeting could be a therapeutic strategy to induce the expression of activating ligands and enhance NB cell susceptibility to NK cell-mediated immunotherapy. Among several mechanisms leading, the BET bromodomain inhibitor JQ1 is a good candidate to down-regulate

MYCN expression/function. The use of JQ1 is very promising in several c-MYC- amplified tumours in which the downregulation of c-MYC leads to cell cycle arrest and apoptosis [161, 169]. Moreover, JQ1 showed anti-angiogenesis activity contributing to arrest of the growth of paediatric sarcoma [170]. JQ1 also dampens the expression of PD-L1, a major player involved in immune checkpoint mechanisms in NB and in other cancers [171, 172]. These data indicate that JQ1 treatment could represent a good strategy enhancing a better lymphocyte-mediated recognition of NB cells by reducing the PD1/PD-L1 immune checkpoint pathway established between lymphocytes and NB cells. Moreover, JQ1 increased the expression of MICA in multiple myeloma, since the downregulation of c-MYC leads to up-modulation of miR-125-5p and the consequent downregulation of its target gene IRF4, known as transcription repressor of MICA [173]. Differently to haematological malignancies, solid tumours, including NB, express lower levels of MICA and MICB and higher levels of ULBPs and ligands for DNAM-1 [125]. Moreover, the shedding of MICA has been associated with an immune escape strategy of NB [168].

Our data showed that JQ1, although efficiently down-modulates the expression of MYCN and strongly reduced the level of reactive oxygen species (ROS), rendered NB cell lines more resistant to NK cells and probably also to T cells (not explored here), due to the shared expression of NKG2D on these lymphocytes. In spite of the cytotoxic activity of JQ1 against NB cells, the inability of the drug to up-regulate the expression of activating ligands, with consequent resistance of NB cells to NK cell-mediated attack, should discourage the use of JQ1 in combination with the perspective NK cell-based immunotherapy of NB.

For this reason, we focused our attention on the use of drugs able to stimulate p53 that, together with c-MYC, is known to regulate NK cell-activating receptor ligands such as ULBP1, ULBP2. Several are the drug-mediated molecular mechanisms underlying the up-regulation of activating ligands, representing efficient strategies aimed to boost the NK cell-based anti-tumour immunotherapy [174]. Stress induced by drugs up-regulate the expression of ligands for NKG2D and DNAM-1 activating receptors, by activating ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) protein kinases [49]. The ATM/ATR pathways are under control of two main checkpoint kinases, that are Chk1 and Chk2 and, through the activation of the master transcription factor p53, lead to cell senescence and induce the expression of ligands for NK cell-activating receptors [34]. In order to evaluate whether drugs commonly use in high-risk NB treatment (cisplatin, etoposide, irinotecan, and topotecan) could sustain the NK cell-mediated antitumor function, we studied the expression of ligands for NK cell-activating receptors on a panel of NB cell lines. No drugs showed a significant immune adjuvant effect confirmed also by

an NK cell functional assay upon cisplatin treatment. This refractoriness was partially related to the abnormal status of ATM, ATR, CHEK1, and CHEK2 genes in the NB cell lines tested. Of note, ATM deletion correlates with lower event-free survival and overall survival in patients affected by NB, independently of MYCN amplification. The altered ATM/ATR induction in NB cells could inhibit the effect mediated by many genotoxic drugs in terms of the induced expression of ligands for NK cell-activating receptors. Moreover, our data showed that both the impaired production of ROS in some NB cell lines and the transient p53 stabilization after drug treatment at pre-apoptotic concentrations could contribute to inefficient induction of NKG2D and DNAM-1 ligands. It cannot be excluded that in our drug treatment conditions the apoptosis induced at 48h and at higher doses could depend also on p53-independent mechanisms occurring in NB, as reported by other authors [175] [176].

A way to bypass ATM-ATR pathway is to directly target p53. It is known that the p53/MDM2/p14(ARF) pathway is often inactivated through MDM2 amplification or p14(ARF) inactivation. Nutlins were the first potent and selective inhibitors of the MDM2–p53 interaction [57], in particular Nutlin-3a was evaluated both *in vitro* and *in vivo* in several types of human cancers, also NB [158]. MDM2–p53 antagonists have been shown to activate the p53 pathway, triggering p53-dependent cell cycle arrest and/or apoptosis, while inducing a reversible cell cycle arrest in normal cells [177]. Several studies showed that Nutlin-3a is less toxic to normal cells than to neoplastic cells [135]. Moreover, Nutlin-3a can induce a cellular senescence [136].

