

1 Targeting MYCN upregulates L1CAM tumor antigen in MYCN-dysregulated
2 neuroblastoma to increase CAR T cell efficacy

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40 Keywords: Adoptive T cell therapy, childhood tumor, combination therapy, MLN8237, MYCN-
41 driven cancer, L1CAM-CAR T cells, drug synergism

42 **Declarations**

43 Ethics approval

44 Ethics approval for generating CAR T cells using T cells from healthy donors was obtained
45 (Charité ethics committee approval EA2/262/20).

46 Consent of publication

47 Not applicable.

48 Availability of data and material

49 Data and materials will be provided by the corresponding author upon reasonable request.

50 Competing interests

51 The authors declare no conflicts of interest.

52 Funding

53 LG is participant in the “Berlin School of Integrative Oncology” and supported by the Wilhelm
54 Sander Stiftung (#2021.115.1). AKü is a participant in the BIH – Charité Advanced Clinician
55 Scientist Pilot Program. SF is a participant in the BIH-Charité Clinician-Scientist Program. All
56 these programs are cofunded by the *Charité – Universitätsmedizin Berlin* and Berlin Institute of
57 Health. This work was supported by the Else Kröner-Fresenius Foundation (#2017_A51 grant to
58 AKü), *Deutsche Krebshilfe* (#701133871 grant to AKü, AGH and ME; #70112951 grant to AKü,
59 ME, JHS, AE) and *Deutsche Forschungsgemeinschaft* (SFB-TRR 338/1 2021 –452881907 to
60 AKü and ME; fellowship grant no. 439441203 to SF). The funders had no role in the study
61 design, data collection and analysis, decision to publish or preparation of the manuscript.

62 Authors’ contributions

63 LG designed and performed experiments, interpreted data and wrote manuscript. LA, SS, AKI,
64 FZ, ML, TS, SF, AV and CC performed experiments. KH and AGH analyzed ChIP seq data.
65 SGW helped with investigation of synergy scores. FW provided the IMR5/75-TR-shMYCN cell

66 line. AGH, FH, GB, ME, AE, UEH, JHS and TB co-conceived study and interpreted data. AKü,
67 JHS and AE acquired funding. AKü conceived and supervised study, KAn co-supervised study.
68 AKü, KAn and KAs revised manuscript. All authors revised and approved the last version of the
69 manuscript.

70 Acknowledgments:

71 We thank Michael C. Jensen for providing the L1CAM-CAR T cell constructs and Hans-Dieter
72 Volk for providing the NSG mice.

73 **List of abbreviations**

CAR	Chimeric antigen receptor
ChIP	Chromatin immunoprecipitation
ELISA	Enzyme-linked immunosorbent assay
E:T	Effector:target
INRG	International neuroblastoma risk grouping
MS	Median survival
NSC	Normalized strand cross-correlation
NSG	NOD/SCID/ γ c ^{-/-}
RSC	Relative strand cross-correlation

74

75 **Abstract**

76 **Background:** Current treatment protocols have only limited success in pediatric patients with
77 neuroblastomas harboring amplifications of the central oncogene, *MYCN*. Adoptive T cell
78 therapy presents an innovative strategy to improve cure rates. However, L1CAM-targeting CAR
79 T cells achieved only limited response against refractory/relapsed neuroblastoma in an ongoing
80 phase I trial to date. Here, we investigate how oncogenic *MYCN* levels influence tumor cell
81 response to CAR T cells, as one possible factor limiting success in trials.

82 **Methods:** High MYCN levels were induced in SK-N-AS cells harboring the normal diploid
83 *MYCN* complement using a tetracycline-inducible system. The inducible MYCN cell model or
84 *MYCN*-amplified neuroblastoma cell lines were cocultured with L1CAM-CAR T cells. CAR T
85 cell effector function was assessed via activation marker expression (flow cytometry), cytokine
86 release and tumor cytotoxicity (biophotonic signal assessment). The cell model was
87 characterized using RNA sequencing, and our data compared to publicly available RNA and
88 proteomic data sets from neuroblastomas. ChIP-sequencing data was used to determine
89 transcriptional *L1CAM* regulation by MYCN using public data sets. Synergism between CAR T
90 cells and the MLN8237 AURKA inhibitor, which indirectly inhibits MYCN activity, was
91 assessed *in vitro* using the Bliss model and *in vivo* in an immunocompromised mouse model.

92 **Results:** Inducing high MYCN levels in the neuroblastoma cell model reduced L1CAM
93 expression and, consequently, L1CAM-CAR T cell effector function (activation, cytokine
94 release and cytotoxicity) *in vitro*. Primary neuroblastomas possessing high *MYCN* levels
95 expressed lower levels of both the *L1CAM* transcript and L1CAM tumor antigen. Indirectly
96 inhibiting MYCN via AURKA using MLN8237 treatment restored L1CAM expression on tumor
97 cells *in vitro* and restored L1CAM-CAR T cell effector function. Combining MLN8237 and
98 L1CAM-CAR T cell treatment synergistically increased neuroblastoma-directed killing in
99 MYCN-overexpressing cells *in vitro* and *in vivo* concomitant with severe *in vivo* toxicity.

100 **Conclusion:** We shed new light on a primary resistance mechanism in MYCN-driven
101 neuroblastoma against L1CAM-CAR T cells via target antigen downregulation. These data
102 suggest that combining L1CAM-CAR T cell therapy with pharmacological MYCN inhibition
103 may benefit patients with high-risk neuroblastomas harboring *MYCN* amplifications.

104 **Background**

105 The International Neuroblastoma Risk Group (INRG) bases neuroblastoma risk stratification on
106 different molecular markers, age group and genetic factors (1). *MYCN* amplification is the most
107 common recurrent genetic aberration, occurring in ~20% of neuroblastomas, in which it alone
108 confers high risk and is associated with poor survival (2). High *MYCN* levels in neuroblastomas
109 lacking *MYCN* amplifications also correlate with poor prognosis (3), and in mouse models, drive
110 tumor development (4), linking oncogenic function to high *MYCN* levels.

111 Aberrant tumor suppressor or oncogene expression has only recently been demonstrated to alter
112 a cancer's ability to shape the host immune response to cancer (5, 6). High-risk *MYCN*-amplified
113 tumors are immunologically “cold”, promoting a T cell-poor environment.
114 Immunohistochemical quantification in archived neuroblastoma samples confirmed that *MYCN*
115 amplification correlates with significantly lower CD4⁺ and CD8⁺ T cell infiltration (7). IFNG
116 signaling and T cell-attracting chemokine (CXCL9, CXCL10) release are negatively regulated in
117 primary neuroblastomas with oncogenic *MYCN* levels and *MYCN*-driven murine neuroblastic
118 tumors, indicating oncogenic *MYCN* levels could hamper T cell infiltration and responsiveness
119 (6).

120 Conventional therapies achieve only limited success against high-risk neuroblastoma, with <30%
121 overall survival (8) that declines to <20% in patients with recurrent disease (9). An innovative
122 therapeutic approach is chimeric antigen receptor (CAR) T cell therapy, which hijacks the
123 immune system to direct T cell effector mechanisms against tumor cells. CARs are synthetic
124 receptors usually constructed by linking a single-chain variable fragment (scFv) from a
125 monoclonal antibody recognizing a tumor-specific protein on the cell surface to a transmembrane
126 domain as well as no (1st generation), one (2nd generation) or more (3rd generation) intracellular T

127 cell costimulatory signaling modules (4-1BB or CD28) and the CD3 ζ T cell signaling domain
128 (10). Our group developed CAR T cells targeting the glycosylated CE7 epitope of L1CAM (11-
129 13), which is specifically expressed on neuroblastoma cells (12). The ongoing phase I trial
130 (NCT02311621) treats children with primary refractory or relapsed neuroblastoma with
131 L1CAM-CAR T cells. Limited responses were observed in the first five enrolled patients, who
132 all had *MYCN*-amplified disease (12).

133 Here we investigated how oncogenic *MYCN* levels influence L1CAM-CAR T cell effector
134 function to better understand the unsatisfactory clinical outcomes achieved in the ongoing
135 clinical trial (NCT02311621) for patients with primary refractory or relapsed neuroblastoma. We
136 used neuroblastoma cell models allowing tight regulation of *MYCN* levels to create normal and
137 oncogenic *MYCN* levels in different molecular cellular backgrounds. *MYCN*-mediated influence
138 on tumor escape from CAR T cell therapy was preclinically analyzed in these models and in
139 combination with indirect pharmacological *MYCN* inhibition as a potential treatment strategy
140 for children with high-risk neuroblastomas harboring *MYCN* amplifications.

141 Material and Methods

142 Mice

143 Male and female NOD/SCID/ γ c^{-/-} (NSG) mice were group-housed according to institutional
144 guidelines, compliant with national and EU regulations for animal use in research. Age- and sex-
145 matched mice were subcutaneously injected (right flank) on day 0 with induced/uninduced 5×10^6
146 SK-N-AS-TR-MYCN tumor cells in 50 μ l MatrigelTM (Corning)/50 μ l phosphate-buffered saline.
147 Mice with induced SK-N-AS-TR-MYCN tumor cells received 0.2mg/ml doxycycline (a more
148 stable form of tetracycline) in 5% sucrose-supplemented drinking water throughout the
149 experiment. When tumors were palpable, mice were ranked by tumor size on the day of CAR T

150 cell treatment, and treatment groups were randomized and contained mice with similar mean
151 tumor sizes. Mice intravenously received 1×10^7 untransduced or L1CAM-28/ ζ CD3 $^+$ T cells in
152 100 μ l phosphate-buffered saline. MLN8237 (15mg/kg/mouse) was administered by oral gavage
153 twice daily within a treatment regimen of 5 days on/2 days off for combination experiments.
154 Mice were sacrificed when tumors reached a 1.500mm 3 mean, according to *Landesamt für*
155 *Gesundheit und Soziales (LAGeSo)* Berlin. Tumor material was processed using a previously
156 described protocol (14).

