





GD2 redirected CAR T and activated NK-cell-mediated secretion of IFN γ overcomes MYCN-dependent IDO1 inhibition, contributing to neuroblastoma cell immune escape

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ABSTRACT

Immune escape mechanisms employed by neuroblastoma (NB) cells include secretion of immunosuppressive factors disrupting effective antitumor immunity. The use of cellular therapy to treat solid tumors needs to be implemented. Killing activity of anti-GD2 Chimeric Antigen Receptor (CAR) T or natural killer (NK) cells against target NB cells was assessed through coculture experiments and quantified by FACS analysis. ELISA assay was used to quantify interferon- γ (IFN γ) secreted by NK and CAR T cells. Real Time PCR and Western Blot were performed to analyze gene and protein levels modifications. Transcriptional study was performed by chromatin immunoprecipitation and luciferase reporter assays on experiments of mutagenesis on the promoter sequence. NB tissue sample were analyzed by IHC and Real Time PCR to perform correlation study. We demonstrate that Indoleamine-pyrrole 2,3-dioxygenase1 (IDO1), due to its ability to convert tryptophan into kynurenines, is involved in NB resistance to activity of immune cells. In NB, IDO1 is able to inhibit the anti-tumor effect displayed by of both anti-GD2 CAR (GD2.CAR) T-cell and NK cells, mainly by impairing their IFN γ production. Furthermore, inhibition of MYCN expression in NB results into accumulation of IDO1 and consequently of kynurenines, which negatively affect the immune surveillance. Inverse correlation between IDO1 and MYCN expression has been observed in a wide cohort of NB samples. This finding was supported by the identification of a transcriptional repressive role of MYCN on IDO1 promoter. The evidence of IDO1 involvement in NB immune escape and its ability to impair NK and GD2.CAR T-cell activity contribute to clarify one of the possible mechanisms responsible for the limited efficacy of these immunotherapeutic approaches. A combined therapy of NK or GD2.CAR T-cells with IDO1 inhibitors, a class of compounds already in phase I/II clinical studies, could represent a new and still unexplored strategy capable to improve long-term efficacy of these immunotherapeutic approaches.

BACKGROUND

Recent advances in oncology have been focused on targeted and immune-mediated therapies through the identification of antigens that are expressed by malignant cells, being critical to their survival, but are absent on normal human tissues. Since solid tumors show a high heterogeneity in the expression of tumor-related antigens, it is difficult to find a target for efficiently eliminating tumor cells. Neuroblastoma (NB) is a malignancy of the sympathetic nervous system, arising from neural crest progenitors that ordinarily develop into sympathetic ganglia and adrenal medulla.¹ Among the different parameters involved in the NB risk classification, MYCN has been the first clinically relevant genetic biomarker. It is an oncogene located on chromosome 2 and its amplification is associated with shorter progression-free survival and worse overall outcome in all stages of disease.²

The use of chimeric antigen receptor (CAR) T-cells is one of the most powerful examples of adoptive cell therapy, but is also very laborious and time-consuming, involving the collection of patient immune cells, their gene-modification and expansion *ex vivo*.³ Despite the unprecedented success of CAR T-cells in B-cell malignancies,⁴ the efficacy of CAR T-cells in solid tumors, including NB, is negatively affected by the presence of a tumor immune-suppressive microenvironment, which may lead to therapeutic resistance.⁵ Indeed, many efforts are now devoted to ameliorate CAR T-cell design and manufacturing to obtain better tumor recognition, prevention of CAR T-cell exhaustion



and improvement of their persistence. Although heterogeneity in clinical presentation and prognosis is a hallmark of NB, tumor cells highly and selectively express the disialganglioside GD2 antigen, already used for targeted immunotherapy with anti-GD2 monoclonal antibodies and GD2.CAR T-cells⁶ (NCT03373097).

Indoleamine-pyrrole 2,3-dioxygenase1 (IDO1) is an intracellular enzyme that mediates the transformation of the essential amino acid tryptophan into immunosuppressive metabolites, such as kynurenines (Kyns). It is overexpressed in several human cancers, including prostate, breast, brain and hematological malignancies.⁷ High expression levels of IDO1 and Kyns in cancer inhibit natural killer (NK)-cell function, prevent activation of effector T-cells and promote the emergence of regulatory T-cells.⁸ Previous findings have also demonstrated that tumor IDO1 activity can inhibit CD19-CAR T-cell therapy in B-cell malignancies.⁹ Here, we show for the first time that MYCN is able to modulate the expression of IDO1, which plays a crucial role in the generation of an immunosuppressive microenvironment, supporting the activation of other escape mechanisms that strongly and negatively affect the efficacy of immune-therapy approaches based on the adoptive transfer of CAR T and NK cells in NB.

MATERIALS AND METHODS

Patients' characteristics

Tumor samples from 18 NB patients diagnosed between 2002 and 2017 at the Bambino Gesù Children's Hospital (Rome, Italy) were used for IHC analysis. All samples were obtained at diagnosis and prior to any therapy.

Written informed parental consent was obtained for each patient in accordance with the Declaration of Helsinki. Clinical data of patients are shown in [table 1](#). Diagnosis and histology were performed according to the International Neuroblastoma Staging System; MYCN status was evaluated following current guidelines.

CAR T-cell generation

Peripheral blood mononuclear cells were obtained from healthy donors following protocol approval by the Institutional Review Board of Bambino Gesù Children's Hospital, Rome, Italy (prot. 969/2015). CAR-T cells were generated as previously described^{10 11}

CAR T-cell coculture assay

For coculture experiments, non-transduced (NT) or GD2.CAR T-lymphocytes were plated with tumor cells at 1×10^5 cells/well in 12-well plates at the indicated Effector:Target (E:T) ratio 1:1. Following 3, 5 and 7 days of incubation at 37°C, adherent tumor cells and T cells were collected and analyzed for assessment of remaining tumor cells and T-cell characterization using PerCP-conjugated anti-CD45, BV421-conjugated anti-CD3, APC-conjugated anti-HLA-DR, PE-Cy7-conjugated anti-CD28, BV605-conjugated anti-CD27 antibodies (BD Biosciences, California, USA). 7AAD antibody was used for dead cells exclusion (BD Biosciences, California, USA). GD2 evaluation was performed with PE-conjugated anti GD2 antibody. CountBright Absolute Counting Beads (Thermo Scientific, Pennsylvania, USA) were used to quantify the number of analyzed cells. At day 7, the cellular compartment of the cell culture supernatants was discarded and a new amount