We show p53 restoration activity by Nutlin-3a therapy can strongly potentiate the ULBP1, ULBP2/5/6, ULBP3 and PVR activating ligands expression in wt p53 NB cells. Real-time PCR and ChIP assays, after Nutlin-3a treatment, revealed an increased transcription of PVR mRNA due to a direct p53 binding on PVR promoter. Nutlin-3a makes MYCN-amplified NB cell lines, more susceptible to NK cell-mediated recognition and lysis: despite amplification of MDM2 is common in MYCN amplified cell lines, nutlin-3 therapy effectively overcomes the p53 inhibition imposed by excess levels of MDM2. Moreover, *in vivo* experiments performed in xenograft NSG mice model and immunohistochemestry analysis, demonstrate the efficacy of Nutlin-3a in inducing the ligands for DNAM-1 NK cell activating receptor.

NK cells are a fundamental component of the innate immune system, capable of recognizing and destroying tumour cells, and they play an important role in adaptive immunity by modulating dendritic cell function as well as controlling antigen-specific T cell responses. Therapies involving NK cells may activate endogenous NK cells or involve transfer of exogenous cells by hematopoietic stem cell transplantation (HSCT) or adoptive cell therapy.

Presently, the *in vivo* adoptive transfer of mature *in vitro* activated and expanded NK cells, in autologous or allogeneic settings, represents a promising approach to treat NB. Nowadays, there are different clinical trials based on the use of mature NK cells combined with some therapeutic agents, such as anti-GD2 (ch14.18/CHO) (http://ClinicalTrials.gov).

We treated NB bearing NSG mice with a combined treatment of mature NK cell expanded from healthy donor and Nutlin-3a and we observed a better overall survival of mice that received a combined treatment compared with mice that received only NK cell treatment. This data suggests Nutlin-3a as a new therapeutic approach to potentiate the function of adoptively transfer NK cells, in autologous or allogenic setting, as a new NK cell-based immunotherapy of NB.

In conclusion we can assert that MYCN overexpression contributes to protect NB cells from NK-cell-mediated recognition and killing, delineating a novel mechanism of tumour escape from the control of the innate immune system. In this context, restoring the p53 transcription factors level and function, through Nutlin-3a use, could pave the way for a promising approaches able to stimulate the activating ligand expression on high-risk NB cells and potentiate the NK cell-mediated cytotoxicity and killing.

Further investigations *in vivo* are warranted to better understand the complex mechanisms involved in NK cell-mediated killing in order to design a translational approach for cancer immunotherapy.

References

1. Louis, C.U. and J.M. Shohet, *Neuroblastoma: molecular pathogenesis and therapy.* Annu Rev Med, 2015. **66**: p. 49-63.
2. Bown, N., *Neuroblastoma tumour genetics: clinical and biological aspects.* J Clin Pathol, 2001. **54**(12): p. 897-910.
3. Raabe, E.H., et al., *Prevalence and functional consequence of PHOX2B mutations in neuroblastoma.* Oncogene, 2008. **27**(4): p. 469-76.
4. Mosse, Y.P., et al., *Identification of ALK as a major familial neuroblastoma predisposition gene.* Nature, 2008. **455**(7215): p. 930-5.
5. Motegi, A., et al., *ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth.* J Cell Sci, 2004. **117**(Pt 15): p. 3319-29.
6. Pugh, T.J., et al., *The genetic landscape of high-risk neuroblastoma.* Nat Genet, 2013. **45**(3): p. 279-84.
7. Brodeur, G.M., *Neuroblastoma: biological insights into a clinical enigma.* Nat Rev Cancer, 2003. **3**(3): p. 203-16.
8. Ruiz-Perez, M.V., A.B. Henley, and M. Arsenian-Henriksson, *The MYCN Protein in Health and Disease.* Genes (Basel), 2017. **8**(4).
9. Brodeur, G.M., et al., *International criteria for diagnosis, staging and response to treatment in patients with neuroblastoma.* Prog Clin Biol Res, 1988. **271**: p. 509-24.
10. Diede, S.J., *Spontaneous regression of metastatic cancer: learning from neuroblastoma.* Nat Rev Cancer, 2014. **14**(2): p. 71-2.
11. Nickerson, H.J., et al., *Favorable biology and outcome of stage IV-S neuroblastoma with supportive care or minimal therapy: a Children's Cancer Group study.* J Clin Oncol, 2000. **18**(3): p. 477-86.
12. Modak, S. and N.K. Cheung, *Neuroblastoma: Therapeutic strategies for a clinical enigma.* Cancer Treat Rev, 2010. **36**(4): p. 307-17.
13. Maris, J.M., et al., *Neuroblastoma.* Lancet, 2007. **369**(9579): p. 2106-20.
14. Brodeur, G.M., et al., *Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage.* Science, 1984. **224**(4653): p. 1121-4.
15. Henriksson, M. and B. Luscher, *Proteins of the Myc network: essential regulators of cell growth and differentiation.* Adv Cancer Res, 1996. **68**: p. 109-82.
16. Kohl, N.E., C.E. Gee, and F.W. Alt, *Activated expression of the N-myc gene in human neuroblastomas and related tumors.* Science, 1984. **226**(4680): p. 1335-7.
17. Meyer, N. and L.Z. Penn, *Reflecting on 25 years with MYC.* Nat Rev Cancer, 2008. **8**(12): p. 976-90.
18. Charron, D., *HLA class II disease associations: molecular basis.* J Autoimmun, 1992. **5 Suppl A**: p. 45-53.
19. Sears, R., et al., *Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability.* Genes Dev, 2000. **14**(19): p. 2501-14.
20. Westermark, U.K., et al., *The MYCN oncogene and differentiation in neuroblastoma.* Semin Cancer Biol, 2011. **21**(4): p. 256-66.
21. Hirning, U., et al., *A comparative analysis of N-myc and c-myc expression and cellular proliferation in mouse organogenesis.* Mech Dev, 1991. **33**(2): p. 119-25.
22. Murphy, D.M., et al., *Global MYCN transcription factor binding analysis in neuroblastoma reveals association with distinct E-box motifs and regions of DNA hypermethylation.* PLoS One, 2009. **4**(12): p. e8154.
23. van Golen, C.M., et al., *N-Myc overexpression leads to decreased beta1 integrin expression and increased apoptosis in human neuroblastoma cells.* Oncogene, 2003. **22**(17): p. 2664-73.
24. Westermann, F., et al., *Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas.* Genome Biol, 2008. **9**(10): p. R150.