157 **Cell lines and models**

158 Neuroblastoma cell lines, SK-N-AS, SK-N-SH and IMR5/75, were cultured in RPMI 1640
159 (ThermoFisher Scientific) while SK-N-BE(2) and SK-N-DZ were maintained in Dulbecco's
160 Modified Eagle Medium (ThermoFisher Scientific). The MYCN-inducible cell models were
161 cultured in the same medium base as parental cell lines, but additionally supplemented with
162 100U/ml penicillin-streptomycin (Gibco), 5 μ g/ml blasticidin and, for SK-N-AS-MYCN,
163 500 μ g/ml G418-BC (Merck) or, for IMR5/75-shMYCN, 50 μ g/ml zeocin (ThermoFisher
164 Scientific) as selection antibiotics (15). Cell models were induced by 2 μ g/ml tetracycline in full
165 medium. Medium for neuroblastoma cell lines and models was supplemented with 10% heat-
166 inactivated fetal calf serum (Sigma-Aldrich), and cultures were maintained at 37°C in 5% CO₂.
167 Neuroblastoma cell lines were transduced with a lentivirus encoding a GFP_firefly luciferase
168 epHIV7 construct, producing a biophotonic light signal (for use in the cytotoxicity assay) and
169 GFP expression. Cell lines were regularly checked for *Mycoplasma* contamination using the cell-
170 based colometric HEK-Blue detection assay (Invivogen) and passaged maximally 20 times. Cell
171 line identity was confirmed by STR fingerprinted (Eurofins, Luxemburg).

172 **CAR T cell generation**

173 Magnetic-activated cell separation isolated CD8⁺ and CD3⁺ T cell populations [CD8⁺ T cell
174 isolation kit as previously described (11, 16) or Pan T Cell Isolation Kit for CD3⁺ selection;
175 Miltenyi Biotec] from human peripheral blood mononuclear cells from healthy donors (Charité
176 ethics approval EA2/262/20). CD8⁺ L1CAM-CAR T cells (used for *in vitro* experiments) were
177 generated and expanded as previously described (16) and supplied with 0.5ng/ml IL15 (Miltenyi
178 Biotec) and 50U/ml IL2 (Novartis). CD3⁺ T cells (used for *in vivo* experiments) were supplied
179 with 0.5ng/ml IL15 (Miltenyi Biotec) and 10ng/μl IL7 (Miltenyi Biotec) (17). CAR constructs
180 were linked downstream to a T2A self-cleaving peptide and truncated EGFR for CAR T cell
181 detection and cetuximab immunomagnetic positive selection (enrichment). Untransduced T cells
182 were used as negative controls (for CAR T cell treatment) in experiments.

183 **Flow cytometric analyses**

184 Cell surface expression of CD3, CD8A, CD4 (all BioLegend) and L1CAM (Miltenyi Biotec)
185 was detected by fluorophore-conjugated monoclonal antibodies on a Fortessa X-20 (BD
186 Biosciences) 4-laser flow cytometer. Truncated EGFR expression was detected using
187 biotinylated cetuximab (Bristol-Myers Squibb) and a phycoerythrin (PE)-conjugated streptavidin
188 antibody (BioLegend). T cell activation was assessed by fluorophore-conjugated monoclonal
189 antibodies detecting CD137 (also known as TNFRSF9; BioLegend) and CD25 (also known as
190 IL2RA; BioLegend). Dead cells were excluded from analyses using the LIVE/DEADTM Fixable
191 Red Dead Cell Stain Kit (Life Technologies). GFP-expressing neuroblastoma cells were
192 identified through the FITC channel. QuantiBRITE PE calibration beads (BD Biosciences) were
193 used to determine L1CAM antigen density on neuroblastoma cells according to manufacturer's
194 instructions. Data was processed using FlowJo_V10 Software (Tree Star Inc.).

195 **Cytotoxicity assay**

196 *In vitro* CAR T cell-mediated tumor cytotoxicity was quantified by luciferase-based reporter
197 assay as previously described (18). For combinatorial treatments, CAR T cells were added to
198 achieve indicated effector:target (E:T) ratios together with MLN8237 (Axon Medchem) inhibitor
199 concentrations (1, 15, 25, 40, 60, 80, 100, 700 and 2,000nM) added from a 10mM stock solution
200 (in DMSO) by the Tecan D300e Digital Dispenser (HP) for accurate volume delivery. Xenolight
201 D-luciferin (PerkinElmer Inc.) was added (0.14mg/ml) after 72h treatment, and the biophotonic
202 signal quantified (Promega GloMax Multi) after 3min. Tumor cell lysis mediated by
203 combination treatment was determined using the formula, %specific lysis = (1-
204 [RLU_{sample}/RLU_{max}])x100%, in relation to untreated tumor cells.

205 **Cytokine assays**

206 IL2 and IFNG release from untransduced and CAR T cells was quantified in media conditioned
207 for 24h by cocultures with neuroblastoma cell lines as previously described (18). Neuroblastoma
208 cell lines were seeded for combination treatments at 5x10⁵ cells/well into 48-well plates with
209 untransduced or L1CAM-CAR T cells (E:T ratio of 1:10) and 40nM of MLN8237.

210 **Quantitative Real-Time PCR (qRT-PCR)**

211 Using the RNeasy Mini Kit (Qiagen), mRNA was isolated from cells and reverse transcribed into
212 cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Gene expression was
213 quantified using FastStart Roche SybrGreen (Roche), the SteponePlus™ Real-Time PCR System
214 (Applied Biosystems) and primer pairs (Eurofins) detecting RNA28S1 (fwd:
215 TTGAAAATCCGGGGAGAG, rev: ACATTGTTCCAACATGCCAG) and L1CAM (fwd:
216 CATCCAGTAGATCCGGAG, rev: CTCAGAGGTTCCAGGGCATC). Delta CT was calculated
217 relative to the *RNA28S1* housekeeping gene using StepOne Software v2.3 (Applied Biosystems)

218 **Droplet digital PCR**

219 *MYCN* copy numbers were determined as previously described (19).

220 **Immunoblotting**

221 Whole tumor cells were lysed 30 min on ice in 15mM HEPES, 150mM NaCl, 10mM EDTA, 2%
222 Triton-X100 with Roche protease inhibitor and phosphatase inhibitor cocktails. The RotiQuant
223 Bradford assay (Roth) determined protein concentrations and 10 or 20 μ g of protein were
224 separated on 10% SDS-PAGE for western blotting. Proteins were detected using mouse
225 monoclonal antibodies detecting *MYCN* (B8.4B; sc-53993, Santa Cruz Biotechnology), *L1CAM*
226 (UJ127.11; ThermoFisher Scientific) or GAPDH (sc-32233, Santa Cruz Biotechnology) together
227 with horseradish peroxidase-conjugated mouse IgG (diluted 1:5,000, Dianova). Proteins were
228 visualized using the Fusion FX system and fold change was calculated using
229 VisionCapt_v16.16d.

230 **RNA Sequencing**

231 Total RNA sequencing libraries were generated as previously described (20). Briefly, RNA was
232 extracted from tumor cells using TrizolTM (ThermoFisher Scientific), followed by enzymatic
233 ribosomal RNA depletion, then transcribed, fragmented and hybridized using the TrueSeq
234 Stranded mRNA kit (Illumina, San Diego, CA, USA). Libraries were sequenced on a HiSeq4000
235 sequencer (Illumina) with a paired-end read length of 2x150nt and a sequencing depth of 100
236 million reads by the Max Delbrueck Center for Molecular Medicine Sequencing Core (Berlin).

237 **Data analysis**

238 Public microarray expression data (R2 genomics and visualization platform, <http://r2.amc.nl/>)
239 from primary neuroblastoma cohorts (21) were re-analyzed to identify the relationship between
240 *MYCN* and *L1CAM* expression. ChIP-seq datasets GSE80151 (22, 23) and GSE94782 (22, 23) were downloaded from the Gene

242 Expression Omnibus. Data quality was controlled (FASTQC 0.11.8) and adapters trimmed
243 (BBMap 38.58) before aligning reads to the human hg19 consensus genome assembly (BWA-
244 MEM 0.7.15, default parameters) and removing duplicate reads (Picard 2.20.4). ChIP-seq
245 mappings were quality controlled with normalized & relative strand cross-correlation (NSC &
246 RSC) (Phantompeakqualtools 1.2.1). Only data with NSC>1.05 and RSC>0.8 were further
247 analyzed, following ENCODE recommendations (24). Reads were extended to 200bp (BigWig
248 tracks in Deeptools 3.3.0) while masking blacklisted regions (ENCODE) and normalizing 10bp
249 bins to counts/million before calling peaks (MACS2 2.1.2, default parameters). Combinatorial
250 treatment (CAR T+MLN8237) synergy scores were calculated using R 1.2.5033 and the
251 SynergyFinder package (25). E:T ratios (CAR T:tumor cells) were transformed into
252 concentrations in MLN8237 IC50 range (1:1 E:T ratio = 1,000nM, 1:2 = 500nM, 1:5 = 200nM,
253 1:10 = 100nM) to analyze tumor cytotoxicity data (3 biological replicates). Data points for CAR
254 T cells or inhibitor alone and in combination were used as default input to plot data and analyze
255 synergism without bias [R using the Bliss model (26)] and visualize the drug combination dose-
256 response landscape.

257 **Statistics**

258 Significant differences in CAR T cell activation, cytokine release and tumor cytotoxicity
259 (compared to untransduced T cells) were determined in paired and unpaired Student's *t*-tests.
260 Gene and protein expression data from neuroblastomas and L1CAM surface expression (*in vitro*)
261 were compared using one-way ANOVA. Mouse cohorts treated with L1CAM-specific CAR T
262 cells or untransduced T cells were compared using Kaplan–Meier survival analysis with log-rank
263 statistics. Statistical analyses were conducted using GraphPad Prism 8 Software (GraphPad).
264 Results were considered significant if $p \leq 0.05$.