Table 1

Patients	INSS	DOB	Diagnosis	Relapse	MycN status
#1	4	17/04/02	03/05/03	no	amp
#2	4	19/11/02	20/06/05	no	amp
#3	4	06/01/05	05/07/06	yes	amp
#4	4	24/05/07	17/08/10	yes	amp
#5	4	20/07/12	16/04/14	no	amp
#6	4	28/02/05	30/08/06	yes	amp
#7	4	05/07/05	30/05/07	yes	amp
#8	4	20/04/06	11/10/07	no	amp
#9	4	28/08/06	11/04/11	no	amp
#10	4	26/03/14	28/10/14	yes	no
#11	4	05/12/96	27/05/03	yes	no
#12	4	30/11/98	03/12/05	yes	no
#13	4	04/02/93	25/02/06	yes	no
#14	4	16/03/05	20/07/06	no	no
#15	4	09/02/02	16/07/02	no	no
#16	4	12/06/02	25/03/03	yes	no
#17	4	09/10/14	27/10/15	no	no
#18	4	05/02/14	22/06/17	no	no

of CAR T cells (1:1 ratio) was added to the culture. The amount of residual tumor cells was measured at day 10.

NK-cell coculture assay

For coculture experiments, NB cells were plated at 1×10^5 cells in 12-well plates. NK cells were added at the indicated ratios. Following 3-day incubation at 37°C, NK cells were collected and assessed by Fluorescence Activated Cell Sorting (FACS) analysis with APC-conjugated anti-CD45, BV786-conjugated anti-CD16, BV711-conjugated anti-CD25, BUV395-conjugated anti-CD56 (BD Biosciences, CA-USA) and PE-conjugated anti-NKp44, PE-Cy7-conjugated anti-CD69 antibodies (Immunological Science, SIC, IT). For apoptosis evaluation, target tumor cells were harvested at the indicated time points and incubated with APC-conjugated anti-CD45 and PE-conjugated anti-Annexin V antibodies following the manufacturers instructions (Immunological Sciences, SIC, IT). K562 cells were marked in Red with PKH26 cell linker (Merck, DE).

Degranulation assay

NB Cell lines were plated at 1×10^5 cells/well in 96-wells plates. NK cells were added at 1:1 E:T ratio and incubated for 3 hours. K562 cells (NK-sensitive target) were used as control of NK-cell activity, as previously published.¹² Thereafter, cells were labeled with PE-conjugated anti-CD56, APC-conjugated anti-CD45 (Immunological Sciences, SIC, IT) and FITC-conjugated anti-CD107a antibody (BD Biosciences, California, USA) for 20 min at 4°C, followed by FACS analysis. For Granzyme B evaluation, cells were labeled with BUV395-conjugated anti-CD56, BUV786-conjugated anti-CD16 (BD Biosciences, California, USA), APC-conjugated anti-CD45 (Immunological Sciences, SIC, IT) for 20 min at 4°C; then, the cells were fixed and permeabilized with Fix/perm buffer (eBioscience, Thermo Scientific) and stained with BV510-conjugated anti-Granzyme B for 30 min, followed by FACS analysis

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as previously described.¹³ Promoter-specific primers sequences and antibodies are described in online supplemental materials and methods section.

Statistical analysis

Data are expressed as average \pm SE of the mean. All statistical tests were carried out using GraphPad Prism V.5.0 for Windows, (GraphPad Software, San Diego, California, USA). Each experiment was performed at least three times. Probability values generated by Student's *t*-test considered to be statistically significant are * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

RESULTS

IDO1 expression impairs anti-GD2.CAR T-cell efficiency

Investigation of IDO1 expression in a wide panel of NB cell lines revealed that IDO1 is not constitutively

expressed. Since IDO1 is preferentially induced by interferon- γ (IFN γ), we stimulated 10 NB cell lines with this cytokine at different time points. We observed that IDO1 was induced in non-MYC amplified NB cell lines on 24 hours of IFN γ treatment (figure 1A). However, IDO1 was upregulated also in MYC amplified cell lines, but only on 48 hours of IFN γ exposure, when MYC starts to be modulated (figure 1B). Indeed IFN γ through the binding of MYC promoter was found to shorten the half-life of MYC mRNA.¹⁴ We also verified by high-performance liquid chromatography (HPLC) analysis that IDO1 protein expression correlates with its enzymatic activity, as shown by the amount of Kyns released in the cell culture supernatants (online supplemental figure S1A,B). To investigate the role of IDO1 in NB microenvironment, we generated two stable IDO1-expressing clones, using the GD2-positive ACN (non-MYC amplified) and IMR-32 (MYC amplified) NB cell lines with a vector carrying IDO1 or an empty vector (Ctrl) (online supplemental figure S1C). The efficiency of transfection was validated by Western blot and IDO1-positive or Ctrl cells were, then, sorted to obtain a clonal population (online supplemental figure S1D). We tested the IDO1-dependent impact of the NB microenvironment on engineered CAR T-cells targeting GD2¹⁰ performing 3, 5 and 7 days coculture experiments with ACN-IDO1 and IMR-32-IDO1 cell lines. We demonstrated that IDO1 overexpression leads to a significant impairment of cytotoxicity of GD2.CAR T-cells when compared with Ctrl conditions (5-day mean values of residual vital tumor cells: ACN-IDO1 $24.6 \times 10^3 \pm 1.1 \times 10^3$ vs ACN-Ctrl $7.5 \times 10^3 \pm 1.4 \times 10^3$; IMR-32-IDO1 $12.1 \times 10^3 \pm 3.3 \times 10^3$ vs IMR-32-Ctrl $7.5 \times 10^3 \pm 0.9 \times 10^3$) (figure 1C (right), figure 1E and H (right), figure 1L). This effect was partially rescued by the addition of the IDO1 inhibitor BMS-986205 (BMS), in both IDO1-expressing clones (5-day mean values of residual vital tumor cells: ACN-IDO1-BMS $16.8 \times 10^3 \pm 1.3 \times 10^3$; IMR-32-IDO1-BMS $7.4 \times 10^3 \pm 2.7 \times 10^3$). The effect of IDO1 inhibitor was also tested in Ctrl conditions (figure 1C (right), figure 1E and H (right), figure 1L). Coculture conditions with NT polyclonally activated T cells were used as negative controls (figure 1C (left), figure 1D and H (left), figure 1L). Furthermore, by the analysis of the expression levels of the activation markers CD27/CD28 and HLA-DR, we observed that IDO1-expressing clones impaired CAR T-cell activity and this effect was partially reversible on addition of the IDO1 inhibitor in both cell lines (figure 1F,M). We also observed that the amount of IFN γ produced by GD2.CAR T-cells in coculture with IDO1-expressing clones was strongly reduced compared with control conditions (figure 1G,N). Since a small number of ACN and IMR-32 Ctrl cells survived, starting to grow between days 5 and 7 of coculture, we hypothesized that these cells could become resistant to CAR T-cell-mediated killing. Indeed, prolonging the time of coculture between GD2.CAR T and NB cells until day 10, after the addition of fresh CAR T-cells, we demonstrated that IDO1-overexpressing cells and Ctrl were not affected by