25. Laurenti, E., et al., *Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity.* Cell Stem Cell, 2008. **3**(6): p. 611-24.
26. Blackwell, T.K., et al., *Binding of myc proteins to canonical and noncanonical DNA sequences.* Mol Cell Biol, 1993. **13**(9): p. 5216-24.
27. Chen, L., et al., *p53 is a direct transcriptional target of MYCN in neuroblastoma.* Cancer Res, 2010. **70**(4): p. 1377-88.
28. Tweddle, D.A., et al., *The p53 pathway and its inactivation in neuroblastoma.* Cancer Lett, 2003. **197**(1-2): p. 93-8.
29. Levine, A.J. and M. Oren, *The first 30 years of p53: growing ever more complex.* Nat Rev Cancer, 2009. **9**(10): p. 749-58.
30. Oliner, J.D., et al., *Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53.* Nature, 1993. **362**(6423): p. 857-60.
31. Chen, L., et al., *ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage.* EMBO J, 2005. **24**(19): p. 3411-22.
32. Takai, H., et al., *Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription.* EMBO J, 2002. **21**(19): p. 5195-205.
33. Sorensen, C.S., et al., *Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A.* Cancer Cell, 2003. **3**(3): p. 247-58.
34. Bartkova, J., et al., *Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints.* Nature, 2006. **444**(7119): p. 633-7.
35. Morelli, M.B., et al., *Axitinib induces DNA damage response leading to senescence, mitotic catastrophe, and increased NK cell recognition in human renal carcinoma cells.* Oncotarget, 2015. **6**(34): p. 36245-59.
36. Antonangeli, F., et al., *Natural killer cell recognition of in vivo drug-induced senescent multiple myeloma cells.* Oncoimmunology, 2016. **5**(10): p. e1218105.
37. He, J., et al., *Crosstalk between MYCN and MDM2-p53 signal pathways regulates tumor cell growth and apoptosis in neuroblastoma.* Cell Cycle, 2011. **10**(17): p. 2994-3002.
38. Gu, L., et al., *MDM2 regulates MYCN mRNA stabilization and translation in human neuroblastoma cells.* Oncogene, 2012. **31**(11): p. 1342-53.
39. Morandi, F., et al., *Human neuroblastoma cells trigger an immunosuppressive program in monocytes by stimulating soluble HLA-G release.* Cancer Res, 2007. **67**(13): p. 6433-41.
40. Raffaghello, L., et al., *Multiple defects of the antigen-processing machinery components in human neuroblastoma: immunotherapeutic implications.* Oncogene, 2005. **24**(29): p. 4634-44.
41. Raffaghello, L., et al., *Mechanisms of immune evasion of human neuroblastoma.* Cancer Lett, 2005. **228**(1-2): p. 155-61.
42. Airoldi, I., et al., *Expression of costimulatory molecules in human neuroblastoma. Evidence that CD40+ neuroblastoma cells undergo apoptosis following interaction with CD40L.* Br J Cancer, 2003. **88**(10): p. 1527-36.
43. Castriconi, R., et al., *Identification of 4Ig-B7-H3 as a neuroblastoma-associated molecule that exerts a protective role from an NK cell-mediated lysis.* Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12640-5.
44. Ljunggren, H.G. and K. Karre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunol Today, 1990. **11**(7): p. 237-44.
45. Fish, J.D. and S.A. Grupp, *Stem cell transplantation for neuroblastoma.* Bone Marrow Transplant, 2008. **41**(2): p. 159-65.
46. Matthay, K.K., et al., *Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group.* N Engl J Med, 1999. **341**(16): p. 1165-73.
47. Yu, A.L., et al., *Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma.* N Engl J Med, 2010. **363**(14): p. 1324-34.
48. Cerboni, C., et al., *The DNA Damage Response: A Common Pathway in the Regulation of NKG2D and DNAM-1 Ligand Expression in Normal, Infected, and Cancer Cells.* Front Immunol, 2014. **4**: p. 508.

