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TGFβR1 Blockade with Galunisertib (LY2157299) Enhances Anti-Neuroblastoma Activity of Anti-GD2 Antibody Dinutuximab (ch14.18) with Natural Killer Cells

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Abstract

Purpose—Immunotherapy of high-risk neuroblastoma using the anti-GD2 antibody dinutuximab induces antibody-dependent cell-mediated cytotoxicity (ADCC). Galunisertib, an inhibitor of TGFβR1, was examined for its ability to enhance the efficacy of dinutuximab in combination with human *ex vivo* activated NK (aNK) cells against neuroblastoma.

Experimental Design—*TGFB1* and *TGFBR1* mRNA expression was determined for 249 primary neuroblastoma tumors by microarray analysis. The ability of galunisertib to inhibit SMAD activity induced by neuroblastoma patient blood and bone marrow plasmas in neuroblastoma cells was tested. The impact of galunisertib on TGFβ1-induced inhibition of aNK cytotoxicity and ADCC *in vitro* and on anti-neuroblastoma activity in NSG mice was determined.

Results—Neuroblastomas express *TGFB1* and *TGFBR1* mRNA. Galunisertib suppressed SMAD activation in neuroblastoma cells induced by exogenous TGFβ1 or by patient blood and bone marrow plasma, and suppressed SMAD2 phosphorylation in human neuroblastoma cells growing in NSG mice. In NK cells treated *in vitro* with exogenous TGFβ1, galunisertib suppressed SMAD2 phosphorylation and restored the expression of DNAM-1, NKp30, and NKG2D cytotoxicity receptors, TRAIL death ligand, the release of perforin and granzyme A, and the direct cytotoxicity and ADCC of aNK cells against NB cells. Addition of galunisertib to adoptive cell therapy with aNK cells plus dinutuximab reduced tumor growth and increased survival of mice injected with two neuroblastoma cell lines or a patient-derived xenograft.

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Conclusion—Galunisertib suppresses activation of SMAD2 in neuroblastomas and aNK cells, restores NK cytotoxic mechanisms, and increases the efficacy of dinutuximab with aNK cells against neuroblastoma tumors.

Keywords

immunotherapy; TGFβR1 inhibitor; dinutuximab; galunisertib; adoptive cell therapy

Introduction

High-risk neuroblastoma (NB) accounts for a disproportionate burden of childhood cancer morbidity and mortality, representing 7% of childhood malignancies but accounting for 15% of all childhood cancer deaths (1). Although event-free survival for patients with high-risk NB has improved with use of the anti-disialoganglioside (anti-GD2) chimeric monoclonal antibody (mAb) dinutuximab (ch14.18) plus IL-2 and GM-CSF immunotherapy, 40% of patients still relapse during or after immunotherapy (2). The reasons for failure of this immunotherapy are not known.

Increasing evidence indicates that the tumor microenvironment (TME) supports tumor growth and survival and regulates immune responses (3). Within the TME, the transforming growth factor beta (TGFβ) family has an important role in tumor immune evasion, leading to tumor progression and metastasis (4, 5). The clinical importance of TGFβ1 in suppressing NK cells is indicated by two studies of patients with breast or squamous cell carcinoma (6, 7). Among the TGFβ family, TGFβ1 is the most commonly up-regulated in tumor cells (5, 8). This ligand binds to TGFβ receptor type I (TGFβR1) which results in its dimerization to TGFβ receptor type II (TGFβR2). This heterodimer then phosphorylates SMAD2 and SMAD3 which complex with SMAD4 to modulate transcription of downstream genes (9, 10). TGFβ1 is known to inhibit the interferon-γ (IFNγ) production, proliferation, and function of natural killer (NK) cells, an important type of immune effector cell expressing the antibody receptor FcγRIIIa (CD16) and mediating ADCC against NB cells (11–13). The NB TME can include transforming growth factor beta 1 (TGFβ1), and higher than median TGFβ1 in *MYCN* non-amplified NB patient tumors correlates with worse event-free survival (14).

Approaches for inhibiting TGFβ-induced signaling include targeting ligand-receptor interactions and intracellular signaling (15). Galunisertib (LY2157299 monohydrate) is a recently developed small-molecule inhibitor of TGFβR1. Galunisertib binds antagonistically to TGFβR1, preventing the intracellular phosphorylation of SMAD2 and SMAD3 (16–18). This agent has demonstrated anti-tumor activity in combination with paclitaxel or sorafenib in xenograft models of breast or hepatocellular carcinoma (17–19). Phase I studies have demonstrated that galunisertib is safe in adult patients with advanced solid tumors (20, 21). However, it is unknown whether galunisertib can augment anti-GD2 antibody therapy or the anti-tumor cytotoxicity of NK cells propagated and activated *ex vivo* with K562.mbIL21 artificial antigen presenting cells (22–24) which we and others are using to generate activated NK (aNK) cells for evaluation in clinical trials of adoptive cell therapy (ClinicalTrials.gov identifier # NCT01787474 and NCT02573896).

We demonstrate that galunisertib significantly restores the cytotoxicity of aNK cells following their inhibition by TGFβ1 *in vitro* and enhances the combination of dinutuximab and aNK cell immunotherapy against NB cell lines and a patient-derived xenograft (PDX) growing in kidneys of NOD-scid gamma (NSG) mice. These findings support the clinical testing of galunisertib in combination with dinutuximab for the immunotherapy of neuroblastoma.

Materials and Methods

Neuroblastoma cells, patient specimens, aNK cells, and reagents

CHLA-255 and CHLA-136 NB cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal bovine serum (FBS). CHLA-255-Fluc and CHLA-136-Fluc cells were transduced with the firefly luciferase (*Fluc*) gene using a lentivirus vector (24). COG-N-415x patient-derived xenograft NB cells expressing mutated ALK (F1174L) and amplified *MYCN* gene were kindly provided by Dr. C. Patrick Reynolds. The correct identity of cells was authenticated using the AmpFLSTR™ Identifiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA). Primary NB tumors were obtained from patients enrolled and consented for Children's Oncology Group (COG) biology and therapeutic protocols. Plasma from whole blood and bone marrow aspirates were obtained from patients with relapsed and refractory neuroblastoma enrolled on the New Approaches to Neuroblastoma Therapy (NANT) Biology Study N2004-05.

NK cells from healthy donors were activated and propagated *ex vivo* using K562.mbIL21 artificial antigen presenting cells (22–24) genetically engineered to express immunostimulatory molecules including CD137 ligand and membrane-bound IL-21 (K562.mbIL21), the latter of which was associated with increased telomere length in cultured NK cells (24). In brief, PBMC were co-incubated at day 0 with irradiated (100 Gy) K562.mbIL21 cells at a ratio of 2:1 in NK cell expansion medium (NKEM) comprised of RPMI1640 and 10% FBS with 50 IU/ml recombinant human IL-2 (PeproTech, Rocky Hill, NJ). On day 7, cultures were replenished with irradiated K562.mbIL21 cells and fresh NKEM. aNK cells were then viably frozen at day 14 in 50% Cryoprotective Medium (Lonza, Walkersville, MD), 25% RPMI-1640, and 25% FBS.