265 **Results**

266 **Oncogenic MYCN expression impairs L1CAM-CAR T cell effector function**

267 Oncogenic *MYCN* amplification has been linked to a T cell-poor tumor microenvironment (6),
268 providing a rationale to analyze how *MYCN* amplification influences CAR T cell efficacy. To
269 study the impact of tightly regulated oncogenic *MYCN* levels, we used the L1CAM⁺ SK-N-AS
270 neuroblastoma cell line (diploid *MYCN*) equipped with tetracycline-inducible *MYCN* expression
271 (15). High *MYCN* levels were confirmed following induction with tetracycline and were
272 comparable to amplified neuroblastoma cell lines harboring ~100 *MYCN* copies per cell (**Figure**
273 **S1A**). We assessed the relative ability of cocultured SK-N-AS-MYCN cells (\pm MYCN induction)
274 to activate effector function in L1CAM-CAR T cells harboring either a 4-1BB (L1CAM-BB/ ζ)
275 or CD28 (L1CAM-28/ ζ) costimulatory domain. Inducing oncogenic *MYCN* levels in the SK-N-
276 AS-MYCN cells reduced activation (lower CD25 and CD137 expression, **Figure 1A**; gating
277 strategy shown in **Figure S1B**) and effector function (IFNG and IL2 release, **Figure 1B**) in
278 cocultured L1CAM-CAR T cells but did not trigger any cytokine release by untransduced T
279 cells, confirming no activation in the negative control and antigen dependency of T cell
280 activation. Transduced CAR T cells were enriched by cetuximab immunomagnetic positive
281 selection (binds the truncated EGFR tag) to 97.5% (L1CAM-BB/ ζ) and 98.6% (L1CAM-28/ ζ ,
282 **Figure S1C**). Viable tumor cells (\pm MYCN induction) were quantified by reporter assay
283 following CAR T cell exposure to assess how *MYCN* influences CAR T cell-mediated
284 cytotoxicity. L1CAM-CAR T cell-dependent tumor cytotoxicity was reduced by high-level
285 *MYCN* induction (**Figure 1C**), regardless of the costimulatory domain used in the CAR
286 construct (4-1BB: $25.8\% \pm 1.2\%$ vs. $36.2\% \pm 3.4\%$; CD28: $60.9\% \pm 6.2\%$ vs. $81.7\% \pm 5.6\%$).
287 Untransduced T cell controls killed $<4\%$ of cocultured tumor cells. To extend our analysis to the

288 *in vivo* situation, subcutaneous tumors derived from inducible SK-N-AS-MYCN tumor cells
289 were initiated in NSG mice (followed by \pm MYCN induction, **Figure S1D**), then injected with
290 L1CAM-28/ ζ CAR T cells, which responded more strongly in *in vitro* testing (**Figure 1D**).
291 MYCN induction alone did not alter tumor growth (**Figure 1E**), indicating that MYCN
292 expression level did not influence growth kinetics in our model. CAR T cell injection had no
293 effect on growth of tumors with oncogenic MYCN levels, but delayed growth in 3 of 5 tumors
294 with normal MYCN levels. Median survival (MS) of mice harboring uninduced tumors and
295 challenged with CAR T cells was significantly longer than mice challenged with untransduced T
296 cells (MS: 60 vs 25 days; $p=0.0048$). Survival was not significantly enhanced (than untransduced
297 T cell controls) after CAR T cell challenge in mice with tumors expressing oncogenic MYCN
298 levels (MS: 29 vs 25 days; **Figure 1F**). Results using this neuroblastoma model with tightly
299 regulable MYCN levels support that oncogenic MYCN levels in neuroblastoma reduces
300 L1CAM-CAR T cell effector functions.

301 **Inducing high-level MYCN expression diminishes L1CAM surface expression on**
302 **neuroblastoma cells**

303 We next sought to unravel the mechanism underlying MYCN-mediated impairment of CAR T
304 cell efficacy. Global transcription profiles were analyzed in the SK-N-AS-MYCN cell model
305 before and 48h after inducing high MYCN levels. Induction increased *MYCN* transcript levels by
306 >6 -fold. Oncogenic MYCN levels upregulated (by almost 2-fold) MAX interactor 1,
307 dimerization protein (MXII1), which competes with MYCN for MAX binding to mediate
308 transcriptional repression (27), and downregulated a number of genes expressed on the cell
309 surface (**Figure 2A**). These including L1 family members, *NFASC*, *CD177* and *CDH6*, as well
310 as our CAR T cell target antigen, *L1CAM*, which was among the most downregulated genes (>2 -

311 fold). Flow cytometric evaluation of L1CAM expression on the SK-N-AS-MYCN cell surface 3
312 days after inducing high MYCN levels showed L1CAM expression to be diminished by 1.7-fold
313 (p=0.0098; **Figure S2A**), and bead-based quantification detected a 10-fold reduction in L1CAM
314 molecules on the SK-N-AS-MYCN cell surface (**Figure 2B**). We explored whether the negative
315 correlation between oncogenic MYCN expression and L1CAM surface expression was
316 recapitulated in other MYCN-regulable neuroblastoma cell models. SK-N-SH-MYCN cells,
317 harboring the same tetracycline MYCN-inducible system (15), displayed significantly fewer
318 (1.8-fold) L1CAM molecules per cell after inducing high MYCN levels (**Figure 2B**). *MYCN*
319 knockdown using tetracycline-inducible shRNA in IMR5/75-shMYCN cells, which harbor a
320 high-level *MYCN* amplification, increased L1CAM cell surface molecules by 1.4-fold (**Figure**
321 **2B**). Changes in L1CAM expression in all 3 inducible models were confirmed on the transcript
322 level (**Figure 2C**). We assessed whether the influence of oncogenic MYCN levels was sustained
323 on target antigen expression over time in the SK-N-AS-MYCN model. L1CAM cell surface
324 expression (assessed before and 3, 7 and 14 days after inducing MYCN) was further reduced to
325 3.6-fold by day 14 of sustained high MYCN levels (**Figure S2B**), and reduced effector cytokine
326 release by L1CAM-CAR T cells correlated with reduced L1CAM surface expression on
327 neuroblastoma cells (**Figure S2C**). Our results from MYCN-regulable cell models confirm that
328 oncogenic MYCN levels reduce L1CAM transcription and expression on the neuroblastoma cell
329 surface.

330 To validate the clinical relevance of our findings from MYCN-regulable neuroblastoma cell
331 models, we re-analyzed datasets for *L1CAM* expression in primary neuroblastoma samples from
332 2 independent patient cohorts, each containing tumors harboring or lacking *MYCN*
333 amplifications. Microarray-based gene expression data profiles from 493 neuroblastomas (28)

334 and RNA sequencing data from 144 neuroblastomas (21) confirmed that *L1CAM* expression was
335 2-fold lower in neuroblastomas harboring *MYCN* amplifications than tumors lacking *MYCN*
336 amplifications in either cohort (**Figure 2D-E**). L1CAM protein abundance was also significantly
337 downregulated ($p=0.0073$) in *MYCN*-amplified primary neuroblastomas in publicly available
338 mass spectrometry sequencing data (21) (**Figure 2E**, $n=34$). We also reanalyzed ChIP
339 sequencing data from the *MYCN*-amplified neuroblastoma cell lines, SK-N-BE(2)-C, Kelly and
340 NGP (22, 23), to assess whether *MYCN* may directly regulate *L1CAM* expression. *MYCN* peaks
341 were identified within the *L1CAM* gene body, suggesting that it may be involved in its regulation
342 (**Figure S3**). In two of three cell lines, we identified *MYCN* binding at the promotor, marked by
343 H3K4me3. *MYCN* peaks also colocalized with an accessible chromatin region in the first intron,
344 as indicated by ATAC-seq, which was also marked by H3K27 acetylation (**Figure S3**). This
345 indicates that *MYCN* binds to a putative intronic enhancer in *L1CAM*, and may be involved in
346 L1CAM regulation. We show that oncogenic *MYCN* levels achieved either through *MYCN*
347 amplification or enhanced expression diminished L1CAM target protein on the neuroblastoma
348 cell surface. This target reduction may be the mechanism behind resistance to L1CAM-CAR T
349 cell therapy.

350 **Exogenous L1CAM surface expression restores *in vitro* CAR T cell effector activity**

351 We explored whether restoring L1CAM expression on neuroblastoma cells is sufficient to rescue
352 *MYCN*-mediated attenuation of CAR T cell function. Constitutive L1CAM surface expression
353 was achieved in the SK-N-AS-*MYCN* model by transducing a lentiviral vector encoding the
354 *L1CAM* transgene. High L1CAM expression, independent of *MYCN* level, was flow
355 cytometrically confirmed in L1CAM-SK-N-AS-*MYCN* cells (**Figure 3A**). L1CAM-CAR T cells
356 were similarly activated (**Figure 3B**), released similar levels of effector cytokines (**Figure 3C**)

357 and mediated cytotoxicity (**Figure 3D**) when cocultured with L1CAM-SK-N-AS-MYCN cells
358 regardless of MYCN induction. CAR constructs using either costimulatory domain achieved
359 similar activities. These data support that enforcing L1CAM expression is sufficient to rescue
360 CAR T cell efficacy against the tumor cells even in the presence of the escape mechanism driven
361 by oncogenic MYCN levels.