49. Soriani, A., et al., *ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype.* Blood, 2009. **113**(15): p. 3503-11.
50. Gasser, S., et al., *The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor.* Nature, 2005. **436**(7054): p. 1186-90.
51. Filippakopoulos, P. and S. Knapp, *The bromodomain interaction module.* FEBS Lett, 2012. **586**(17): p. 2692-704.
52. Filippakopoulos, P., et al., *Histone recognition and large-scale structural analysis of the human bromodomain family.* Cell, 2012. **149**(1): p. 214-31.
53. Schnepp, R.W. and J.M. Maris, *Targeting MYCN: a good BET for improving neuroblastoma therapy?* Cancer Discov, 2013. **3**(3): p. 255-7.
54. Barone, G., et al., *New strategies in neuroblastoma: Therapeutic targeting of MYCN and ALK.* Clin Cancer Res, 2013. **19**(21): p. 5814-21.
55. Esposito, M.R., et al., *Neuroblastoma treatment in the post-genomic era.* J Biomed Sci, 2017. **24**(1): p. 14.
56. Carr, J., et al., *Increased frequency of aberrations in the p53/MDM2/p14(ARF) pathway in neuroblastoma cell lines established at relapse.* Cancer Res, 2006. **66**(4): p. 2138-45.
57. Vassilev, L.T., et al., *In vivo activation of the p53 pathway by small-molecule antagonists of MDM2.* Science, 2004. **303**(5659): p. 844-8.
58. Gamble, L.D., et al., *MYCN sensitizes neuroblastoma to the MDM2-p53 antagonists Nutlin-3 and MI-63.* Oncogene, 2012. **31**(6): p. 752-63.
59. Van Maerken, T., et al., *Pharmacologic activation of wild-type p53 by nutlin therapy in childhood cancer.* Cancer Lett, 2014. **344**(2): p. 157-65.
60. Goldberg, J.L. and P.M. Sondel, *Enhancing Cancer Immunotherapy Via Activation of Innate Immunity.* Semin Oncol, 2015. **42**(4): p. 562-72.
61. Cho, D., et al., *Cytotoxicity of activated natural killer cells against pediatric solid tumors.* Clin Cancer Res, 2010. **16**(15): p. 3901-9.
62. Asai, O., et al., *Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation.* J Clin Invest, 1998. **101**(9): p. 1835-42.
63. Olson, J.A., et al., *NK cells mediate reduction of GVHD by inhibiting activated, alloreactive T cells while retaining GVT effects.* Blood, 2010. **115**(21): p. 4293-301.
64. Schulz, G., et al., *Detection of ganglioside GD2 in tumor tissues and sera of neuroblastoma patients.* Cancer Res, 1984. **44**(12 Pt 1): p. 5914-20.
65. Ruggeri, L., et al., *Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value.* Blood, 2007. **110**(1): p. 433-40.
66. Scott, A.M., J.D. Wolchok, and L.J. Old, *Antibody therapy of cancer.* Nat Rev Cancer, 2012. **12**(4): p. 278-87.
67. Seidel, D., et al., *Disialoganglioside-specific human natural killer cells are effective against drug-resistant neuroblastoma.* Cancer Immunol Immunother, 2015. **64**(5): p. 621-34.
68. Hartmann, J., et al., *Clinical development of CAR T cells-challenges and opportunities in translating innovative treatment concepts.* EMBO Mol Med, 2017. **9**(9): p. 1183-1197.
69. Ritchie, D.S., et al., *Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia.* Mol Ther, 2013. **21**(11): p. 2122-9.
70. Fang, F., W. Xiao, and Z. Tian, *NK cell-based immunotherapy for cancer.* Semin Immunol, 2017. **31**: p. 37-54.
71. Cheng, M., et al., *NK cell-based immunotherapy for malignant diseases.* Cell Mol Immunol, 2013. **10**(3): p. 230-52.
72. Braumuller, H., et al., *T-helper-1-cell cytokines drive cancer into senescence.* Nature, 2013. **494**(7437): p. 361-5.
73. Mittica, G., et al., *Adoptive immunotherapy against ovarian cancer.* J Ovarian Res, 2016. **9**(1): p. 30.