Anti-GD2 chimeric mAb ch14.18/dinutuximab was provided by the National Cancer Institute-Frederick. Human TGFβ1 (R&D Systems, Minneapolis, MN) was reconstituted at 10 μg/ml in sterile 4 mM HCl containing 0.1% BSA. Aliquots were kept at −80 °C and discarded after 3 months. Galunisertib was provided by Eli Lilly and Company (Indianapolis, IN). For *in vivo* experiments, galunisertib was freshly suspended in a formulated vehicle (1% carboxymethylcellulose sodium salt, 0.5% SDS, 0.085% povidone, and 0.05% antifoam Y-30 emulsion) and kept at 4° C for up to one week. Galunisertib was dissolved in DMSO at 10 mM and kept at −20° C as a stock solution for *in vitro* experiments.

Gene expression analysis

Affymetrix Human Exon Array data (manuscript in preparation, see https://ocg.cancer.gov/programs/target/research) of 249 primary NB tumor specimens obtained at diagnosis was normalized by quantile normalization and summarized using robust multichip average (Affymetrix Power Tools software package version 1.12). This dataset includes samples from 219 patients with high-risk (68 with amplified and 151 with non-amplified MYCN) and 30 with low-risk primary tumors. The transcript level data of core probe sets for each sample were averaged based on gene symbol annotations provided by the manufacturer (17,422 unique genes). To identify relative expression of genes in neuroblastomas, the percentile values of *TGFBR1, TGFB1, TGFBR2, TGFB2, TBX21, IFNG*, *NTRK1, and MYCN* were computed from the cumulative distribution function for each sample's gene profile. As an independent dataset, Agilent single-color expression profiles of 478 samples were downloaded from the GEO GSE16716 dataset. Patients with stage 4S disease in this latter dataset (n=62) were excluded from analysis to allow comparison with our Human Exon Array data. Expression profiles from the resulting cohort of 416 tumors from patients with high-risk (n=135), intermediate-risk (n=34), or low-risk (n=247) NB were used to assess expression of *TGFBR1, TGFB1, TGFBR2, TGFB2, TBX21, IFNG*, as well as *NTRK1* and *MYCN* as internal controls. Concordant results were obtained between our NB dataset and the GEO GSE16716 dataset.

Neuroblastoma patient plasma SMAD activity assay

The Cignal lenti SMAD Reporter, purchased from SA Biosciences (Frederick, MD), is comprised of lentiviral particles containing the *firefly luciferase* gene under the control of SMAD transcription response elements. CHLA-255 cells expressing a high level of renilla luciferase (CHLA-255-hRL) were transduced by this SMAD reporter lentivirus and the stable CHLA255hRL-SmadFluc cell line was established by puromycin selection. To validate detection of SMAD activity, 5×10^4 CHLA255hRL-SMADFluc cells were seeded into each well of a 96-well plate overnight and then cultured with various doses of galunisertib with and without recombinant human TGFβ1 (10 ng/ml). Beetle luciferin (7 μl, 5 mg/ml) (Promega, Madison, WI) was added to each well for 5 minutes in the dark. Activation of SMAD transcription response elements by TGFβ1 or patient plasma resulted in expression of firefly luciferase which when activated by luciferin was measured as luminescence using the GloMax Multi-Detection System (Promega, Sunnydale, CA, model #E8032). To determine whether NB patient blood or bone marrow plasma could activate SMAD2, samples from 17 high-risk NB patients (1:10 dilution with 1% FBS-IMDM) were added into wells with or without galunisertib for 18 or 36 hours.

Western blotting for pSMAD2

Cell pellets were lysed with 1× RIPA buffer including 1× Halt protease and phosphatase inhibitor cocktail (Thermo Fisher, Grand Island, NY). Proteins (40 μg) were analyzed on a Novex 4–20% Tris-Glycine gel (Life Technologies, Carlsbad, CA) using Novex Tris-Glycine SDS running buffer (Invitrogen/Thermo Fisher). Proteins were transferred to a pure nitrocellulose membrane (Bio-Rad, Hercules, CA) in Novex Tris-Glycine Transfer buffer (Invitrogen/Thermo Fisher) plus 20% methanol. The following antibodies were used: rabbit

polyclonal anti-phospho-SMAD2 (Ser465/467) antibody (Cell Signaling, Danvers, MA); rabbit polyclonal anti-SMAD2/3 antibody (Cell Signaling); rabbit polyclonal anti-β-Actin (N-21) antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA). Antibody-reactive proteins were detected with HRP-labeled goat anti-rabbit IgG (Bio-Rad) and ECL substrate (Denville Scientific, Metuchen, NJ). NewBlot Nitro Stripping Buffer (Li-Cor, Lincoln, NB) was used to strip the anti-phospho-SMAD2 antibody before addition of antibodies for detection of other proteins.

Immunohistochemistry staining of phospho-SMAD2 in human NB tumors formed in NSG mice

Tumor tissues from kidneys of NSG mice in different treatment groups were placed into formalin (Fisher Scientific Company LLC, Kalamazoo, MI) for 2 days, and then into 75% ethanol at 4 °C. Formalin-fixed, paraffin-embedded sections in Leica BOND-MAX™ (Leica Microsystems Inc., Bannockburn, IL) were heated for 30 minutes in Bond™ Epitope Retrieval Solution 2 (No. AR9640; Leica Biosystems Newcastle Ltd, Benton Ln, Newcastle Upon Tyne, UK). Sections then were incubated for 2 hours at room temperature with anti-phospho-SMAD2 antibody (Ser465/467) (rabbit polyclonal antibody, EMD Millipore) at a dilution of 1:500 followed by a poly-horseradish peroxidase-conjugated goat anti-rabbit antibody (Leica, Buffalo Grove, IL). Kidney tissue of a normal NSG mouse was stained as a negative control.

Flow cytometry

Cell surface staining was performed as previously described (24, 25). Briefly, cells were washed twice in FACS buffer (PBS with 0.1% NaN3 and 0.1% bovine serum albumin) and centrifuged for 10 minutes at 400 × g. Antibodies listed in Supplemental Table 1 were added in the dark at 4°C using concentrations previously determined by titration. Isotype-matched irrelevant mAbs were used to define non-specific staining. Cells were incubated at 4 °C for 90 minutes and washed twice in FACS buffer. Dead cells were excluded according to positivity for DAPI. Cell aggregates were excluded from analysis by gating out events exhibiting high forward and side light scatter. Flow cytometry analysis was performed using a BD LSR II flow cytometer with DIVA software (BD Biosciences, San Jose, CA) and FCS Express software (DeNovo Software, Los Angeles, CA). The Stain Index was calculated using values of median fluorescence intensity (MFI) and robust standard deviation (rSD) as follows: (MFI of viable aNK cells stained with specific antibody - MFI of viable aNK cells stained with an isotype-matched irrelevant antibody) / (2 × rSD of the isotype control).