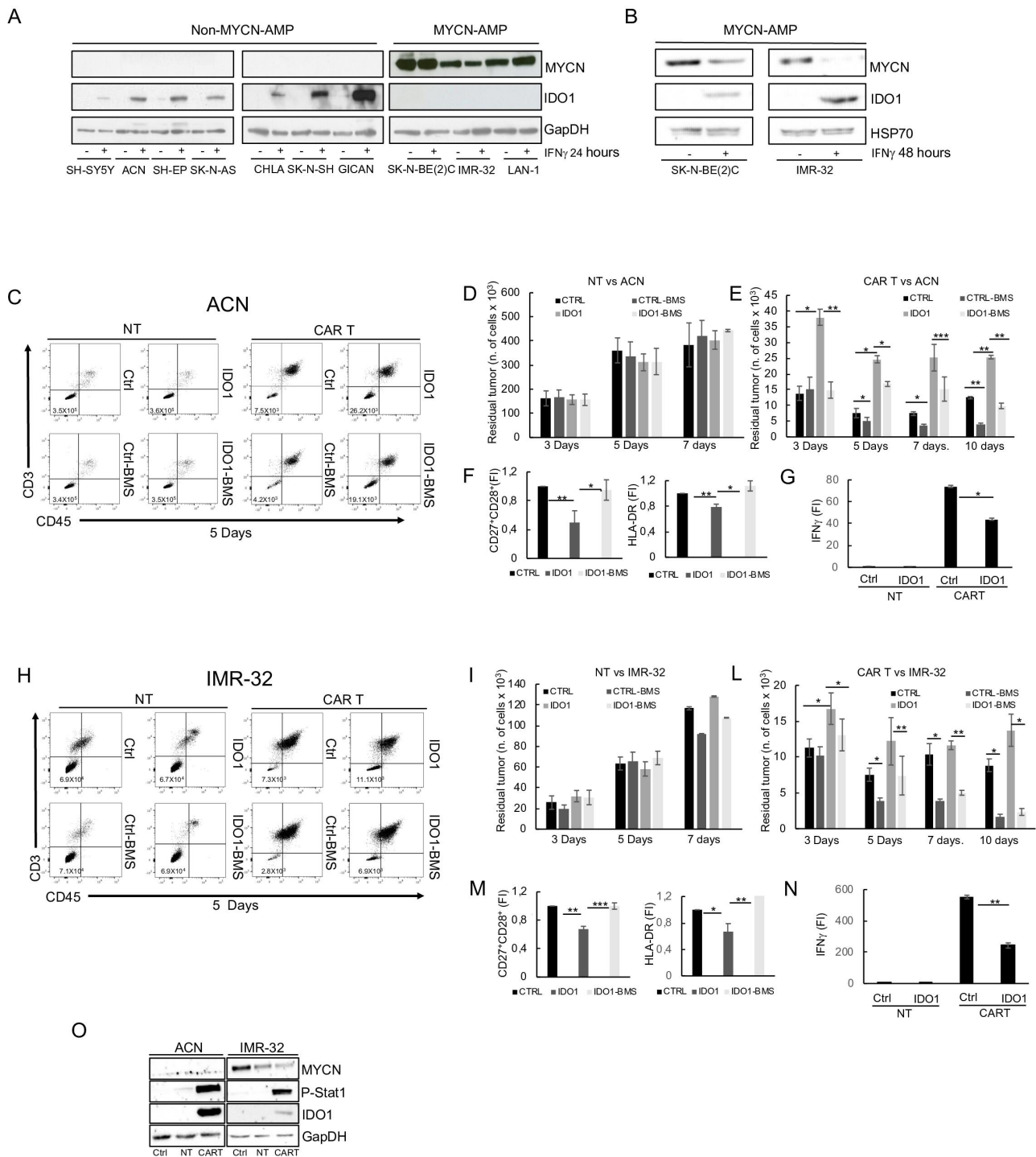


Figure 1 MYCN-dependent IDO1 modulation affects GD2.CAR-T cells efficacy. WB analysis of non-MYCN amplified NB cell lines (non-MYCN-AMP) (A) and MYCN-amplified cell lines (MYCN-AMP) (B) for the expression of MYCN and IDO1, on 24 or 48 hours of IFN γ stimulation (100 ng/mL). GAPDH or hsp-70 were used as loading control. (C) Representative plots at day 5 of coculture between either acn Ctrl cell line (Ctrl) or IDO1-overexpressing NB cell line (IDO1) and either nt cells (left) or GD2.CAR T-cells (right), without IDO1 inhibitor BMS-986205 (upper panels) and with BMS-986205 (lower panels, Ctrl-BMS and IDO1-BMS). (D, E) residual tumor cells were identified as CD3-CD45- population, after 7AAD-positive cells exclusion, and counted through the addition of counting beads at day 3, 5, 7 and 10. Data are expressed as mean \pm SEM (F). Evaluation of CAR T-cells activity in coculture with acn Ctrl, IDO1 and IDO1-BMS at day 5, through the measure of the intensity mean values (FI) of CD27+CD28+ (left) and HLA-DR (right) in the CD3+CD45+ population. (G) ELISA assay for IFN γ quantification performed in the supernatants of 24-hours coculture between either acn Ctrl or IDO1 and either nt or GD2.CAR T-cells. (H, I, L-N) GD2.CAR-T cells coculture with IMR-32 Ctrl, Ctrl-BMS, IDO1 and IDO1-BMS performed as in C-G. (O) WB analysis of IDO1 and P-Stat1 expression in acn and IMR32 after coculture with either nt or CAR-T cells, compared with parental cell lines alone as negative control (Ctrl). GAPDH antibody was used as loading control. CAR, chimeric antigen receptor; IDO1, indoleamine-pyrrole 2,3-dioxygenase 1; IFN γ , interferon- γ ; NB, neuroblastoma; SEM, SE of the mean. * p <0.05; ** p <0.01; *** p <0.001

CAR T-cell killing activity, maintaining their proliferative status. The presence of IDO1-inhibitor in the coculture counteracts the mechanism of resistance due to IDO1 induction, as shown by the low amount of residual live tumor cells (figure 1E,L). To verify whether this mechanism was due to the induction of IDO1 expression by CAR T-cell-produced IFN γ , we analyzed IDO1 expression in the targeted tumor cell lines. We demonstrated that IDO1 is induced in both ACN and IMR-32 Ctrl cells in coculture with GD2.CAR T-cells, but not in coculture with NT cells (figure 1O). The HPLC analysis for the detection of Kyns in the supernatants of each coculture conditions revealed that CAR T-produced IFN γ is able to induce IDO1 enzymatic activity also in Ctrl conditions although with different kinetics in the two NB lines (online supplemental figure S1F,H). Furthermore, also the coculture with NT cells is able to induce the production of Kyns with a lower extent when compared with coculture with CAR T-cell, although the low level of IDO1 protein is not detectable by western blot (online supplemental figure S1E,G). In the same conditions, STAT1 resulted highly phosphorylated, as direct effect of the presence of IFN γ in the supernatant of CAR T-cells.¹⁵ This mechanism seems to be favored by IFN γ -dependent MYCN down-modulation (figure 1O).¹⁴ This finding corroborates the hypothesis of the existence of a loop through which the IDO1-dependent resistance of NB cells to GD2.CAR T-cell treatment is further increased by the IFN γ produced by these effector cells through the induction of IDO1 expression in the targeted tumor.