74. Overacre-Delgoffe, A.E., et al., *Interferon-gamma Drives Treg Fragility to Promote Anti-tumor Immunity.* Cell, 2017. **169**(6): p. 1130-1141 e11.
75. Yang, Y., et al., *Phase I Study of Random Healthy Donor-Derived Allogeneic Natural Killer Cell Therapy in Patients with Malignant Lymphoma or Advanced Solid Tumors.* Cancer Immunol Res, 2016. **4**(3): p. 215-24.
76. Miller, J.S., et al., *Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer.* Blood, 2005. **105**(8): p. 3051-7.
77. Fang, F., W. Xiao, and Z. Tian, *Challenges of NK cell-based immunotherapy in the new era.* Front Med, 2018.
78. Caligiuri, M.A., *Human natural killer cells.* Blood, 2008. **112**(3): p. 461-9.
79. Ritz, J., et al., *Characterization of functional surface structures on human natural killer cells.* Adv Immunol, 1988. **42**: p. 181-211.
80. Sivori, S., et al., *p46, a novel natural killer cell-specific surface molecule that mediates cell activation.* J Exp Med, 1997. **186**(7): p. 1129-36.
81. Walzer, T., et al., *Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3384-9.
82. Galy, A., et al., *Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset.* Immunity, 1995. **3**(4): p. 459-73.
83. Fehniger, T.A., et al., *CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity.* Blood, 2003. **101**(8): p. 3052-7.
84. Ferlazzo, G., et al., *Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16606-11.
85. Koka, R., et al., *Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice.* J Exp Med, 2003. **197**(8): p. 977-84.
86. Bakker, A.B., et al., *NK cell activation: distinct stimulatory pathways counterbalancing inhibitory signals.* Hum Immunol, 2000. **61**(1): p. 18-27.
87. Maher, S.G., et al., *Interferon: cellular executioner or white knight?* Curr Med Chem, 2007. **14**(12): p. 1279-89.
88. Smyth, M.J., et al., *Activation of NK cell cytotoxicity.* Mol Immunol, 2005. **42**(4): p. 501-10.
89. Zamai, L., et al., *Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells.* J Exp Med, 1998. **188**(12): p. 2375-80.
90. Parham, P. and K.L. McQueen, *Alloreactive killer cells: hindrance and help for haematopoietic transplants.* Nat Rev Immunol, 2003. **3**(2): p. 108-22.
91. Kim, S., et al., *In vivo natural killer cell activities revealed by natural killer cell-deficient mice.* Proc Natl Acad Sci U S A, 2000. **97**(6): p. 2731-6.
92. Boudreau, J.E. and K.C. Hsu, *Natural killer cell education in human health and disease.* Curr Opin Immunol, 2018. **50**: p. 102-111.
93. Fernandez, N.C., et al., *A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules.* Blood, 2005. **105**(11): p. 4416-23.
94. Anfossi, N., et al., *Human NK cell education by inhibitory receptors for MHC class I.* Immunity, 2006. **25**(2): p. 331-42.
95. Leibson, P.J., *The regulation of lymphocyte activation by inhibitory receptors.* Curr Opin Immunol, 2004. **16**(3): p. 328-36.
96. Uhrberg, M., et al., *Human diversity in killer cell inhibitory receptor genes.* Immunity, 1997. **7**(6): p. 753-63.
97. Chan, H.W., et al., *DNA methylation maintains allele-specific KIR gene expression in human natural killer cells.* J Exp Med, 2003. **197**(2): p. 245-55.
98. Santourlidis, S., et al., *Crucial role of DNA methylation in determination of clonally distributed killer cell Ig-like receptor expression patterns in NK cells.* J Immunol, 2002. **169**(8): p. 4253-61.
99. Stewart, C.A., et al., *Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors.* Proc Natl Acad Sci U S A, 2005. **102**(37): p. 13224-9.