NK cytotoxicity assay

Frozen K562.mbIL21-expanded aNK cells (24) were cultured in 10% FBS-RPMI1640 with 100 U/ml of IL-2 for 24 hours, and then were pre-treated in individual wells of 6-well plates for 48 hours with (a) 5 μM galunisertib, (b) 10 ng/ml human TGFβ1, (c) galunisertib for 30 minutes followed by TGFβ1, or (d) 24 hours with TGFβ1 followed by treatment with galunisertib for an additional 24 hours. For the aNK cytotoxicity assay, NB cell lines (CHLA-255-Fluc and CHLA-136-Fluc) were labeled with calcein-AM for 30 minutes and washed once (26). 1×10^4 labeled CHLA-255-Fluc or CHLA-136-Fluc were seeded into individual wells of a 96-well plate, and 5×10^3 aNK cells pre-treated as described above were

added to NB cells at a ratio of 1:2. Dinutuximab was added to the indicated wells at a concentration of 1 μg/ml for the ADCC assay. The plate was incubated at 37° C in 5% CO_2 for six hours, and surviving tumor cells were quantified as calcein-containing cells using a digital imaging microscopy system (DimScan) (26).

Luminex assay

Granzyme A and perforin were measured using a custom-plex bead array from EMD Millipore (Billerica, MA) following the manufacturer's instructions with a Luminex-200 instrument (Luminex Corporation, Austin, TX) as described previously (24).

Intra-renal neuroblastoma model and treatment of NSG mice

All *in vivo* experiments utilized 4–6 week old male and female NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice, which were bred in-house, genotyped for colony maintenance, and housed in a pathogen-free environment. Mice were implanted using our previously described intra-renal xenograft model of NB (27). Briefly, 1×10^6 cells from NB cell lines or PDX cells in 100 μl PBS were injected in the left kidney of mice. Tumor growth in mice injected with luciferase-expressing cell lines was assessed by bioluminescence imaging using a Xenogen IVIS® 100 instrument (IVIS Lumina XR System, Caliper Life Sciences, Hopkinton, MA). All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Usage Committee of Children's Hospital Los Angeles.

Mice in groups receiving aNK cells and dinutuximab were intravenously injected with 1×10^7 aNK cells (immediately after thawing) plus 15 μg mAb twice per week per mouse for four weeks, as described previously (24). IL-2 (2 μg/mouse) and IL-15 (4.9 μg/mouse) were injected intraperitoneally at the same time as dinutuximab and aNK cells. Mice in galunisertib treatment groups were gavaged BID with 75 mg/kg of galunisertib (16) suspended in formulated vehicle as described above. Mice not receiving galunisertib were gavaged BID with the formulated vehicle solution alone.

Statistical Analysis

Data were analyzed using Stata statistical software (version 11.2) and are represented as means ± standard deviation unless otherwise stated. ANOVA was performed to determine the significance of observed differences. Analysis of tumor bioluminescence data transformed the photon flux for each mouse using the log (flux + 1) transformation and then area under the growth curve (AUC) was calculated. The AUC values were used in the analysis to compare differences in tumor photon flux between treatment groups. Mouse survival time was defined as the length of time (in days) from the tumor injection date until the end of the study or time of sacrifice due to disease progression. Censored normal regression was utilized to examine whether any difference in survival time existed due to treatments. The censored Wilcoxon test was used to examine the difference in the survival curves among the different treatment groups. A p value of < 0.05 was considered statistically significant.

Results

TGFB1 and *TGFBR1* genes are expressed by high-risk human NB tumors and TGFβ activity is present in blood and bone marrow plasma from NB patients

Exon gene expression in 249 primary NB tumors was analyzed using Affymetrix human exon arrays (17,422 genes). *TGFBR1* and *TGFBR2* genes were expressed at high levels relative to all genes analyzed, and the median expression of *TGFB1* was above the 30th percentile (Fig. 1). *MYCN* expression was at the 100th percentile in the MYCN-amplified group of tumors, and *NTRK1* expression was high in the low-risk group, as expected. mRNA expression was at less than the 5th percentile for *interferon-γ* (*IFNG*) and above the 30th percentile for its controlling transcriptional regulator *TBX21* (the human ortholog of the mouse *Tbet/Tbx21* gene). Median percentile expression of *TGFB1* and *TGFB2* and high percentile expression of their receptors with low expression of *IFNG* suggests activity of the TGFβ signaling pathway in NBs.

Next, we used the NB SMAD reporter cell line CHLA-255hRL-SmadFluc to determine whether blood and bone marrow plasmas from NB patients activate the SMAD pathway and whether this activation could be suppressed by the TGFβR1 inhibitor, galunisertib. First, we performed serial 5-fold dilutions of galunisertib and found that a clinically achievable dose of 5 μM (1.8 μg/ml) (21, 28) gave maximum inhibition of SMAD activity induced by 10 ng/ml of TGFβ1 without cell toxicity (Fig. 1B). Then, using 5 μM galunisertib and the CHLA255hRL-SmadFluc reporter cell line, we observed that SMAD activity was induced by blood and bone marrow plasma obtained from 17 NB patients (Fig. 1C) and that this activity could be inhibited by galunisertib. These results suggest that TGFβ1 and/or TGFβ2 are present in blood and bone marrow from NB patients.

Galunisertib decreases suppression of aNK cells by TGFβ1

Having established that galunisertib reduces SMAD activity in NB cells, we examined whether it might reduce the effects of TGFβ1 on aNK cells. *Ex vivo*-propagated aNK cells were cultured with galunisertib (5 μM), TGFβ1 (15 ng/ml), or the combination of galunisertib with TGFβ1 for 18 hours and lysates were subjected to immunoblot assay to detect phospho-SMAD2, total SMAD2, and β-actin. Fig. 2A shows strong induction of SMAD2 phosphorylation in aNK cells by TGFβ1 and strong inhibition of this phosphorylation by galunisertib.