362 **MLN8237 treatment enhances neuroblastoma L1CAM expression to boost L1CAM-CAR T**
363 **cell efficacy**

364 We investigated whether pharmacologically inhibiting MYCN activity could produce the same
365 effect as enforcing L1CAM expression, and restore L1CAM-CAR T cell neuroblastoma
366 cytotoxicity. The alpha-helical structure of the MYCN transcription factor has prevented
367 development of direct MYCN-targeting agents so far. MLN8237 is a small molecule that
368 indirectly inhibits MYCN by targeting the aurora A kinase (AURKA) to drive MYCN
369 degradation (**Figure 4A**) (29). MLN8237 treatment (80, 800nM) dose-dependently reduced
370 MYCN levels in SK-N-AS-MYCN cells (\pm MYCN induction, **Figure S4A**). MYCN reduction in
371 SK-N-AS-MYCN cells (\pm MYCN induction) treated with the lower MLN8237 dose enhanced
372 L1CAM surface expression by 1.2-fold, while treatment with 800nM MLN8237 enhanced
373 L1CAM surface expression by 1.4-fold (low MYCN levels) and 1.6-fold (high MYCN levels,
374 **Figure 4B**). Since oncogenic MYCN levels appeared to impact CAR T cells utilizing CD28
375 costimulation slightly more strongly (**Figure 1**) and L1CAM-28/ ζ CAR T cells were previously
376 shown to be more effective against solid tumors (30), further testing was performed only with
377 this CAR T cell product. MLN8237 treatment did not alter IL2 or IFNG cytokine release from
378 CAR T cells exposed to SK-N-AS-MYCN (\pm MYCN induction) cells (**Figure S4B**). The
379 combined effect of MLN8237 treatment (8 doses, range: 15-2,000nM) with L1CAM-CAR T

380 cells (5 E:T ratios, range: 1:1-1:20) was tested on cocultured SK-N-AS-MYCN cells (\pm MYCN
381 induction), using the Bliss independence model (26) to determine additive, synergistic or
382 antagonistic effects. We have chosen the Bliss model as per definition both drugs used here, act
383 independently against tumor cells and there is no drug-drug interaction (31). The combination of
384 both low-dose treatments enhanced tumor cytotoxicity against tumor cells with oncogenic
385 MYCN levels. (**Figures 4C-D**). To illustrate effects on SK-N-AS-MYCN cells with oncogenic
386 MYCN levels more clearly, we depict each therapy alone and in combination at the peak synergy
387 score (E:T = 1:10, 40nM MLN8237, **Figure 4E**), based on absolute inhibitory values of
388 individual concentrations used in combinational treatment (**Figure S4C-D**). At the peak synergy
389 score, combining treatments significantly increased tumor cytotoxicity ($74.7 \pm 6.1\%$), while single
390 treatments with CAR T cells ($29.7 \pm 11.1\%$, $p=0.0037$) or MLN8237 ($46.4 \pm 11.0\%$, $p=0.018$)
391 achieved only limited cytotoxicity. Combining MLN8237 treatment with CAR T cells did not
392 provide a significant benefit against SK-N-AS-MYCN cells with normal MYCN levels.
393 Pharmacologically inhibiting MYCN activity works in concert with L1CAM-CAR T cell-
394 directed cytotoxicity against SK-N-AS-MYCN cells with oncogenic MYCN levels, resulting in
395 significantly enhanced tumor cell lysis *in vitro*.

396 We tested the combined L1CAM-CAR T cell and MLN8237 treatment against xenograft SK-N-
397 AS-MYCN tumors with maintained oncogenic MYCN levels (doxycycline in drinking water) in
398 our immunodeficient NSG mouse model. Once palpable tumors were detected, T cells (L1CAM-
399 28/ ζ CAR or untransduced control T cells) were intravenously injected once into mice that either
400 received MLN8237 or not by oral gavage twice daily in a cotreatment course of up to 90 days
401 (**Figure 4F**). Inhibiting MYCN activity alone delayed tumor growth in 3 of 5 mice and improved
402 median survival (MS) to 43 days, while L1CAM-CAR T cells alone did not delay tumor growth

403 or improve survival compared to control mice treated with untransduced T cells (MS=29 days
404 and 25 days, respectively; **Figure 4G; Figure S4E**). Combining L1CAM-CAR T cells with
405 MLN8237 eradicated tumors in 3 of 5 mice and improved MS to 42 days. However, MLN8237
406 treatment caused severe toxicity in mice (discomfort not caused by tumor growth), resulting in
407 the need to sacrifice 4 mice (1 treated with untransduced T cells + MLN8237 3 days after T cell
408 injection, 2 treated with L1CAM-CAR T cells + MLN8237 14 days after T cell injection; 1
409 treated with MLN8237 after 25 days of treatment) and preventing statistical analysis of the
410 combination effect on survival. This experiment revealed that although MLN8237 causes severe
411 toxicity *in vivo*, overall survival appeared to improve by combining L1CAM-CAR T cells with
412 inhibition of MYCN activity.

413 *MYCN* amplification may create a different cell background environment than raising *MYCN*
414 expression to oncogenic levels alone in the *MYCN*-inducible model. We extended testing of
415 combined treatment to 3 neuroblastoma cell lines harboring different *MYCN* copy numbers,
416 IMR5/75 (112 copies), SK-N-DZ (130 copies) and SK-N-BE(2) with 487 copies **Figure S1A**),
417 all considered *MYCN*-amplified. MLN8237 treatment significantly increased flow cytometrically
418 detected L1CAM surface expression in IMR5/75 (2.0-fold) and SK-N-DZ (1.9-fold) cells, but
419 not in SK-N-BE(2) cells (**Figure 5A**). L1CAM-CAR T cell-directed cytotoxicity was improved
420 by MLN8237 cotreatment in IMR5/75 and SK-N-DZ cells, but not SK-N-BE(2) tumor cells, as
421 would be expected by their lack of L1CAM target enhancement by MLN8237 (**Figure 5B**).
422 MLN8237 does not appear to inhibit *MYCN* activity well in SK-N-BE(2), suggesting there may
423 be some variability in the efficacy of co-treatment depending on the drug selected. Collectively,
424 our findings demonstrate that combining inhibition of *MYCN* activity with L1CAM-CAR T cell
425 therapy could increase the efficacy of L1CAM-CAR T cell therapy for patients with *MYCN*-

426 amplified neuroblastoma by counteracting the MYCN-directed tumor escape mechanism that
427 downregulates L1CAM target expression on the tumor.

428 Discussion

429 Here we show that oncogenic MYCN levels in neuroblastoma impair L1CAM-CAR T cell
430 efficacy by downregulating L1CAM target antigen expression on neuroblastoma cells.
431 Combining the indirect MYCN inhibitor, MLN8237, with CAR T cells enhanced L1CAM-CAR
432 T cell-directed cytotoxicity *in vitro* in neuroblastoma cells expressing oncogenic MYCN levels
433 caused by induced upregulation in the diploid *MYCN* background or *MYCN* amplifications.
434 Combined inhibition of MYCN activity and L1CAM-CAR T cell treatment also delayed
435 neuroblastoma outgrowth in mice, in a background of tumor-unrelated toxicity to the MLN8237
436 inhibitor.

437 The presence of tumor infiltrating lymphocytes in many tumor entities positively correlates with
438 improved clinical outcome [reviewed in (32), (7, 33)]. A T cell-poor microenvironment, with
439 reduced IFNG signaling and chemokine activity (CXCL9 and CXCL10), characterizes
440 oncogene-driven, *MYCN*-amplified neuroblastoma (6). This tumor environment is expected in
441 the first five patients treated in the ongoing phase I clinical trial investigating L1CAM-CAR T
442 cells, since *MYCN* amplifications were documented in diagnostic samples from all five patients
443 (12), driving our aim to investigate how oncogenic MYCN levels impact CAR T cell efficacy.

444 Here we demonstrate that oncogenic MYCN levels in neuroblastoma cells impair activation
445 (reduced CD25 and CD137 molecules on T cells) and cytotoxic potential of L1CAM-CAR T
446 cells to reduce effector function. Particularly IFNG effector cytokine release by L1CAM-CAR T
447 cells declined severely with high MYCN levels, adding an immunosuppressive function to
448 *MYCN* oncogene-driven tumor cells already previously shown to have a poor IFNG pathway

449 activity by Layer *et al.* (6). Here, raising MYCN to oncogenic levels caused faster outgrowth in
450 xenotransplanted tumors treated with L1CAM-CAR T cells and reduced mouse survival. Since
451 *MYCN*-amplified tumors are known to harbor myeloid-derived suppressor cells and tumor-
452 associated macrophages, which are immunosuppressive (34, 35), the tumor microenvironment
453 could be contributing to the negative effect exerted on L1CAM-CAR T cells via oncogenic
454 MYCN levels in the tumor cells. This cannot be tested in an immunocompromised NSG mouse
455 model and is, therefore, a limitation in our study. We demonstrated that oncogenic MYCN drives
456 L1CAM target antigen reduction and identified an inverse correlation of L1CAM surface
457 expression and MYCN overexpression or *MYCN* amplification in neuroblastoma cell lines and
458 patient data. This was in contrast to data from Rached *et al.* who reported that *L1CAM*
459 knockdown reduced MYCN expression in *MYCN*-amplified IMR-32 neuroblastoma cells, with
460 reductions in proliferation, migration and tumor sphere formation (36). CAR T cell effector
461 function strongly depends on abundance of target antigen and is impaired when antigen
462 expression levels decline below a certain threshold (37-39). Watanabe *et al.* demonstrated that
463 CD20-CAR T cells effectively lysed tumor cells expressing ~200 CD20 molecules/cell but
464 required ~5,000 CD20 molecules/cell to trigger effector cytokine production and T cell
465 proliferation (40). Our results confirm that antigen density is pivotal for optimal L1CAM-CAR T
466 cell efficacy, since reducing L1CAM molecules/cell from ~4,000 to ~500 by inducing oncogenic
467 MYCN levels also diminished cytokine release and tumor cytotoxicity. This finding shows, to
468 our knowledge, the first evidence that MYCN contributes to tumor escape from L1CAM-specific
469 CAR T cells by downregulating the target antigen decreasing responsiveness or even causing
470 primary resistance to L1CAM-CAR T cell therapy.