IDO1-dependent resistance of NB cell lines to NK-cell-mediated killing activity is due to NK-produced IFN γ

In order to investigate other strategies capable to induce antitumor activity, we focused our attention on NK cells, which have already been used in the treatment of several diseases, including NB.^{16,17} However, so far, an efficient NK-cell-mediated destruction of NB could not be demonstrated, suggesting the existence of mechanisms allowing tumor evasion of host immunity.¹⁸ Also in this context, we hypothesized a pivotal role for IDO1. In order to validate the role exerted by IDO1 on NK-cell activity, we performed long-term coculture experiments. NK cells obtained from healthy donors were cocultured with K562, as positive control, and ACN-Ctrl or ACN-IDO1 either in the presence or in the absence of IDO1 inhibitor, evaluating the NK-cell activation status by the mean of fluorescence of CD16 (dull), CD25, CD69 and NKp44 markers. K562 cells were killed already after 24 hours of coculture (online supplemental figure S2A,B). The cytofluorimetric analysis showed that NK cells targeting Ctrl cells were highly activated when compared with NK cells in contact with ACN-IDO1 (CD16 p=0.009; CD25 p=0.036; CD69 p=0.043; NKp44 p=0.001) and IMR-32-IDO1 (CD16 p=0.018; CD25 p=0.005; CD69 p=0.000021; NKp44 p=0.0008) cells. We demonstrated that the addition of IDO1 inhibitor in cocultures partially restores the activation status of NK cells when compared with IDO1-overexpressing

condition (ACN: CD16 p=0.039, CD25 p=0.017, CD69 p=0.052, NKp44 p=0.036 IMR-32: CD16 p=0.031, CD25 p=0.023, CD69 p=0.036, NKp44 p=0.03) (figure 2A). The killing activity of NK cells in each culture condition was evaluated analyzing apoptosis of the targeted tumor cells at different time points. Both ACN-IDO1 and IMR-32-IDO1 clones showed a lower level of apoptosis when compared with ACN and IMR-32-Ctrl on 24 hours of coculture, as measured by Annexin V analysis. This effect was partially recovered after 3 days suggesting that ACN and IMR-32-Ctrl cells became resistant to NK killing activity. To support this hypothesis, we added fresh NK cells (2X) in all coculture conditions observing a total rescue of NB cell viability. The addition of IDO1 inhibitor partially restores the susceptibility of NB cells to NK-cells-mediated killing activity (figure 2B,C). Since NK-cells are strong producers of IFN γ , through WB analysis of target cells, we demonstrated that ACN and IMR-32 in both Ctrl or IDO1-overexpressing conditions became endogenously positive for IDO1 expression on 24 and 48 hours, respectively (figure 2D). These findings sustain the evidence of a loop driven by NK-cell produced IFN γ able to induce IDO1 expression in NB cells, which, in turn, become resistant to NK-cell killing activity.