TGFβ1 also decreased aNK cell expression of the cytotoxicity receptors DNAM-1, NKp30, and NKG2D (Fig. 2B), as reported by others for human NK cells cultured for two to seven days with IL-2 or IL-15 (3, 29). TGFβ1 also decreased aNK cell expression of the membrane-bound form of the death ligand TRAIL (Fig. 2B), which we have previously shown to supplement the cytotoxicity of aNK cells against NB cells (30). Our experiments did not demonstrate that TGFβ1 affected expression of NKp46, NKG2A, NKG2C, or CD16 (data not shown). The approximately 3-fold decrease in the Stain Index for DNAM-1, NKp30, NKG2D, and TRAIL induced by TGFβ1 was inhibited by galunisertib when added either 30 minutes before or 24 hours after commencing incubation with TGFβ1 (Fig. 2B). Among the receptors for TRAIL, TRAIL receptor 2 (TRAIL-R2) was expressed by the NB

cell lines CHLA-255-Fluc and CHLA-136-Fluc and by PDX cells COG-N-415x (Fig. 2C). There was little or no expression of TRAIL-R1, TRAIL-R3, or TRAIL-R4 (data not shown). Ligands for DNAM-1 (CD112, CD155) were also expressed by CHLA-255-Fluc and CHLA-136-Fluc cells and by PDX cells COG-N-415x (Fig. 2C). The ligand for NKp30 (B7-H6) was expressed by the cell lines but not the PDX cells. Among ligands for NKG2D (MICA, MICB, ULBP1, ULBP2/5/6, ULBP3), CHLA-255-Fluc cells demonstrated clear expression of only ULBP3, CHLA-136-Fluc cells expressed little or none of the ligands, and COG-N-415x cells expressed low levels of MICA and ULBP2/5/6 with minimal expression of the other three ligands. Importantly, neither TGFβ1 nor galunisertib affected the expression of these ligands or of GD2 on the NB cell lines *in vitro* (Supplemental Fig. S1).

Since perforin and granzymes are important for NK cell cytotoxicity (31, 32), we next examined the effect of TGFβ1 and galunisertib on aNK cell release of perforin and granzyme A. As expected, TGFβ1 (10 ng/ml) reduced, by 30% or more ($p \leq 0.02$), the release of both perforin and granzyme A from aNK cells cultured alone or from aNK cells cultured in direct contact with NB cells (Fig. 2D). TGFβ1-induced inhibition of perforin and granzyme A secretion was prevented by 5 μM of galunisertib (Fig. 2D) ($p < 0.05$). Intracellular immunostaining for perforin and granzyme A expression indicated no effect of TGFβ1 or of galunisertib (Supplementary Fig. S2), suggesting that TGFβ1 inhibits the release of perforin and granzyme A from aNK cells rather than inhibiting their intracellular expression. Taken together, these results indicate that galunisertib inhibits multiple suppressive effects of TGFβ1 on the cytotoxic mechanisms of aNK cells.

Having established that galunisertib significantly inhibits effects of TGFβ1 on aNK cells, we examined whether it also prevents inhibition of their cytotoxicity by TGFβ1. aNK cells that have been propagated using K562.mbIL21 cells are known to be highly cytotoxic, and this cytotoxicity against multi-drug sensitive and resistant NB cell lines *in vitro* is increased by dinutuximab (24). We found that aNK cytotoxicity against CHLA-255-Fluc (moderately multi-drug sensitive) and CHLA-136-Fluc NB cells (multi-drug resistant) (33) was inhibited by TGFβ1 but this inhibitory effect was significantly reversed by addition of 5 μM galunisertib thirty minutes before (Fig. 3). Importantly, addition of galunisertib 24 hours after TGFβ1 also inhibited the reduction in aNK cytotoxicity by TGFβ1. These findings demonstrate that galunisertib inhibits the suppressive effects of TGFβ1 on aNK cell-mediated direct cytotoxicity and ADCC against NB cells.

Galunisertib decreases phosphorylation of SMAD2 in NB xenografts in NSG mice

To evaluate the inhibitory effect of galunisertib on the TGFβ pathway in NB tumors, intra-renal tumors were examined for phospho-SMAD2 on day 36, 21, or 27 from mice injected in their left kidneys with CHLA-255-Fluc, CHLA-136-Fluc, or patient-derived xenograft COG-N-415x cells, respectively. These mice were injected intravenously twice a week with dinutuximab mixed with K562.mbIL21-propagated aNK cells, starting three days after intra-renal injection of NB cells. Immunohistochemistry demonstrated phospho-SMAD2 in nuclei of tumors that were untreated or treated with dinutuximab and aNK cells (Fig. 4A, 4B, 4C, brown color). Compared to untreated tumors, phospho-SMAD2 was decreased in NBs treated with galunisertib and was decreased the greatest in tumors treated with the

combination of galunisertib, dinutuximab, and aNK cells. These results indicate that galunisertib is able to penetrate human NB xenografts where it reduces SMAD2 pathway activation.

Galunisertib enhances anti-NB activity of dinutuximab plus human aNK cells in NSG mice

The ability of galunisertib to inhibit growth of human NB tumors and improve survival of NSG mice treated with dinutuximab plus human aNK cells was tested in mice bearing tumors formed by intra-renal injection of CHLA-255-Fluc, CHLA-136-Fluc, and COG-N-415x cells. K562.mbIL21-propagated aNK cells were mixed with dinutuximab and injected intravenously twice weekly, starting three days after intra-renal injection of NB cells. Bioluminescence imaging was performed for mice injected with luciferase-expressing CHLA-255-Fluc or CHLA-136-Fluc cells, and decreases in bioluminescence were observed in mice treated with the combination of dinutuximab, aNK cells and galunisertib (Fig. 5A). In contrast, galunisertib alone had no discernable effect on luciferase signals. In a meta-analysis of area under the curve (AUC) for these experiments involving a total of 52 mice and both of the NB cell lines, the combination of galunisertib with dinutuximab and aNK cells significantly reduced tumor growth compared to the untreated control group (Supplementary Fig. S3, $p = 0.0003$ in the meta-analysis, and $p = 0.003$ and 0.02 for CHLA-255-Fluc and CHLA-136-Fluc cells, respectively). Treatment with dinutuximab plus aNK cells without galunisertib exhibited a trend towards tumor growth reduction (p value of meta-analysis = 0.08), and treatment with galunisertib alone did not significantly decrease tumor growth ($p = 0.41$). Importantly, the combination of galunisertib with dinutuximab and aNK cells significantly extended survival of mice injected with CHLA-255-Fluc, CHLA-136-Fluc, or COG-N-415x NB cells compared to either untreated mice or mice treated with dinutuximab and NK cells (Figs. 5B, 5C, and 5D). Taken together, these data indicate that the addition of galunisertib significantly enhances the anti-tumor effect of dinutuximab and aNK cells in NSG mice implanted with NB cell lines or a PDX.

Discussion

Using microarray gene expression profiling of 249 untreated primary NBs from patients, we show *TGFBR1, TGFBR2, TGFB1*, and *TGFB2* expression both in high-risk tumors that have either amplified or non-amplified *MYCN* and in low-risk NBs. We also show very low expression of *IFNG* in these tumors, consistent with TGFβ-mediated suppression of NK cells (13). A previous study of 61 NBs of all clinical stages using conventional RT-PCR and electrophoresis also showed expression of *TGFBR1, TGFBR2*, and *TGFB1* (34). Additionally, we show for the first time that bone marrow and blood plasmas from patients induce SMAD signaling in a reporter NB cell line and that galunisertib, a small molecule inhibitor of the TGFβR1 signaling pathway that is in phase I and II clinical trials (15), blocks this activity. In agreement with these findings, we show that phospho-SMAD2, which accumulates in cell nuclei downstream of TGFβR1 signaling (35), can be detected in untreated NBs growing in NSG mice. Treatment of mice with galunisertib inhibited phosphorylation of SMAD2 in these tumors, which indicates its activity in the tumor microenvironment.