471 Our analyses showed that targeting MYCN with MLN8237 upregulates L1CAM surface
472 expression on neuroblastoma cell lines dependent on MYCN copy numbers, subsequently
473 improving L1CAM-CAR T cell efficacy. We also demonstrate how the Bliss model, only
474 applied to data from drug combinations to date, can be modified to calculate synergism between
475 CAR T cells and pharmacological inhibitors. The combination therapy delayed tumor outgrowth
476 and seemed to improve overall survival of mice harboring tumors with high-level MYCN.
477 However, survival could not be statistically analyzed because too many mice needed to be
478 removed from the experiment due to toxicity. Discomfort of mice was only seen in MLN8237-
479 treated animals (single treatment or in combination with untransduced T cells or CAR T cells),
480 suggesting severe side effects of this drug *in vivo*. Our observation is in line with results by
481 Mossé et al., who detected high toxicities, including myelosuppression, mucositis, neutropenia
482 and depression among others in a recent phase II trial, where patients with recurrent/refractory
483 solid tumors or leukemia received MLN8237 as a single agent (41). These toxicities have not
484 been observed in the first preclinical evidence in mice, where MLN8237 produced a complete
485 response against pediatric tumors and acute lymphoblastic leukemia independent of *MYCN* status
486 (42). Alternative drug combinations with L1CAM-CAR T cells could be used, like indirect MYC
487 family inhibitors, I-BET726 and JQ1, that show reduced immunogenicity profiles in
488 neuroblastoma cells harboring *MYCN* amplifications, suggesting other MYC family inhibitors
489 could have the same effect as MLN8237 but with lower toxicity profiles (43). These indirect
490 inhibitors of MYCN, as well as next-generation AURKA inhibitors (e.g. LY3295668) are
491 already used in clinical trials (NCT03936465, NCT04106219) making them available for future
492 combination testing with L1CAM-CAR T cells in clinical trials.

493 Here we demonstrate that oncogenic MYCN levels impair L1CAM-CAR T cell effector function
494 by reducing target molecules expressed on the neuroblastoma cells, providing a route to tumor
495 immune escape. We provide preclinical evidence that pharmacologically inhibiting MYCN
496 function restores L1CAM target expression on neuroblastoma cells. These findings offer the
497 rationale for a future clinical trial to test the combination of MYCN-targeting drugs with
498 L1CAM-CAR T cells in children with *MYCN*-amplified neuroblastoma.

499 **Figure Legends**

500 **Fig. 1: L1CAM-CAR T cell display impaired effector function when stimulated with MYCN^{ind}**
501 **neuroblastoma cell model *in vitro* and *in vivo*. A.** CD25 and CD137 surface molecule expression on
502 viable CD8⁺ L1CAM-CAR and untransduced (UT) T cells after 24 h coculture with SK-N-AS-MYCN^{non-}
503 ^{ind/ind} tumor cells (effector to target ratio (E:T) of 1:5, n=3 L1CAM-BB/ζ, n=5 L1CAM-28/ζ) measured by
504 flow cytometry. **B.** IL2 and IFNG cytokine release by CAR T cells cocultured with SK-N-AS-MYCN^{non-}
505 ^{ind/ind} cells for 24 h was analyzed using ELISA (E:T 1:5; n=5 biological replicates with each in technical
506 triplicates). **C.** SK-N-AS-MYCN^{non-ind} tumor cells were stably transduced with GFP_ffluc and cocultured
507 with L1CAM-CAR T cells (E:T 1:5). Tumor cell lysis was determined by a luciferase-based reporter
508 assay relative to an untreated coculture after 48 h (n=4 biological replicates with each in technical
509 triplicates). **D.** Scheme of experimental set-up. **E.** Tumor growth curves of NSG mice harboring either
510 SK-N-AS-MYCN^{non-ind} or -MYCN^{ind} tumors treated with untransduced (UT) and L1CAM-28/ζ CAR T
511 cells (all groups n=5). Each line represents changes in tumor volume of an individual mouse over time of
512 the experiment. Zero (“0”) indicates start of treatment. **F.** Kaplan-Meier survival curve of NSG mice
513 harboring either SK-N-AS-MYCN^{non-ind} or -MYCN^{ind} tumors treated with untransduced (UT) and
514 L1CAM-28/ζ CAR T cells (all groups n=5; log-rank Mantel-Cox test) Zero (“0”) indicates start of
515 treatment. mean ± SD, students T-test, ns = not significant, *, p≤0.05; **, p≤0.01.; UT=untransduced

516 **Fig. 2: MYCN overexpression correlates with reduced L1CAM expression on neuroblastoma cells**
517 **and in patient cohorts.** **A.** MA-Plot of RNA-sequencing data using DESeq2 of fold change (\log_2) of
518 MYCN induction in SK-N-AS-MYCN^{non-ind} versus MYCN^{ind} cells after 48 h of tetracycline treatment.
519 Red dots represent up- or downregulated genes upon MYCN induction (n=3 biological replicates, M (log
520 ratio), A (mean average)). **B.** L1CAM cell surface expression quantified on SK-N-AS-MYCN^{non-ind}, -
521 MYCN^{ind}, SK-N-SH-MYCN^{non-ind}, -MYCN^{ind}, IMR5/75-shMYCN^{amp} and -MYCN^{tet} tumor cells. Data
522 shows L1CAM molecules per cell (n=3 biological replicates). **C.** Relative expression *LICAM* mRNA
523 levels in SK-N-AS-MYCN^{non-ind/ind}, SK-N-SH-MYCN^{non-ind/ind} and IMR5/75-shMYCN^{amp/tet} tumor cells
524 relative to housekeeper h28S. **D.** Gene-expression data from two patient cohort represents \log_2 fold
525 change of *LICAM* expression in a cohort of 498 neuroblastoma patients, without (*MYCN*^{non-amp} n=401)
526 and with *MYCN* amplification (*MYCN*^{amp} n=92, n=5 no *MYCN* status available, not included, ANOVA
527 p=4.53e-30) and in a cohort of 144 neuroblastoma patients, without (*MYCN*^{non-amp} n=104) and with *MYCN*
528 amplification (*MYCN*^{amp} n=40, ANOVA p=2.63e-12) (21). **E.** Patient data from Hartlieb et al. represents
529 \log_2 change of L1CAM protein expression in a cohort of 34 neuroblastoma patients, without (*MYCN*^{non-amp}
530 n=22) and with *MYCN* amplification (*MYCN*^{amp} n=12, ANOVA p=7.31e-3) (21).

531 **Fig. 3: Stable L1CAM expression restores CAR T cell effector function against tumor cells with**
532 **MYCN overexpression.** **A.** L1CAM surface expression was analyzed on SK-N-AS-MYCN tumor cells
533 engineered to express L1CAM under a strong constitutively active promoter EF1A (L1CAM⁺SK-N-AS-
534 MYCN). Cells were either cultured without (*MYCN*^{non-ind}) or with 2 μ g/ml tetracycline (*MYCN*^{ind}).
535 Exemplary flow cytometry staggered histogram plots per condition and dot blots representing L1CAM
536 MFI are shown (n=3 biological replicates). **B.** Activation marker expression levels on L1CAM-CAR T
537 cells after 24 h coculture with L1CAM⁺SK-N-AS-MYCN^{non-ind} or -MYCN^{ind} cells (E:T 1:5; n=3
538 biological replicates). **C.** Quantification of cytokine release after a 24 h coculture of L1CAM⁺SK-N-AS-
539 MYCN^{non-ind}-MYCN^{ind} tumor cells with L1CAM-specific CAR T cells (E:T 1:5; n=3 biological replicates
540 in technical triplicates). **D.** L1CAM⁺SK-N-AS-MYCN^{non-ind/ind} tumor cell lysis assay after coculture with

541 L1CAM-specific CAR T cell was determined by a luciferase-based reporter assay relative to an untreated
542 coculture after 48 h (E:T 1:5, n=3 biological replicates in technical triplicates). Mean \pm SD, students T-
543 test, ns = not significant.

544 **Fig. 4: Indirect MYCN inhibition by MLN8237 increases L1CAM expression and improves**
545 **L1CAM-CAR T cell effector function against MYCN^{ind} neuroblastoma cells *in vitro* and *in vivo*. A.**
546 Schematic overview of the mechanism of action of Aurora A kinase inhibitor MLN8237. **B.** L1CAM
547 surface expression of SK-N-AS-MYCN^{non-ind} and -MYCN^{ind} tumor cells after treatment with 80 and
548 800nM MLN8237 for 72 h measured by flow cytometry. Dot blots represent MFI of L1CAM (n=3
549 biological replicates). **C.** and **D.** Bliss synergism model showing the heatmap indicating antagonism (red)
550 and synergism in blue for SK-N-AS-MYCN^{non-ind/ind} tumor cells that were treated with L1CAM-CAR T
551 cells and MLN8237 in different concentrations (n=3 in biological replicates). **E.** Cytolytic activity of
552 L1CAM-28/ ζ CAR T cells against SK-N-AS-MYCN^{non-ind} -MYCN^{non-ind} NB tumor cells (E:T 1:10) with
553 or without 40nM MLN8237 (n=3 biological replicates in technical triplicates). **F.** Scheme of experimental
554 set-up. **G.** Kaplan-Meier curve of NSG mice harboring SK-N-AS-MYCN^{ind} tumors treated with
555 untransduced (UT) and L1CAM-28/ ζ CAR T cells alone or in combination with MLN8237 (all groups
556 n=5). Zero ("0") indicates start of treatment. mean \pm SD, students T-test, *, p \leq 0.05; **, p \leq 0.01.