The role of IDO1 is inversely correlated to MYCN expression

For better deconvoluting the relationship existing between IDO1 and MYCN expression, we started modulating the expression of MYCN in TET21/N, an NB-cell line engineered to inhibit MYCN expression on doxycycline (Doxy) addition, this strategy resulting into either the total ablation of MYCN or its overexpression, when needed. As shown in figure 3A, the low amount of IDO1 induced by IFN γ is strongly regulated by doxy-dependent MYCN ablation, already 16 hours after IFN γ stimulation (figure 3A, left) as sustained by Kyns production (figure 3A, right). In order to reproduce these data in two more physiologically representative NB contexts, we inhibited MYCN expression in an MYCN-overexpressing cell line (SK-N-BE(2)C), followed by IFN γ addition (48 hours). We demonstrated that MYCN interference allows the expression of IDO1 when compared with the control condition transfected with scr oligonucleotides at both protein and mRNA levels (figure 3B). On the other hand, the overexpression of MYCN in ACN (non-MYCN-overexpressing cell line), is able to inhibit the protein and mRNA expression of IFN γ -induced IDO1 in NB cells, compared with the control transfected with an empty vector (Ctrl) (figure 3C). In all the experimental conditions, the amount of Kyns produced and released in the supernatants directly correlates with the level of IDO1 protein expression (figure 3B,C, lower panels). In the same experimental conditions, we evaluated the effect of MYCN-dependent IDO1 modulation on a NK-cell mediated immune escape mechanism controlled by the enzyme. Notably, we observed that the interference with MYCN expression in MYCN-overexpressing cell lines (SK-N-BE(2)C), inducing IDO1 expression on IFN γ addition,

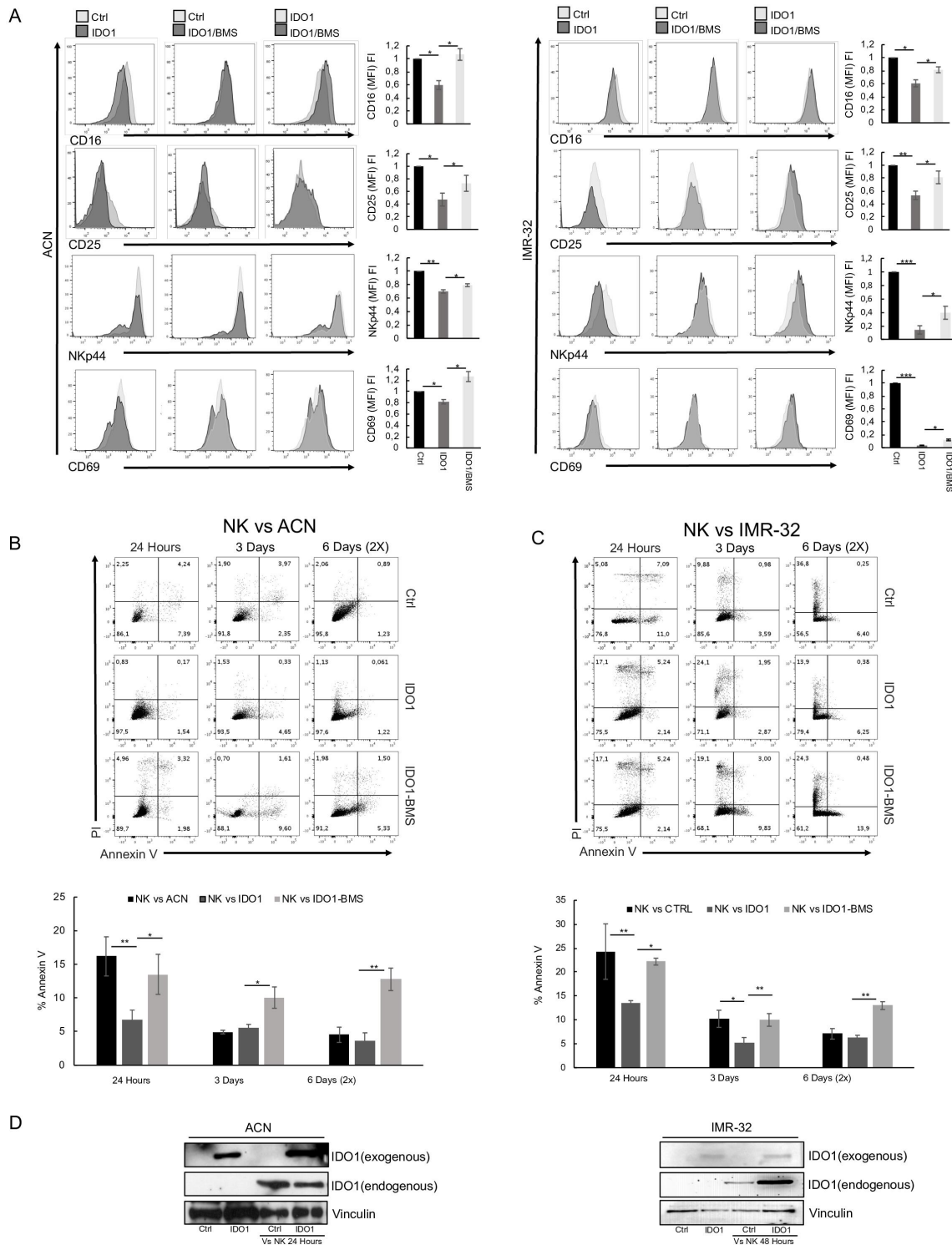


Figure 2 IDO1 expression induced by NK-produced IFN γ results in NB cells resistance to NK killing activity. (A) Representative histograms of CD16, CD25, CD69 and NKp44 mean of fluorescence intensity (MFI) in natural killer (NK) cells on 3 days of coculture with acn (left) and IMR-32 (right) in Ctrl, IDO1 and IDO1-BMS conditions. Graphs (on the right of each panel) show the mean values (\pm SEM) (fold of induction, FI). (B) representative plots of FACS analysis of annexin V expression in CD45-population to evaluate apoptosis of acn in Ctrl, IDO1 and IDO1-BMS conditions after 24 hours, 3 days and 6 days of coculture with NK cells. Notably, at day 3, fresh NK cells were added and the apoptotic level was re-evaluated at day 6 (2X). The lower graph summarizes annexin V expression in acn Ctrl, IDO1 and IDO1/BMS conditions, after coculture with NK cells, at 24 hours, 3 and 6 days (2x). (C) IMR-32 coculture experiments, performed as in B. (D) WB analysis of IDO1 expression (endogenous) after 24 hours, in acn (left), and 48 hours, in IMR-32 (right), of coculture with NK cells. Cell lysates from Ctrl and IDO1 conditions were loaded as control. exogenous IDO1 expression was validated and vinculin antibody was used for loading control. IDO1, indoleamine-pyrrole 2,3-dioxygenase1; IFN γ , interferon- γ ; NB, neuroblastoma; SEM, SE of the mean. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