100. Braud, V.M., et al., *HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C.* Nature, 1998. **391**(6669): p. 795-9.
101. Moretta, L., et al., *Human natural killer cell function and receptors.* Curr Opin Pharmacol, 2001. **1**(4): p. 387-91.
102. Gutierrez-Franco, J., et al., *Characterization of B7H6, an endogenous ligand for the NK cell activating receptor NKp30, reveals the identity of two different soluble isoforms during normal human pregnancy.* Immunobiology, 2018. **223**(1): p. 57-63.
103. Clarkson, S.B. and P.A. Ory, *CD16. Developmentally regulated IgG Fc receptors on cultured human monocytes.* J Exp Med, 1988. **167**(2): p. 408-20.
104. Clynes, R.A., et al., *Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets.* Nat Med, 2000. **6**(4): p. 443-6.
105. Ravetch, J.V. and B. Perussia, *Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions.* J Exp Med, 1989. **170**(2): p. 481-97.
106. Lanier, L.L., *Up on the tightrope: natural killer cell activation and inhibition.* Nat Immunol, 2008. **9**(5): p. 495-502.
107. Stern, M., et al., *Pre-emptive immunotherapy with purified natural killer cells after haploidentical SCT: a prospective phase II study in two centers.* Bone Marrow Transplant, 2013. **48**(3): p. 433-8.
108. Tsukerman, P., et al., *Expansion of CD16 positive and negative human NK cells in response to tumor stimulation.* Eur J Immunol, 2014. **44**(5): p. 1517-25.
109. Raulet, D.H., *Roles of the NKG2D immunoreceptor and its ligands.* Nat Rev Immunol, 2003. **3**(10): p. 781-90.
110. Upshaw, J.L., et al., *NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells.* Nat Immunol, 2006. **7**(5): p. 524-32.
111. Marcais, A., et al., *Regulation of mouse NK cell development and function by cytokines.* Front Immunol, 2013. **4**: p. 450.
112. Horng, T., J.S. Bezbradica, and R. Medzhitov, *NKG2D signaling is coupled to the interleukin 15 receptor signaling pathway.* Nat Immunol, 2007. **8**(12): p. 1345-52.
113. Cosman, D., et al., *ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor.* Immunity, 2001. **14**(2): p. 123-33.
114. Raulet, D.H. and N. Guerra, *Oncogenic stress sensed by the immune system: role of natural killer cell receptors.* Nat Rev Immunol, 2009. **9**(8): p. 568-80.
115. Pende, D., et al., *Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin.* Eur J Immunol, 2001. **31**(4): p. 1076-86.
116. Raulet, D.H., et al., *Regulation of ligands for the NKG2D activating receptor.* Annu Rev Immunol, 2013. **31**: p. 413-41.
117. Baragano Raneros, A., B. Suarez-Alvarez, and C. Lopez-Larrea, *Secretory pathways generating immunosuppressive NKG2D ligands: New targets for therapeutic intervention.* Oncoimmunology, 2014. **3**: p. e28497.
118. Mincheva-Nilsson, L. and V. Baranov, *Cancer exosomes and NKG2D receptor-ligand interactions: impairing NKG2D-mediated cytotoxicity and anti-tumour immune surveillance.* Semin Cancer Biol, 2014. **28**: p. 24-30.
119. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.
120. Textor, S., et al., *Human NK cells are alerted to induction of p53 in cancer cells by upregulation of the NKG2D ligands ULBP1 and ULBP2.* Cancer Res, 2011. **71**(18): p. 5998-6009.
121. Waldhauer, I. and A. Steinle, *Proteolytic release of soluble UL16-binding protein 2 from tumor cells.* Cancer Res, 2006. **66**(5): p. 2520-6.
122. Waldhauer, I., et al., *Tumor-associated MICA is shed by ADAM proteases.* Cancer Res, 2008. **68**(15): p. 6368-76.