557 **Fig. 5: MLN8237 enhances L1CAM surface expression on MYCN-amplified tumor cell lines *in vitro*.**
558 **A.** Flow cytometry analysis representing L1CAM expression of IMR5/75, SK-N-BE(2) and SK-N-DZ
559 cell lines treated with 80 and 800nM MLN8237 for 72 h. Dot blots represent normalized MFI of L1CAM
560 of IMR5/75, SK-N-BE(2) and SK-N-DZ n=4. **B.** Combinational therapy of L1CAM-specific CAR T cells
561 (L1CAM-28/ ζ ; E:T 1:10) and 40nM MLN8237. Biophotonic signal of IMR5/75, SK-N-BE(2) and SK-N-
562 DZ cells was measured to analyze cytotoxic potential of therapies alone and in combination (n=3
563 biological replicates in technical replicates). mean \pm SD, students T-test, ns= not statistically significant,
564 *, p \leq 0.05.

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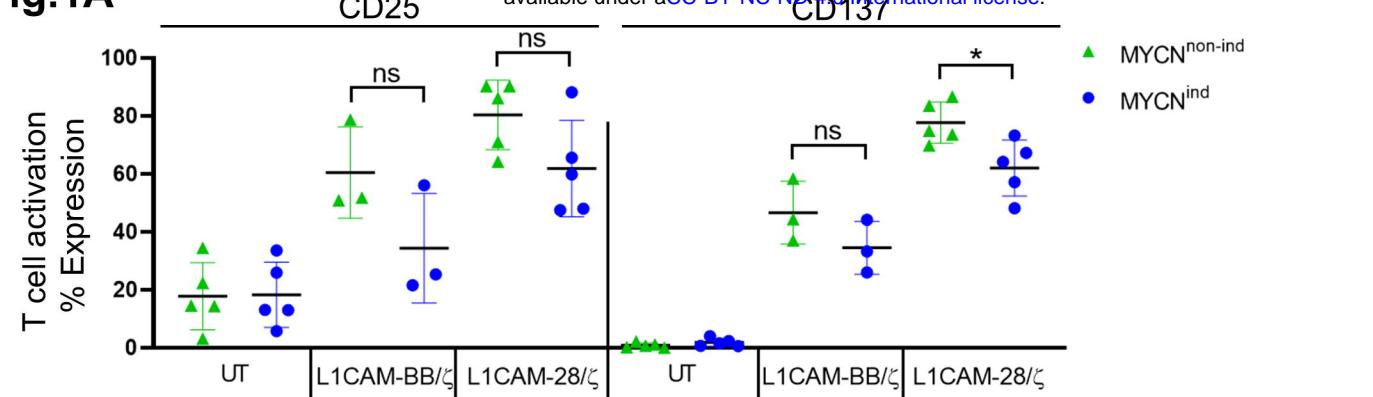
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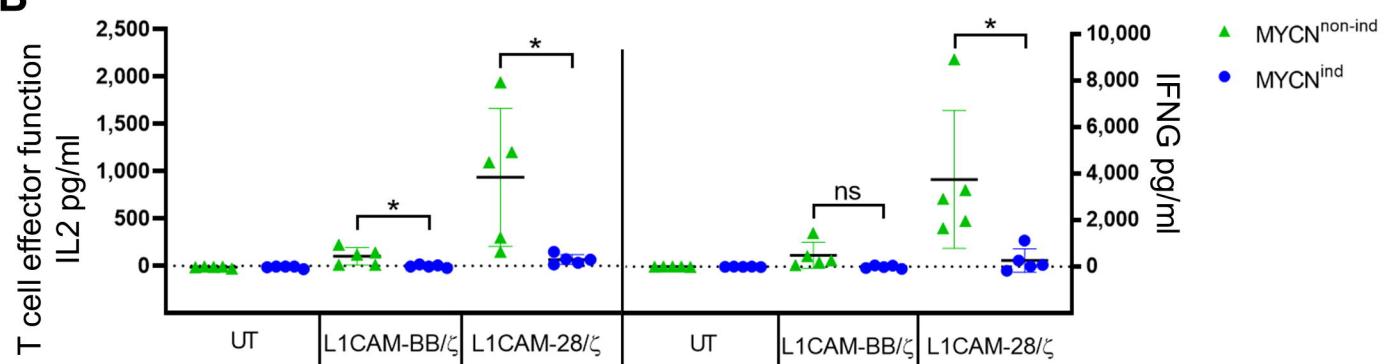
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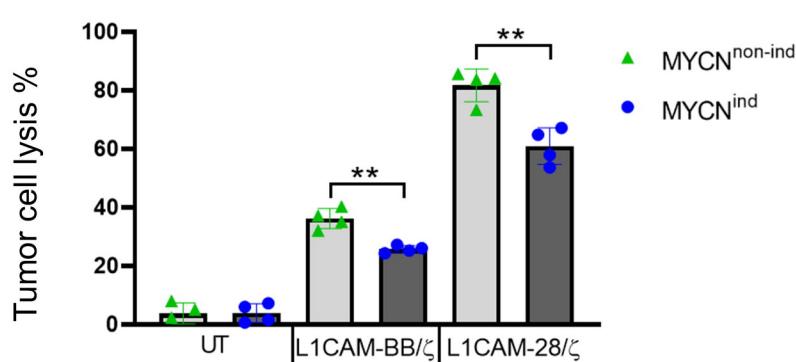
Fig.1A



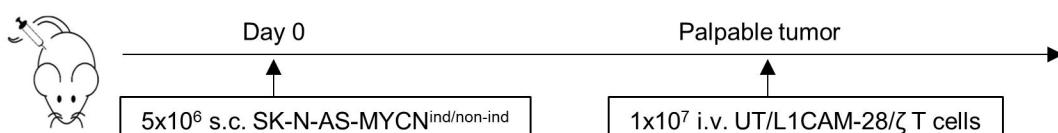
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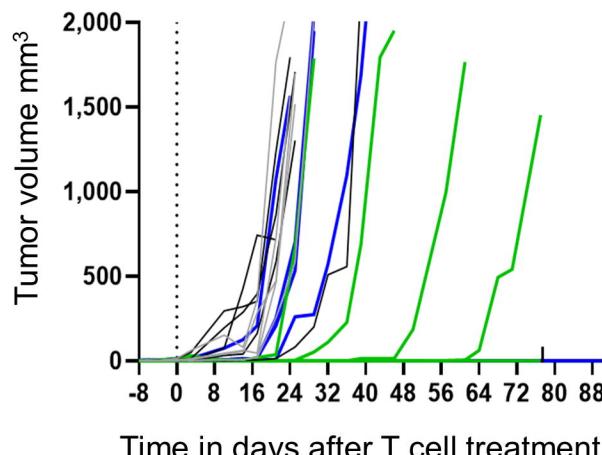
C



D



E



F

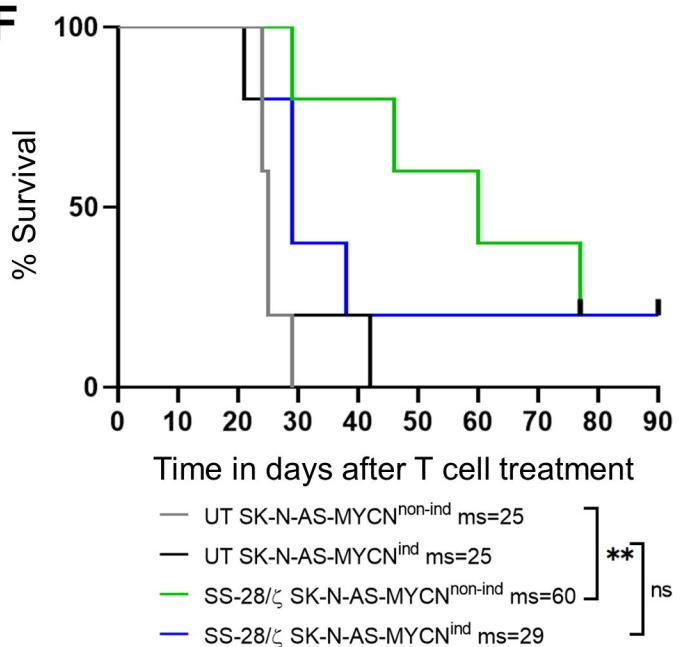
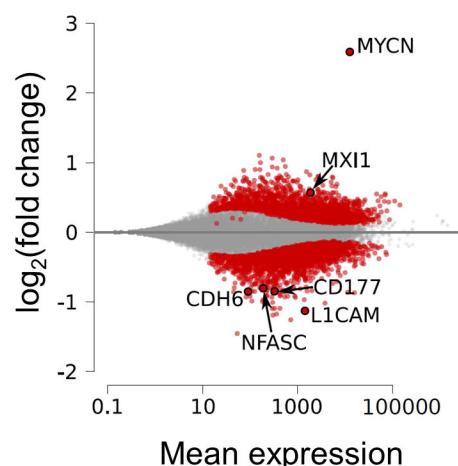
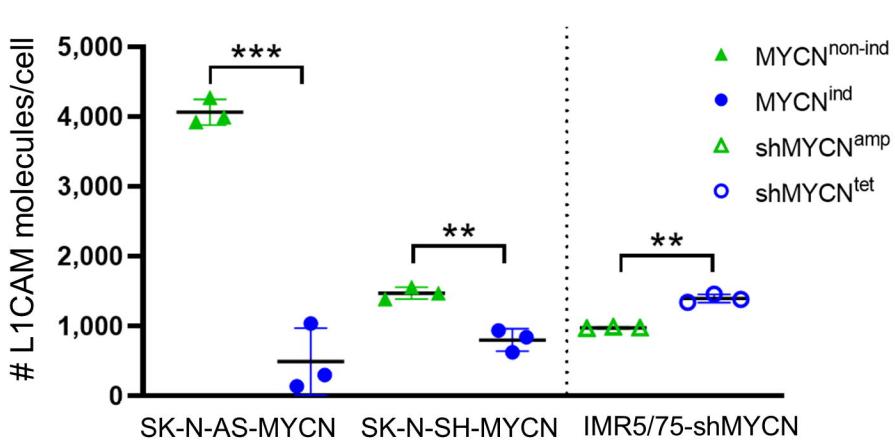