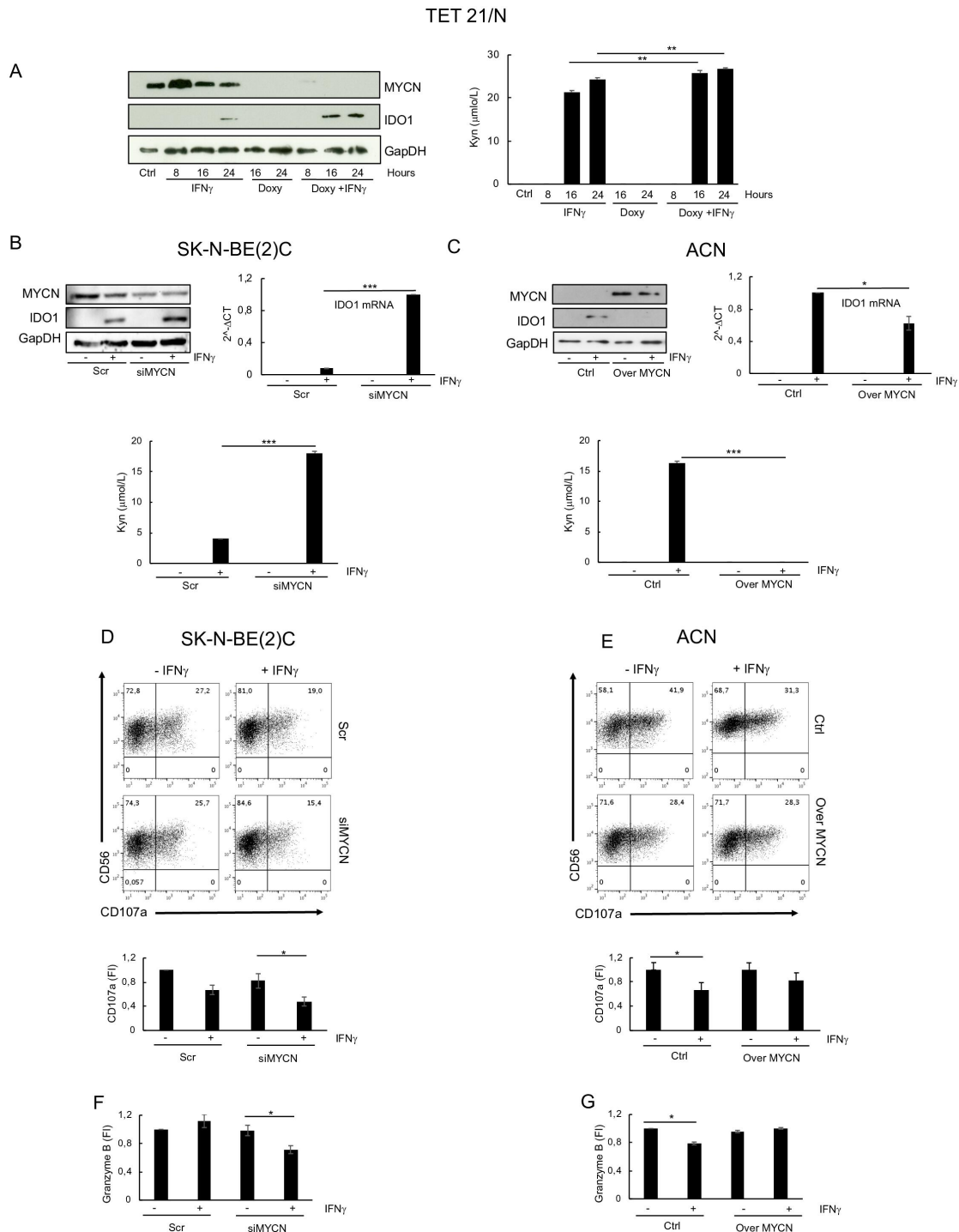


Figure 3 MYCN modulation regulates IDO1 protein and mRNA and affects IDO1-dependent NK-mediated degranulation activity. (A) WB analysis of MYCN and IDO1 expression in TET21/N cell line after treatment with doxycycline (10 ng/mL at different time points) and IFN γ , either alone or in combination. GAPDH antibody was used for loading control. The graph (right) represents Kyns concentration, detected by HPLC, in the same experimental conditions. (B) WB analysis of MYCN and IDO1 expression in SK-N- BE(2)C cell line silenced for MYCN expression through RNA interference (left). Expression of IDO1 mRNA detected by Real time PCR in the same conditions (right). The lower graph shows Kyns concentration detected by HPLC. (C) Detection of MYCN and IDO1 proteins through WB analysis in acn cell line overexpressing the MYCN protein (left). IDO1 mRNA level (right) and Kyns production (lower) in the same experimental conditions. (D) Representative plots (upper panels) and mean values (lower graph) of degranulation activity of NK cells after coculture with SK-N-BE(2)C in the same condition as in B, detected by measuring CD107a in CD45+56+ population through FACS analysis. (E) Representative plots (upper panels) and mean values (lower panels) of degranulation activity of NK cells after coculture with acn, in the same condition as in C, showed as expression of CD107a on NK cells. (F, G) Granzyme B evaluation in the same experimental conditions as in E, in CD45+CD56+ cells after 7AAD-positive cells exclusion. IDO1, indoleamine-pyrrole 2,3-dioxygenase1; HPLC, high-performance liquid chromatography; IFN γ , interferon- γ ; Kyns, kynurenines; NK, natural killer. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

resulted in the reduction of NK-cell degranulation activity against the tumor, as demonstrated by the lower CD107a expression on NK-cells (figure 3D). On the other hand, in ACN cell line, the overexpression of MYCN, repressing IDO1 expression and activity although stimulated with IFN γ , did not protect the tumor from the NK-cell killing activity (figure 3E). To confirm these data in the same experimental conditions, we measured the NK-cell intracytoplasmic accumulation of GranzymeB, showed as intensity of fluorescence (fold of induction). We demonstrated that the accumulation of GranzymeB follows the same trend of CD107a expression (figure 3F,G). These data demonstrate how the expression of MYCN is able to regulate the NK-cell-mediated immune response through the control of IDO1 expression.