We and others have previously demonstrated that aNK cells can be propagated using K562.mbIL21 cells to numbers that should be sufficient for adoptive cell therapy in humans (22–24). Here, we demonstrate for the first time that TGFβ1 inhibits both direct killing and ADCC of NB cells by such aNK cells *in vitro* and that galunisertib can substantially reverse this inhibition. Restoration of ADCC by galunisertib did not involve modulation of CD16 expression. Instead, our findings show that galunisertib treatment of aNK cells *in vitro* inhibits TGFβ1-induced SMAD2 phosphorylation and significantly restores expression of DNAM-1, NKp30, NKG2D, and TRAIL and release of perforin and granzyme A, which could contribute to the observed reversal of TGFβ1-mediated inhibition of cytotoxicity. The ability of a specific TGFBR1 inhibitor to inhibit SMAD2 phosphorylation, an indicator of TGFBR1 activity, and to reverse multiple downstream effects of TGFβ1 suggests that TGFBR1-mediated signals are key regulators of multiple components of the cytotoxicity of aNK cells and that this regulation may be reversible upon specific treatment.

Importantly, addition of galunisertib to dinutuximab and aNK not only prevented *in vivo* phosphorylation of SMAD2 but enhanced the immunotherapy and survival of NSG mice bearing tumors formed by two human NB cell lines or a PDX. Although, several small-molecule agents that bind and inhibit TGFβR1 have been shown to inhibit TGFβ induced signaling (4, 16, 36–38), our study is the first to demonstrate that a TGFBR1 inhibitor in clinical trials (15), galunisertib, is able to prevent and reverse the suppressive effects of TGFβ1 on activated, non-transformed NK cells propagated in a manner nearly identical to that being tested in adoptive cell therapy trials of acute myeloid leukemia (ClinicalTrials.gov identifier # NCT01787474) and of NB (Identifier # NCT02573896).

In pre-clinical studies of cancer, galunisertib has been reported to enhance the activity of paclitaxel or sorafenib but to have limited activity by itself (17, 18). Treatment with galunisertib alone inhibited the growth of only 2 of 13 lung and prostate carcinoma cell lines grown in *nude* mice (39) and had little or no effect against triple-negative breast cancer xenografts even though pSMAD2 was decreased (17). Galunisertib enhanced paclitaxel treatment of breast cancer stem cells by blocking paclitaxel-induced interleukin-8 transcription and associated cell proliferation, and treatment of breast cancer xenografts with both drugs prevented reestablishment of tumors (17). Galunisertib decreased phosphorylation of SMAD2 in hepatocellular carcinoma cells, limiting their invasive properties *ex vivo*, and potentiating sorafenib-mediated apoptosis and a decrease in proliferation *in vitro* (18). Our study, which did not show galunisertib alone to be active against NBs growing in NSG mice, is in agreement with these previous reports. Taken together, these findings indicate that combining galunisertib with other therapeutic modalities is necessary for realizing its anti-cancer potential.

Our study is the first to show that galunisertib can act as a combinatorial agent with immunotherapy, enhancing mAb and NK cell-based treatment of human tumor xenografts. We show that galunisertib administered to NSG mice according to a previously established pharmacokinetic/pharmacodynamic protocol (16) enhances the anti-NB effect of dinutuximab with adoptively transferred aNK cells. These findings provide preclinical support for testing galunisertib in combination with dinutuximab in clinical trials for neuroblastoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Anti-cancer functions of natural killer (NK) cells may be suppressed in the tumor microenvironment. The cytokine TGFβ1 can be a major effector of this suppression. Galunisertib, a small molecule inhibitor of TGFβR1, enhances anti-tumor activity when combined with paclitaxel or sorafenib in xenograft models of breast or hepatocellular carcinoma and is being tested clinically. However, the effect of galunisertib on TGFβ suppression of NK cell function has not been investigated. We demonstrate that galunisertib reverses TGFβ1-induced suppression of direct cytotoxicity and anti-GD2 antibody-dependent cell-mediated cytotoxicity of human *ex vivo* propagated and activated NK (aNK) cells against neuroblastoma cells *in vitro*. Furthermore, galunisertib enhances aNK adoptive cell therapy with the anti-GD2 monoclonal antibody dinutuximab against neuroblastoma cell lines and a neuroblastoma patient-derived xenograft growing in NSG mice. These data suggest that galunisertib may improve anti-GD2 antibody-based immunotherapy of neuroblastoma.

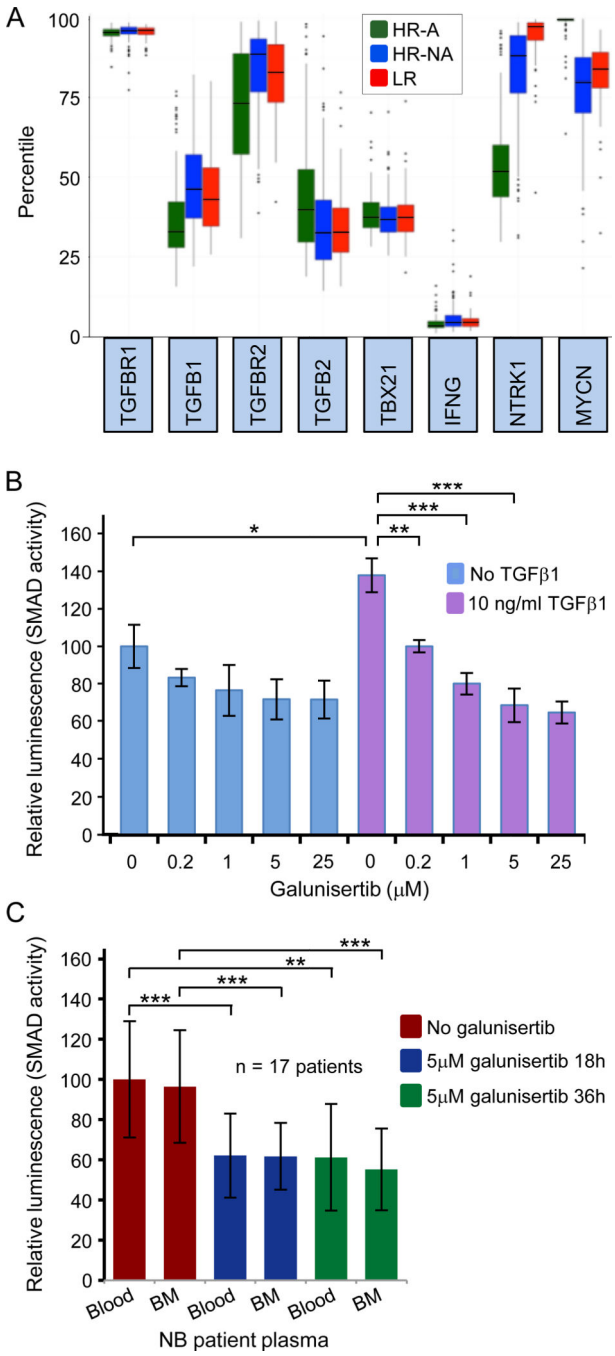

Figure 1.