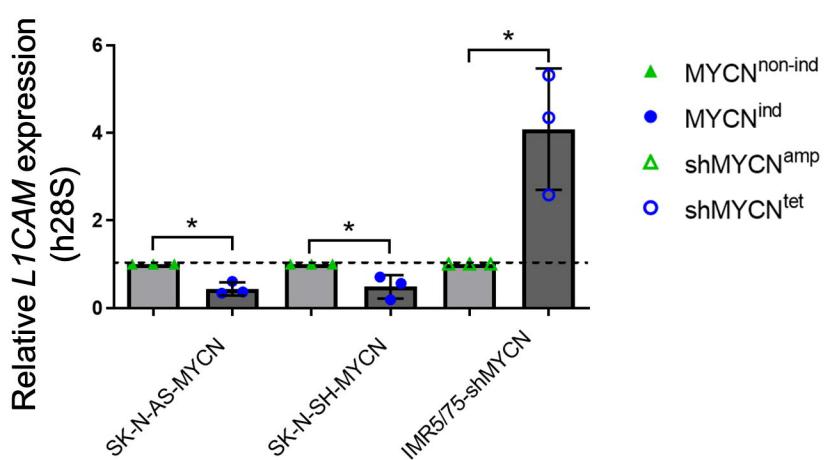
Fig.2A



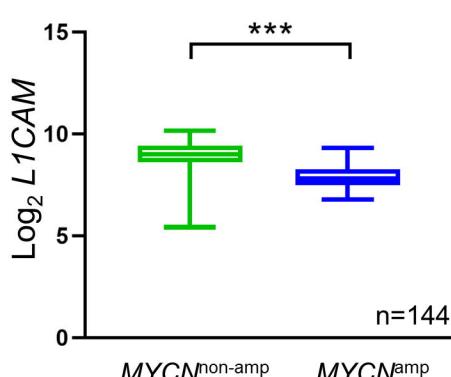
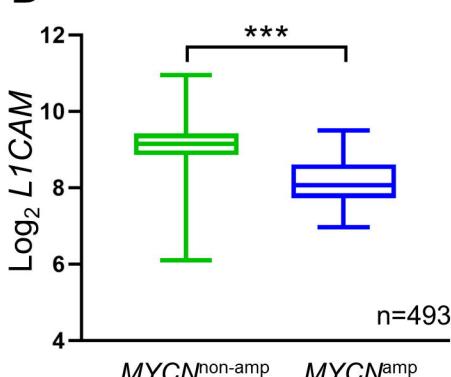
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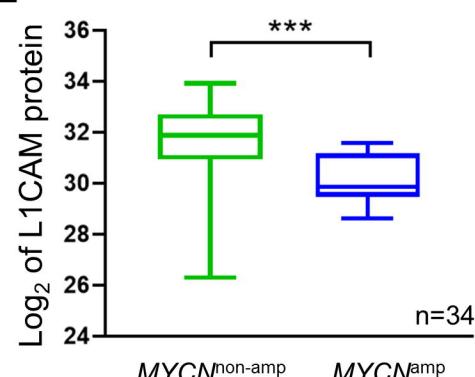
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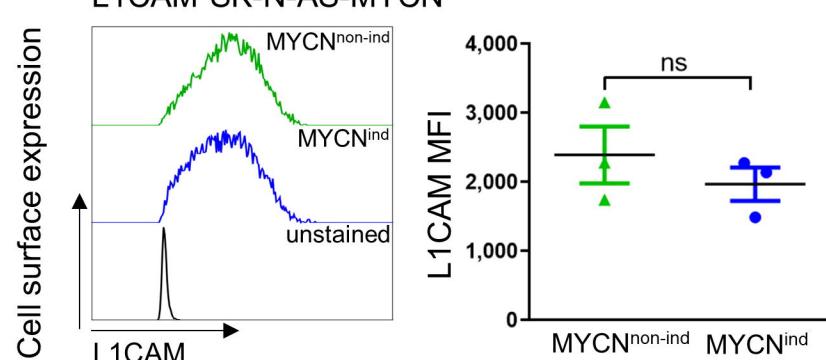


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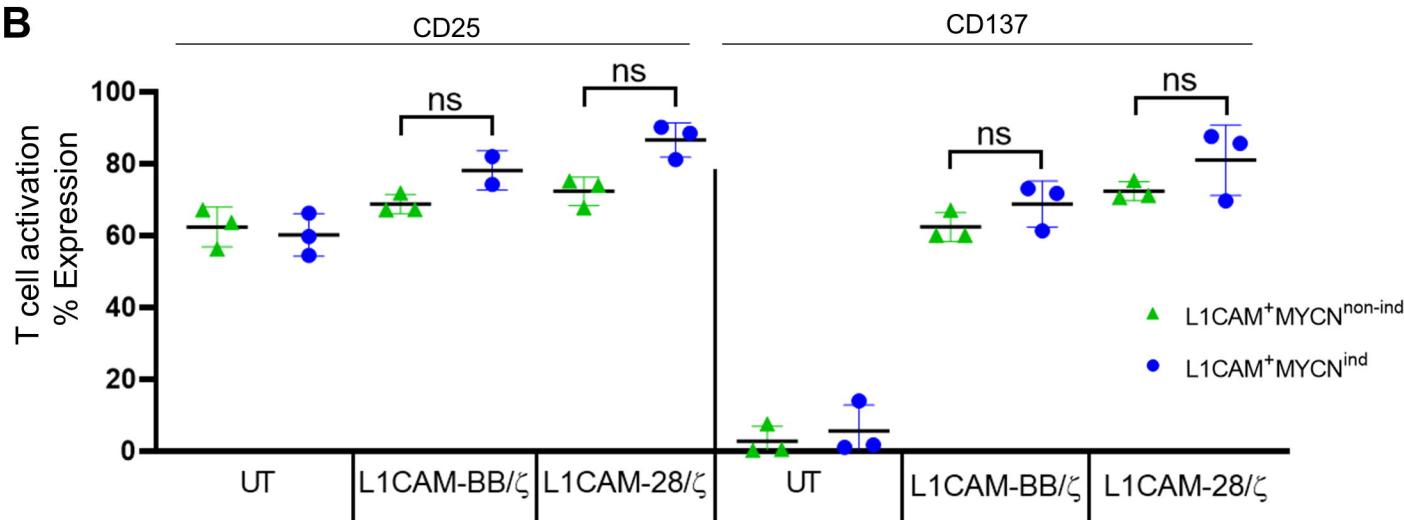


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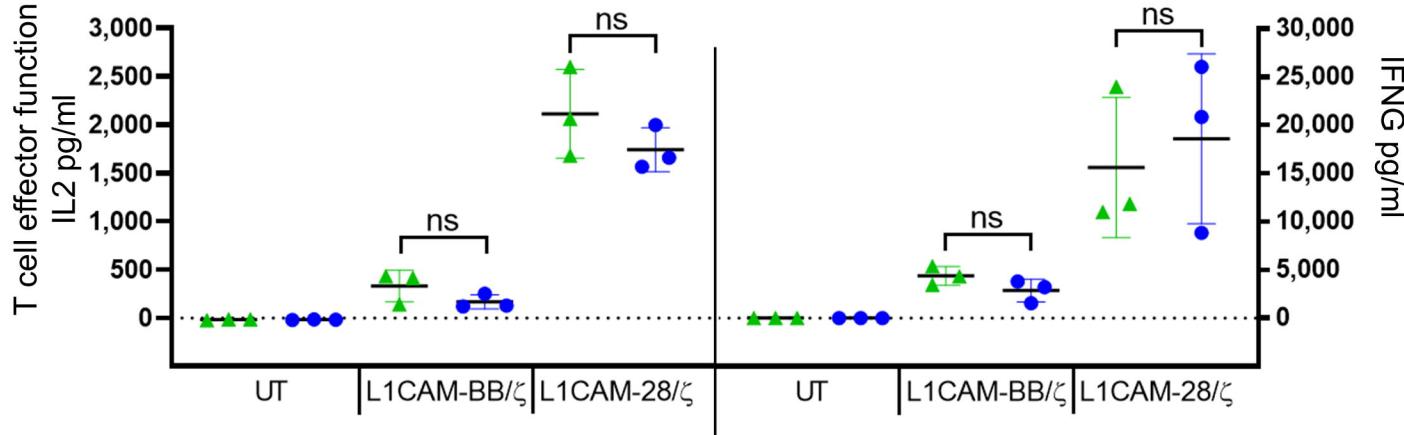