MYCN is a transcriptional repressor of IDO1 expression

In order to further investigate the relationship existing between MYCN and IDO1, we analyzed NB tissue samples collected at the time of diagnosis from a cohort of patients affected by advanced NB (table 1, figure 4). Immunohistochemistry assay revealed that the expression of IDO1 was negative in MYCN-overexpressing samples and positive in MYCN-negative NB samples, confirming an inverse correlation between the two molecules (figure 4A). The same trend of inverse correlation was demonstrated through the quantification of *IDO1* and *MYCN* mRNA by Real time PCR in the available NB samples ($R=-0.7857$; $p=2.7\times 10^{-2}$) (figure 4B). In addition, we extended the analysis querying a public data base (r2.amc.nl/; Tumor Neuroblastoma–Kocak–649-RPM-ag44kcwof) of 649 NB samples confirming a statistically significant ($R=0.3334$; $p=2.12\times 10^{-18}$) inverse correlation between MYCN and IDO1 (GEO:gse45547) (figure 4C). These data prompted us to study the promoter sequence of IDO1 to find the e-box sequences available to MYCN binding assuming that MYCN is able to transcriptionally control IDO1 expression. The CATGTG e-box sequence (from -553 to -560) was utilized for ChIP assay. The experiments were performed in non-MYCN-overexpressing NB cell lines (SH-SY5Y and ACN) and in an MYCN-overexpressing cell line (SK-N-BE(2)C) with or without IFN γ stimulation, to verify the capability of MYCN to bind the IDO1 promoter and to verify whether IFN γ treatment could affect the enrichment of the transcription factor on the promoter sequence. In SH-SY5Y and ACN cell lines, we were able to validate our hypothesis. Indeed, MYCN binds IDO1 promoter and its enrichment is reduced on 24 hours of IFN γ exposure, but it is not modified in the MYCN-overexpressing cell line SK-N-BE(2)C (figure 4D). Prolonging IFN γ stimulation until 48 hours, we observed a reduced amount of MYCN on IDO1 promoter in IMR-32 cell line, an MYCN-overexpressing cell line. In addition, we observed an enrichment of phospho-STAT1, a known transcription factor responsible of the IFN γ -dependent IDO1 transcription (figure 4E).¹⁵ To validate the binding specificity of MYCN to the IDO1 promoter, we mutated the e-box sequence. We used a homologous murine IDO1

promoter containing two e-box sequences (from -411 to -405; from -721 to -715) that were mutated and used for a luciferase assay performed in HEK 293T cells to perform cotransfection experiments. We observed that the repression of the transcriptional activity exerted by MYCN is lost when the e-box sequences were mutated (figure 4F). We confirmed this finding also in the SK-N-BE(2)C cell line, where MYCN is endogenously active (figure 4G).

DISCUSSION

The immune suppressive tumor microenvironment in solid tumors is one of the most important factors impairing the efficacy of immunotherapy. Since insights into the immune evasion mechanisms promoted by the tumor microenvironment are crucial for improving adoptive cell therapies, including NK and CAR T-cells, we hypothesized a pivotal role of IDO1 in GD2-redirection CAR T-cell therapy and NK cells for the treatment of NB. The rationale at the basis of this hypothesis is the known IDO1 ability of impairing T-cell and NK-cell activity, through tryptophan consumption and the accumulation of the immune suppressive catabolites, Kyns. In our in vitro experiments, we were able to reproduce the response of NK and CAR T-cells, demonstrating that the IFN γ released by these cell populations is able to induce IDO1 expression also in MYCN-amplified cell lines, the more aggressive NB tumors. We also propose a negative loop through which in both NB tumor contexts (MYCN-amplified and non-MYCN amplified), IDO1-dependent immune evasion mechanisms are induced and sustained by NK and CAR T-cells IFN γ production. A more important role for IFN γ in shaping the immunological phenotype of developing tumors via escape mechanisms is recently becoming evident.¹⁹ These immune functions provide selective pressure promoting the outgrowth of tumor cells that have acquired immune evasive capacities. Once formed, tumors may become susceptible to the influence of other components of the immune system, among which secreted molecules such as IFN γ or interleukin-12 and cellular component, including cancer-associated fibroblasts and/or tumor-associated macrophages. In the cancer immunoediting mechanism, IDO1 has been reported to play an important immunomodulating role in the escape phase: it is strongly susceptible to IFN γ , increasing the immune-suppressive polarization within the tumor microenvironment. The expression and function of IDO1 have not been previously investigated in NB and our findings show an important role for IDO1 in both MYCN-amplified and non-MYCN-amplified NB, but with difference in the timing of induction of IDO1 activation. In accordance with recent findings,²⁰ we demonstrated that the expression of IDO1 is more susceptible to IFN γ stimulation when MYCN is absent, but can be induced also in MYCN amplified cells when the duration of stimulation and amount of produced cytokines are higher. We gained insight into the molecular mechanism showing that MYCN transcriptionally represses IDO1 expression