Expression of *TGFBR1* and *TGFB1* genes by human NB tumors and inhibition of SMAD activity in patient blood and bone marrow plasma by the TGFβR1 inhibitor galunisertib. A, expression levels of genes for 249 primary NB tumors were determined using microarray whole genome analysis (68 patients with high risk MYCN-amplified disease, 151 with high risk non-amplified disease, and 30 with low risk disease). High-risk tumors with *MYCN* amplification (HR-A) exhibited *MYCN* gene expression with a median percentile value at the 100th percentile. B, effect of galunisertib on SMAD activity in reporter cells induced by

10 ng/ml TGFβ1. Galunisertib was examined in serial 5-fold dilutions from 25 μM to 0.20 μM. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. C, effect of galunisertib on SMAD activation in reporter cells induced by blood or bone marrow plasma from 17 NB patients. Blood and bone marrow plasma were diluted ten-fold with IMDM and added into wells containing the reporter cell line CHLA255hRL-SmadFluc with or without 5 μM galunisertib for 18 or 36 hours. The mean induction of SMAD activity by the 17 blood plasmas was assigned a value of 100% (SD ± 28.9).

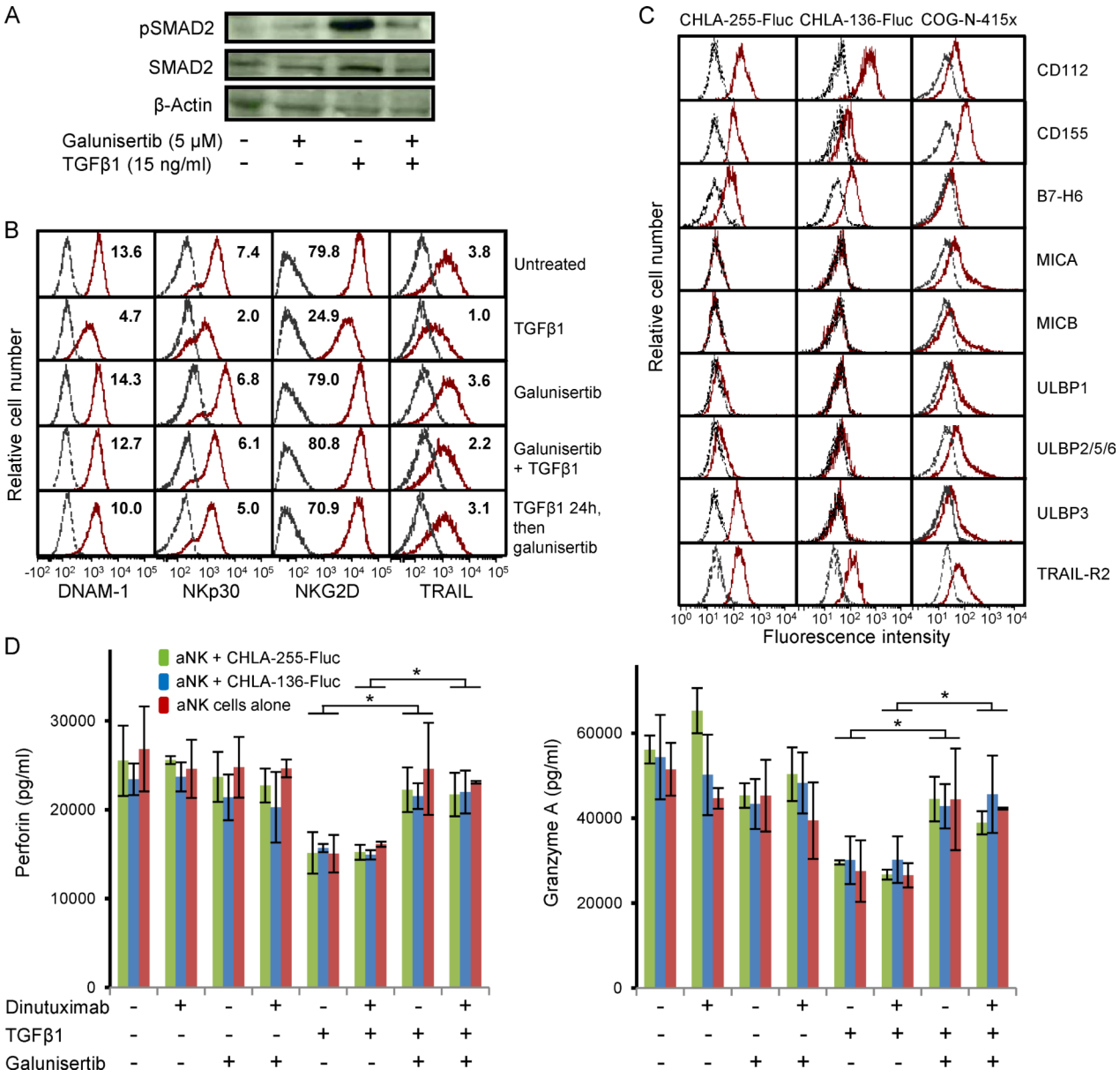

Figure 2.

Effect of galunisertib on TGFβ1-induced SMAD2 phosphorylation, down-regulation of cytotoxicity receptors and TRAIL, and inhibition of release of perforin and granzyme A in aNK cells. A, aNK cells cultured with IL-2 (10 ng/ml) were treated with galunisertib alone (5 μM), TGFβ1 alone (15 ng/ml), or TGFβ1 and galunisertib for 18 hours. Whole lysates from aNK cells were subjected to pSMAD2, total SMAD2, and β-actin immunoblot assay. B, down-regulation by TGFβ1 of DNAM-1, NKp30, NKG2D, and TRAIL, and reversal by galunisertib. aNK cells were pretreated for 48 hours with TGFβ1 (10 ng/ml) alone, with galunisertib (5 μM) alone, or with TGFβ1 and galunisertib. An additional group received TGFβ1 alone for 24 hours, followed by addition of galunisertib for another 24 hours. Using 9-color flow cytometry, viable aNK cells were identified according to CD56 and CD16 expression and absence of CD3, CD14, and CD19 expression, and then their levels of

cytotoxicity receptors and of TRAIL were determined. Stain Index values are given in each histogram overlay. The presented results are similar to those for aNK cells propagated from three additional human donors. Red lines, specific antibody; black lines, isotype control. C, expression of ligands for DNAM-1 (CD112 and CD155), NKp30 (B7-H6), and NKG2D (MICA, MICB, ULBP1, ULBP2/5/6, and ULBP3) and of TRAIL-R2 on NB cell lines and on PDX cells (COG-N-415x). D, suppression of perforin and granzyme A secretion from aNK cells by TGFβ1 is inhibited by galunisertib. After 6 hours of co-culturing aNK cells with NB cells, perforin and granzyme A were quantified in the supernatant of each treatment condition using a Luminex multiplexed microbead assay (*, $p < 0.05$).