123. Biassoni, R. and M.S. Malnati, *Human Natural Killer Receptors, Co-Receptors, and Their Ligands.* Curr Protoc Immunol, 2018. **121**(1): p. e47.
124. Zhang, Z., et al., *DNAM-1 controls NK cell activation via an ITT-like motif.* J Exp Med, 2015. **212**(12): p. 2165-82.
125. Bottino, C., et al., *Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule.* J Exp Med, 2003. **198**(4): p. 557-67.
126. Reymond, N., et al., *DNAM-1 and PVR regulate monocyte migration through endothelial junctions.* J Exp Med, 2004. **199**(10): p. 1331-41.
127. Sullivan, D.P., M.A. Seidman, and W.A. Muller, *Poliovirus receptor (CD155) regulates a step in transendothelial migration between PECAM and CD99.* Am J Pathol, 2013. **182**(3): p. 1031-42.
128. Shibuya, A., et al., *DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes.* Immunity, 1996. **4**(6): p. 573-81.
129. Shibuya, K., et al., *Physical and functional association of LFA-1 with DNAM-1 adhesion molecule.* Immunity, 1999. **11**(5): p. 615-23.
130. Wang, W., et al., *Modulation of the poliovirus receptor expression in malignant lymphocytes by epigenetic alterations.* J Immunother, 2011. **34**(4): p. 353-61.
131. Kussie, P.H., et al., *Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain.* Science, 1996. **274**(5289): p. 948-53.
132. Shangary, S., et al., *Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition.* Proc Natl Acad Sci U S A, 2008. **105**(10): p. 3933-8.
133. Shangary, S., et al., *Reactivation of p53 by a specific MDM2 antagonist (MI-43) leads to p21-mediated cell cycle arrest and selective cell death in colon cancer.* Mol Cancer Ther, 2008. **7**(6): p. 1533-42.
134. Vassilev, L.T., *p53 Activation by small molecules: application in oncology.* J Med Chem, 2005. **48**(14): p. 4491-9.
135. Carvajal, D., et al., *Activation of p53 by MDM2 antagonists can protect proliferating cells from mitotic inhibitors.* Cancer Res, 2005. **65**(5): p. 1918-24.
136. Efeyan, A., et al., *Induction of p53-dependent senescence by the MDM2 antagonist nutlin-3a in mouse cells of fibroblast origin.* Cancer Res, 2007. **67**(15): p. 7350-7.
137. Xue, W., et al., *Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas.* Nature, 2007. **445**(7128): p. 656-60.
138. Shen, H. and C.G. Maki, *Persistent p21 expression after Nutlin-3a removal is associated with senescence-like arrest in 4N cells.* J Biol Chem, 2010. **285**(30): p. 23105-14.
139. Thompson, T., et al., *Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis.* J Biol Chem, 2004. **279**(51): p. 53015-22.
140. Mitani, N., Y. Niwa, and Y. Okamoto, *Surveyor nuclease-based detection of p53 gene mutations in haematological malignancy.* Ann Clin Biochem, 2007. **44**(Pt 6): p. 557-9.
141. Kojima, K., et al., *MDM2 antagonists induce p53-dependent apoptosis in AML: implications for leukemia therapy.* Blood, 2005. **106**(9): p. 3150-9.
142. Secchiero, P., et al., *Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL).* Blood, 2006. **107**(10): p. 4122-9.
143. Stuhmer, T., et al., *Nongenotoxic activation of the p53 pathway as a therapeutic strategy for multiple myeloma.* Blood, 2005. **106**(10): p. 3609-17.
144. Coll-Mulet, L., et al., *MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells.* Blood, 2006. **107**(10): p. 4109-14.
145. Canner, J.A., et al., *MI-63: a novel small-molecule inhibitor targets MDM2 and induces apoptosis in embryonal and alveolar rhabdomyosarcoma cells with wild-type p53.* Br J Cancer, 2009. **101**(5): p. 774-81.
146. Miyachi, M., et al., *Restoration of p53 pathway by nutlin-3 induces cell cycle arrest and apoptosis in human rhabdomyosarcoma cells.* Clin Cancer Res, 2009. **15**(12): p. 4077-84.

147. Michaelis, M., et al., *Reversal of P-glycoprotein-mediated multidrug resistance by the murine double minute 2 antagonist nutlin-3.* Cancer Res, 2009. **69**(2): p. 416-21.
148. Van Maerken, T., et al., *Antitumor activity of the selective MDM2 antagonist nutlin-3 against chemoresistant neuroblastoma with wild-type p53.* J Natl Cancer Inst, 2009. **101**(22): p. 1562-74.
149. Peirce, S.K. and H.W. Findley, *The MDM2 antagonist nutlin-3 sensitizes p53-null neuroblastoma cells to doxorubicin via E2F1 and TAp73.* Int J Oncol, 2009. **34**(5): p. 1395-402.
150. Chen, L., et al., *p53 is nuclear and functional in both undifferentiated and differentiated neuroblastoma.* Cell Cycle, 2007. **6**(21): p. 2685-96.
151. Barbieri, E., et al., *MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death.* Mol Cancer Ther, 2006. **5**(9): p. 2358-65.
152. Ribas, J., J. Boix, and L. Meijer, *(R)-roscovitine (CYC202, Seliciclib) sensitizes SH-SY5Y neuroblastoma cells to nutlin-3-induced apoptosis.* Exp Cell Res, 2006. **312**(12): p. 2394-400.
153. Patterson, D.M., et al., *Effect of MDM2 and vascular endothelial growth factor inhibition on tumor angiogenesis and metastasis in neuroblastoma.* Angiogenesis, 2011. **14**(3): p. 255-66.
154. Van Maerken, T., et al., *Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma.* Cancer Res, 2006. **66**(19): p. 9646-55.
155. Fontana, L., et al., *Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM.* PLoS One, 2008. **3**(5): p. e2236.
156. Mistry, A.R. and C.A. O'Callaghan, *Regulation of ligands for the activating receptor NKG2D.* Immunology, 2007. **121**(4): p. 439-47.
157. Slack, A., et al., *The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 731-6.
158. Chen, L. and D.A. Tweddle, *p53, SKP2, and DKK3 as MYCN Target Genes and Their Potential Therapeutic Significance.* Front Oncol, 2012. **2**: p. 173.
159. Norris, M.D., et al., *Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma.* N Engl J Med, 1996. **334**(4): p. 231-8.
160. Brandetti, E., et al., *MYCN is an immunosuppressive oncogene dampening the expression of ligands for NK-cell-activating receptors in human high-risk neuroblastoma.* Oncoimmunology, 2017. **6**(6): p. e1316439.
161. Delmore, J.E., et al., *BET bromodomain inhibition as a therapeutic strategy to target c-Myc.* Cell, 2011. **146**(6): p. 904-17.
162. Nanbakhsh, A., et al., *c-Myc regulates expression of NKG2D ligands ULBP1/2/3 in AML and modulates their susceptibility to NK-mediated lysis.* Blood, 2014. **123**(23): p. 3585-95.
163. Gaymes, T.J., et al., *FLT3 and JAK2 Mutations in Acute Myeloid Leukemia Promote Interchromosomal Homologous Recombination and the Potential for Copy Neutral Loss of Heterozygosity.* Cancer Res, 2017. **77**(7): p. 1697-1708.
164. Amin, P.J. and B.S. Shankar, *Sulforaphane induces ROS mediated induction of NKG2D ligands in human cancer cell lines and enhances susceptibility to NK cell mediated lysis.* Life Sci, 2015. **126**: p. 19-27.
165. Okita, R., et al., *MHC class I chain-related molecule A and B expression is upregulated by cisplatin and associated with good prognosis in patients with non-small cell lung cancer.* Cancer Immunol Immunother, 2016. **65**(5): p. 499-509.
166. Veneziani, I., et al., *Neuroblastoma Cell Lines Are Refractory to Genotoxic Drug-Mediated Induction of Ligands for NK Cell-Activating Receptors.* J Immunol Res, 2018. **2018**: p. 4972410.
167. Marcus, A., et al., *Recognition of tumors by the innate immune system and natural killer cells.* Adv Immunol, 2014. **122**: p. 91-128.
168. Raffaghello, L., et al., *Downregulation and/or release of NKG2D ligands as immune evasion strategy of human neuroblastoma.* Neoplasia, 2004. **6**(5): p. 558-68.
169. Garcia, P.L., et al., *JQ1 Induces DNA Damage and Apoptosis, and Inhibits Tumor Growth in a Patient-Derived Xenograft Model of Cholangiocarcinoma.* Mol Cancer Ther, 2018. **17**(1): p. 107-118.
170. Bid, H.K., et al., *The Bromodomain BET Inhibitor JQ1 Suppresses Tumor Angiogenesis in Models of Childhood Sarcoma.* Mol Cancer Ther, 2016. **15**(5): p. 1018-28.