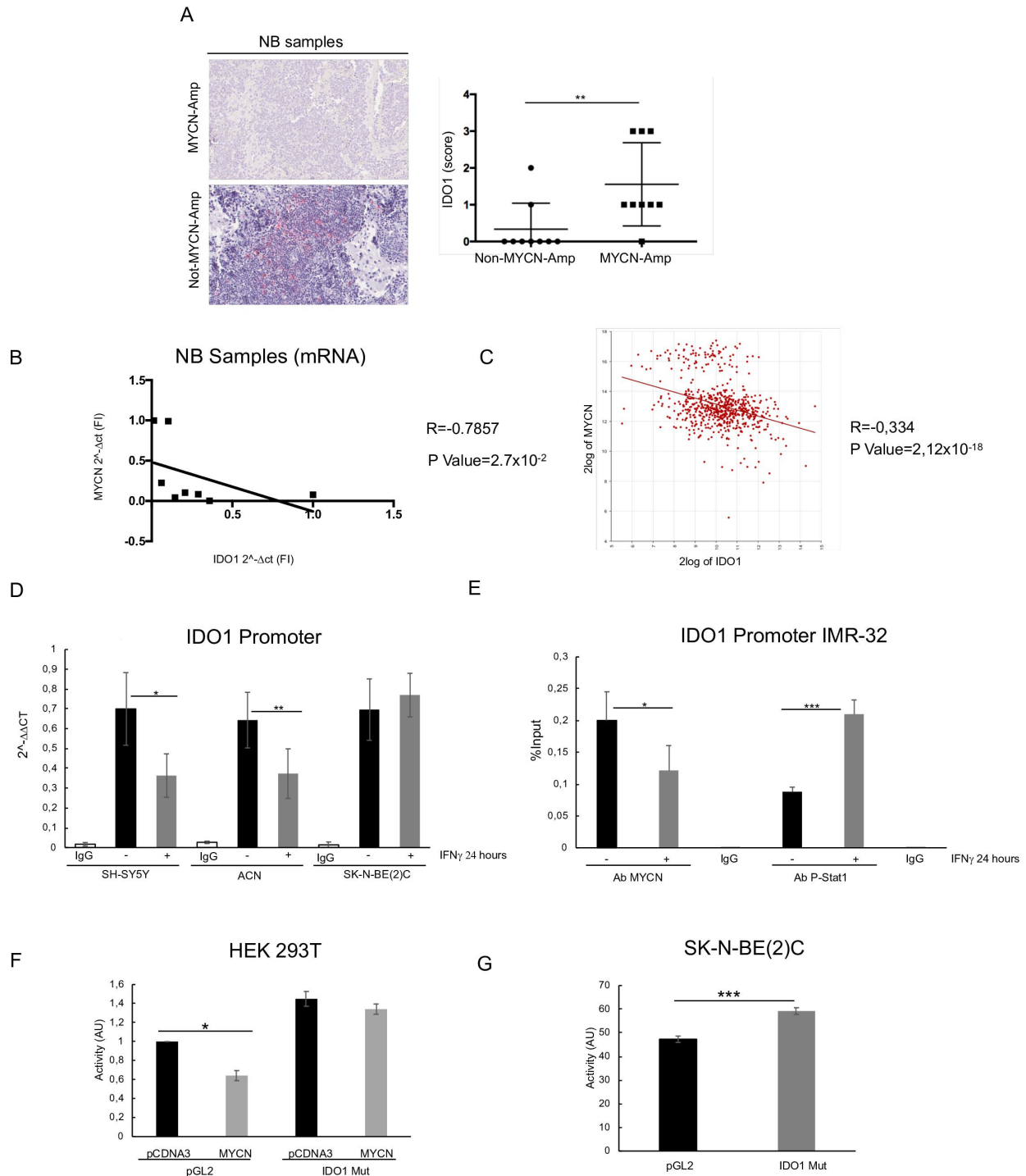


Figure 4 MYCN negatively regulates IDO1 expression. (A) Representative immunohistochemistry (IHC) analysis of IDO1 expression in NB tissues obtained from patients with or without MYCN amplification at time of diagnosis (left) (MYCN-AMP and non-MYCN-AMP, respectively). red staining for IDO1; patients characteristics at diagnosis are summarized in the table on the left. The graph on the right quantifies IDO1 protein expression, evaluated in MYCN-AMP and non-MYCN-AMP stage IV human NB tissue samples by IHC, using an intensity score (% of positive cells) of 0 to 3. (B) Correlation between *IDO1* and *MYCN* mRNA expression extracted from NB samples analyzed by real time PCR. (C) correlation between *IDO1* and *MYCN* mRNA expression in a public dataset (r2.amc.nl/; tumor Neuroblastoma–Kocak–649-RPM-ag44kcwof) of 649 Nb samples. (D) Chromatin immunoprecipitation (ChIP) assay of MYCN on IDO1 promoter in SH-SY5Y, ACN and SK-N-BE(2)C cell lines with or without 24 hours of IFN γ treatment. (E) ChIP assay of MYCN and P-Stat1 on IDO1 promoter in IMR-32 cell line with or without 48 hours of IFN γ stimulation. (F) luciferase assay performed in HEK 293T cell line cotransfected with pcDNA3-MYCN overexpressing plasmid and pGL2 plasmid carrying IDO1 promoter portion containing mutated E-box sequences. pcDNA3-empty vector (pcDNA3) and pGL2 empty vector (WT) were used as control. (G) Luciferase assay performed in SK-N-BE(2)C (MYCN-AMP) cotransfected with pGL2 or pGL2-IDO1 Mut vector. IDO1, indoleamine-pyrrole 2,3-dioxygenase1; IFN γ , interferon- γ ; NB, neuroblastoma. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

specifically binding the e-box sequence located on the IDO1 promoter. Thus, MYCN downregulation, due to the high amount of available IFN γ in the tumor microenvironment, favors the reduction of MYCN repressive activity. In stage IV NB tissues analyzed at the time of diagnosis, we confirmed the data observed in NB cell lines showing an inverse correlation between the expression of MYCN and IDO1. Nevertheless, the potential impact of immunosuppressive factors like IDO1, abundant in the tumor microenvironment has not yet been investigated as an opportunity to further improve the direct antitumor activity and to enhance the immune-stimulatory potential of more recent antitumor approaches with CAR-NK cells. We hypothesize that the combination of IDO1 inhibitors with CAR-T cell/NK cell therapy could preventively overcome the negative effect played by the release of high amount of IFN γ in the tumor microenvironment. For these reasons, we tested a selective IDO1 inhibitor (BMS-986205) that has been evaluated in early stage clinical trials²¹ and the results obtained in our in vitro study in combination with NK and GD2.CAR T-cell administration are promising. Based on the evidence of a strong involvement of the IFN γ pathway in response to NK or GD2.CAR T-cell therapy, we can even hypothesize a combinatory treatment with an anti-IFN γ monoclonal antibody such as Emapalumab,²² recently approved by the US Food and Drug Administration, after the exclusion of a possible interference of this compound with CAR T or NK-cell cytotoxicity, in order to obtain tumor control in patients with refractory or relapsed NB and improve outcome for these patients.

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