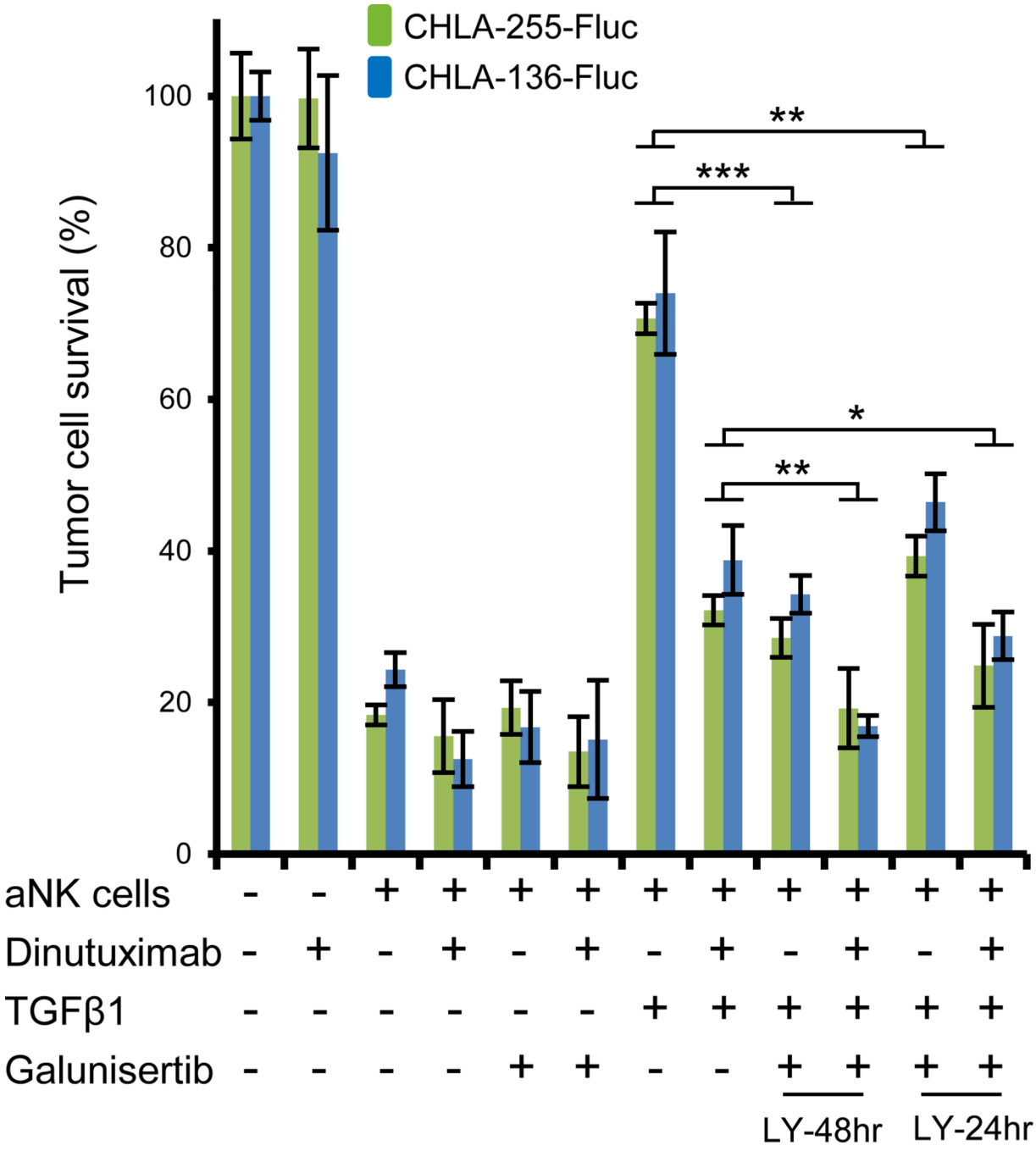

Figure 3.

Effect of TGFβ1 and galunisertib on human aNK cytotoxicity and ADCC against NB cells. A, aNK cells sustained with IL-2 (10 ng/ml) were pretreated 48 hours with TGFβ1 alone (10 ng/ml), galunisertib alone (5 μM), TGFβ1 and galunisertib, or TGFβ1 for 24 hours followed by addition of galunisertib for an additional 24 hours. aNK cells were then cultured with calcein-AM-labeled NB cell lines CHLA-255-Fluc or CHLA-136-Fluc at a 1:2 E:T ratio for 6 hours. aNK cell-mediated killing of NB cells was assayed by digital imaging microscopy.

Galunisertib (5 μM) by itself had no cytotoxic effect (data not shown). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

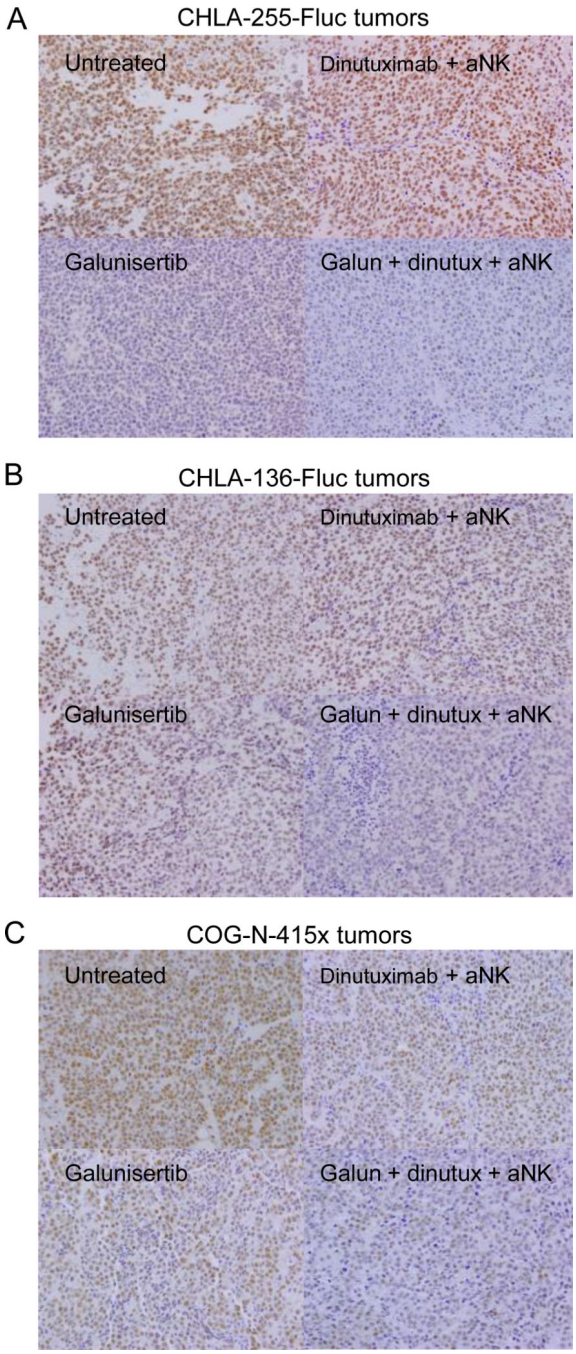

Figure 4.