171. Casey, S.C., et al., *MYC regulates the antitumor immune response through CD47 and PD-L1.* Science, 2016. **352**(6282): p. 227-31.
172. Melaiu, O., et al., *PD-L1 Is a Therapeutic Target of the Bromodomain Inhibitor JQ1 and, Combined with HLA Class I, a Promising Prognostic Biomarker in Neuroblastoma.* Clin Cancer Res, 2017. **23**(15): p. 4462-4472.
173. Abruzzese, M.P., et al., *Inhibition of bromodomain and extra-terminal (BET) proteins increases NKG2D ligand MICA expression and sensitivity to NK cell-mediated cytotoxicity in multiple myeloma cells: role of cMYC-IRF4-miR-125b interplay.* J Hematol Oncol, 2016. **9**(1): p. 134.
174. Cifaldi, L., et al., *Boosting Natural Killer Cell-Based Immunotherapy with Anticancer Drugs: a Perspective.* Trends Mol Med, 2017. **23**(12): p. 1156-1175.
175. Li, Y. and A. Nakagawara, *Apoptotic cell death in neuroblastoma.* Cells, 2013. **2**(2): p. 432-59.
176. Nicolai, S., et al., *Neuroblastoma: oncogenic mechanisms and therapeutic exploitation of necroptosis.* Cell Death Dis, 2015. **6**: p. e2010.
177. Cheok, C.F., et al., *Translating p53 into the clinic.* Nat Rev Clin Oncol, 2011. **8**(1): p. 25-37.
178. Ardolino, M et al., *DNAM-1 ligand expression on Ag-stimulated T lymphocytes is mediated by ROS-dependent activation of DNA-damage response: relevance for NK-T cell interaction.* Blood 2011 117(18):4778-86
179. Soriani, A et al*., Reactive oxygen species- and DNA damage response-dependent NK cell activating ligand upregulation occurs at transcriptional levels and requires the transcriptional factor E2F1.* J Immunol 2014 193(2):950-60
180. Qiu, H et al., *JQ1 suppresses tumor growth through downregulating LDHA in ovarian cancer.* Oncotarget 2015, 30;6(9):6915-30.
181. Hussong, M et al., *The bromodomain protein BRD4 regulates the KEAP1/NRF2-dependent oxidative stress response.* Cell Death Dis 2014 24;5:e1195.

LIST OF PUBBLICATIONS

The BET-Bromodomain inhibitor JQ1 renders neuroblastoma cells more resistant to NK cell-mediated recognition and killing by downregulating ligands for NKG2D and DNAM-1 receptors
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Under review

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