B



C



D

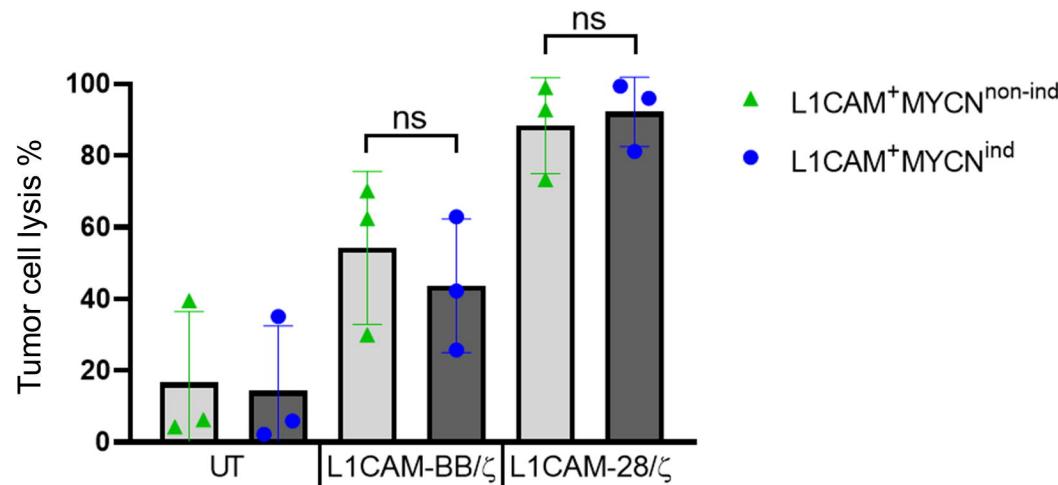
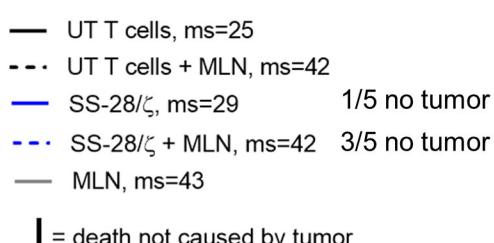
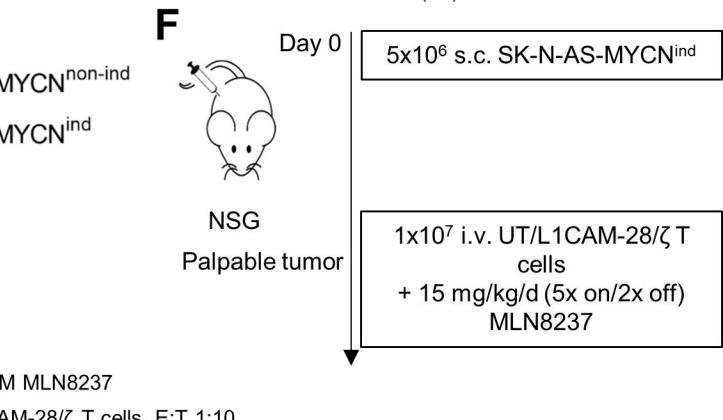
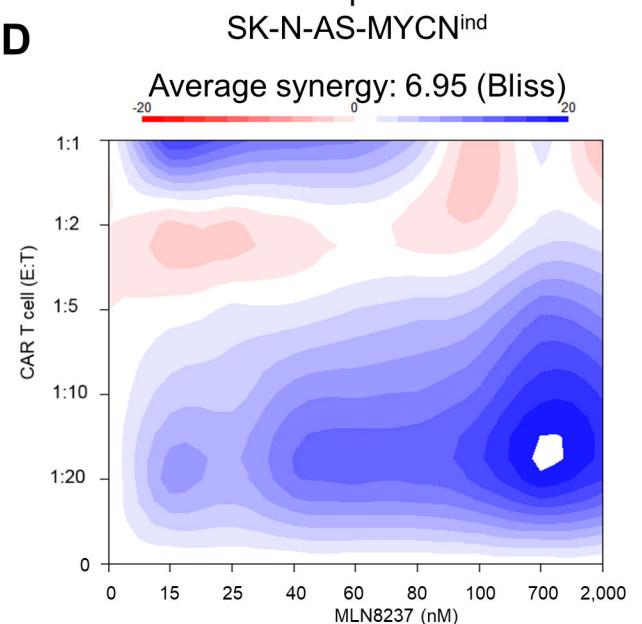
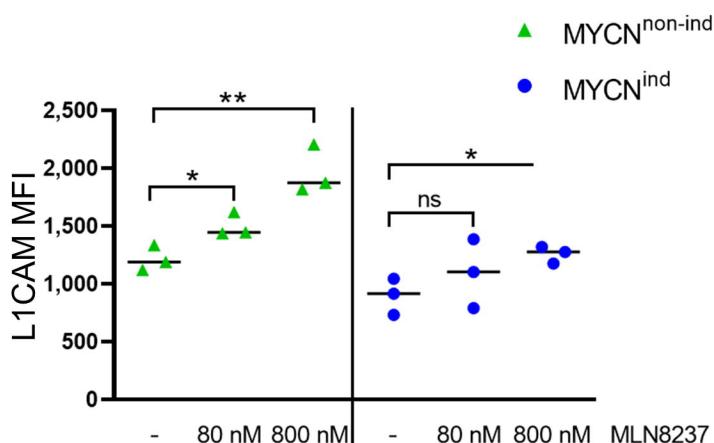
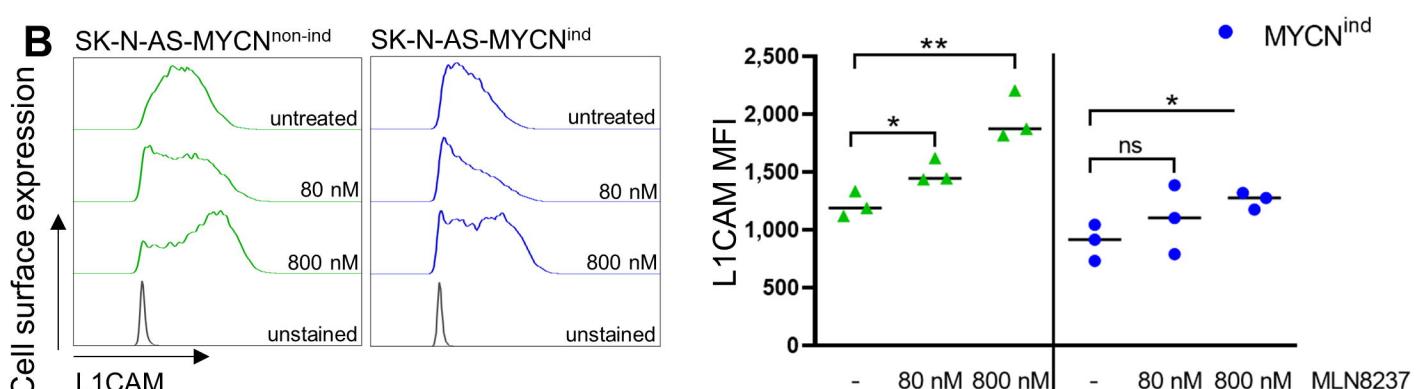
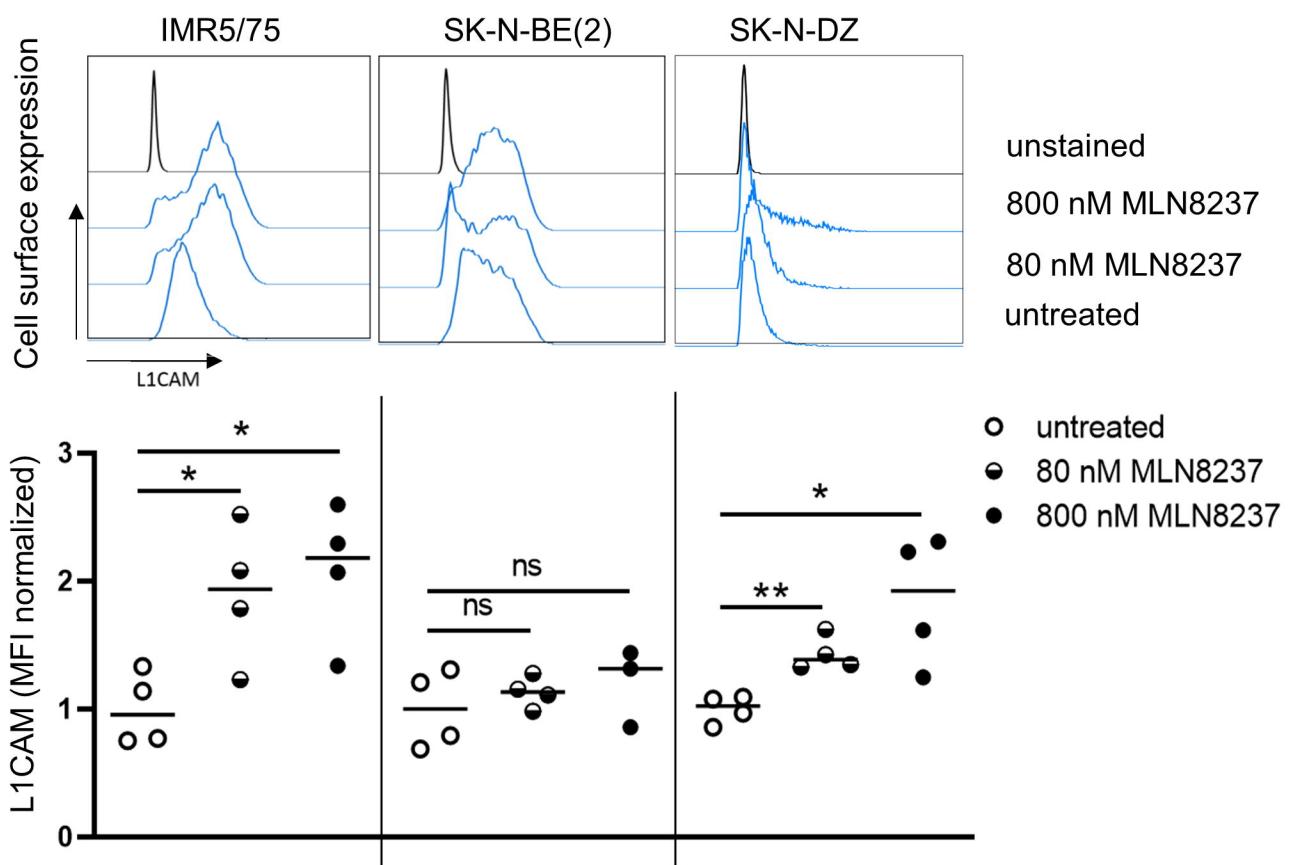


Fig.4A



— = death not caused by tumor

Fig.5A



B