Effect of galunisertib on SMAD2 phosphorylation in human NB tumors growing in NSG mice. A, tumors derived from CHLA-255-Fluc cells. B, tumors derived from CHLA-136-Fluc cells. C, tumors from COG-N-415x cells. NB cells (1×10^6 CHLA-255-Fluc or CHLA-136-Fluc cell lines or COG-N-415x PDX cells) were injected into the left kidney of each NSG mouse on day 0. Formulated galunisertib (75 mg/kg) was gavaged twice a day from days 3–10 and then five days per week from days 13–31. aNK cells (1×10^7) plus dinutuximab (15 μg/mouse) were injected intravenously twice a week from day 3 along with

IL-2 (2 μg) and IL-15 (4.9 μg/mouse) intraperitoneally. Paraffin-embedded tumor sections (5 μm) were prepared and immunostained with rabbit polyclonal anti-pSMAD2 as described in *Materials and Methods*. Tumors from the four treatment groups (untreated, aNK + dinutuximab, galunisertib alone, or aNK + dinutuximab + galunisertib) were harvested at 36 and 21 days after injection of CHLA-255-Fluc and CHLA-136-Fluc, respectively, and 28 days after injection of COG-N-415x PDX cells. Brown = phosho-SMAD2; blue = hematoxylin; galun = galunisertib; dinutux = dinutuximab.

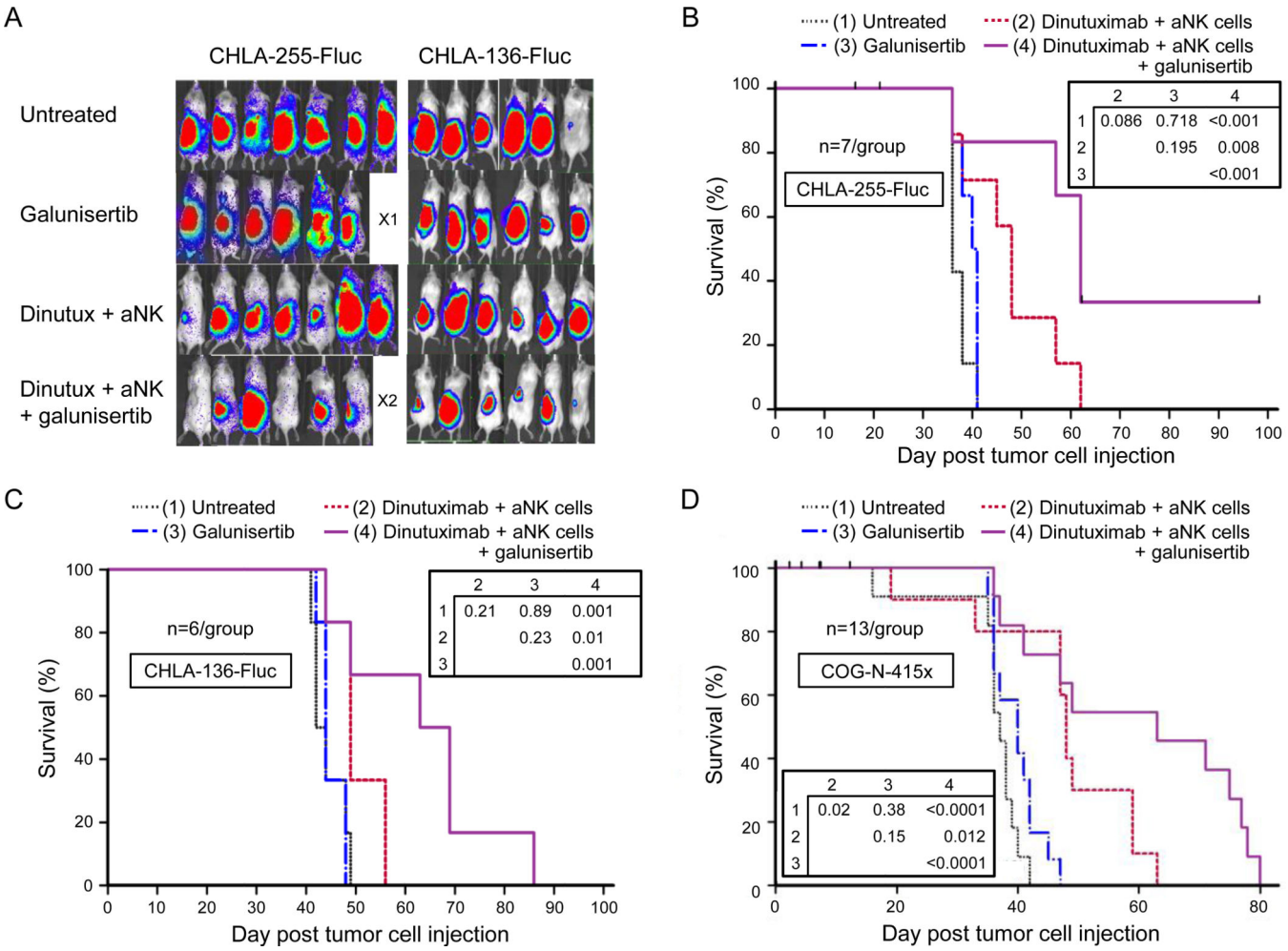

Figure 5.

Effect of galunisertib on growth of NB tumors and survival of NSG mice. Mice were treated as in figure 4. A, bioluminescence imaging on day 23 for mice bearing CHLA-255-Fluc or CHLA-136-Fluc cells. One mouse injected with CHLA-136-Fluc cells in the untreated group exhibited a weak signal but had a large tumor when sacrificed on day 49. Mouse X1 died on day 16 during anesthesia for imaging; mouse X2 died on day 21 from unknown reasons with no observable tumor. B, Kaplan Meier survival plot for mice injected with CHLA-255-Fluc cells. p values comparing treatment groups are shown in the inserted box. C, Kaplan Meier survival plot for mice injected with CHLA-136-Fluc cells. D, Kaplan Meier survival plot for mice injected with PDX COG-N-415x cells.