

# NKG2D-Dependent Antitumor Effects of Chemotherapy and Radiotherapy against Glioblastoma

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Source / Izvornik: **Clinical Cancer Research, 2018, 24, 882 - 895**

Journal article, Accepted version

Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

<https://doi.org/10.1158/1078-0432.CCR-17-1766>

Permanent link / Trajna poveznica: <https://urn.nsk.hr/urn:nbn:hr:184:482828>

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Download date / Datum preuzimanja: **2025-01-17**



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Weiss, Tobias; Schneider, Hannah; Silginer, Manuela; Steinle, Alexander; Pruschy, Martin N; Polic, Bojan; Weller, Michael; Roth, Patrick (2018). *NKG2D-dependent anti-tumor effects of chemotherapy and radiotherapy against glioblastoma*. *Clinical Cancer Research*, 24(4):882-895

DOI: 10.1158/1078-0432.CCR-17-1766

<http://clincancerres.aacrjournals.org/lookup/doi/10.1158/1078-0432.CCR-17-1766>

<https://repository.medri.uniri.hr/islandora/object/medri%3A3163>

1 **NKG2D-dependent anti-tumor effects of chemotherapy and radiotherapy against**  
2 **glioblastoma**

3

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17 **Conflict of interest:** The authors declare no potential conflicts of interest.

18

19 **Running title:** NKG2D-dependent anti-glioma effects of chemoradiotherapy

20

21 **Keywords:** glioblastoma, NKG2D, temozolomide, irradiation, immunotherapy

22

23 **Funding:** This study was supported by grants from the Gertrud-Hagmann Foundation  
24 and the Swiss Cancer League (KFS-3478-08-2014) to PR and “Hochspezialisierte  
25 Medizin Zurich” (HSM-2) to MW and PR.

26 **Statement of translational relevance:**

27 Temozolomide and radiotherapy are the adjuvant standard of care for patients with  
28 glioblastoma. This manuscript demonstrates an unprecedented role of the NKG2D-  
29 dependent immune pathway for the efficacy of these anti-cancer therapies against  
30 glioblastoma. Both treatment modalities induce immune-stimulatory NKG2D ligands  
31 also in unfavorable but clinically relevant settings of MGMT overexpression, TMZ  
32 resistance and at tumor recurrence. This promotes the role of the NKG2D system as an  
33 attractive immunotherapeutic target in glioblastoma at primary diagnosis and at  
34 recurrence. Furthermore, it provides a strong rationale for future combination studies of  
35 conventional radiochemotherapy and NKG2D-based immunotherapy.

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52 **Abstract**

53 Purpose: NKG2D is a potent activating immune cell receptor and glioma cells express  
54 the cognate ligands (NKG2DL). These ligands are inducible by cellular stress and  
55 temozolomide (TMZ) or irradiation (IR), the standard treatment of glioblastoma, could  
56 affect their expression. However, a role of NKG2DL for the efficacy of TMZ and IR has  
57 never been addressed.

58 Experimental Design: We assessed the effect of TMZ and IR on NKG2DL *in vitro* and *in*  
59 *vivo* in a variety of murine and human glioblastoma models including glioma-initiating  
60 cells and a cohort of paired glioblastoma samples from patients before and after  
61 therapy. Functional effects were studied with immune cell assays. The relevance of the  
62 NKG2D system for the efficacy of TMZ and IR was assessed *in vivo* in syngeneic  
63 orthotopic glioblastoma models with blocking antibodies and NKG2D knockout mice.

64 Results: TMZ or IR induced NKG2DL *in vitro* and *in vivo* in all glioblastoma models and  
65 glioblastoma patient samples had increased levels of NKG2DL after therapy with TMZ  
66 and IR. This enhanced the immunogenicity of glioma cells in a NKG2D-dependent  
67 manner, was independent from cytotoxic or growth inhibitory effects, attenuated by O<sup>6</sup>-  
68 methylguanine-DNA-methyltransferase (MGMT) and required the DNA  
69 damage response. The survival benefit afforded by TMZ or IR relied on an intact  
70 NKG2D system and was decreased upon inhibition of the NKG2D pathway.

71 Conclusion: The immune system may influence the activity of conventional cancer  
72 treatments with particular importance of the NKG2D pathway in glioblastoma. Our data  
73 provide a rationale to combine NKG2D-based immunotherapies with TMZ and IR.

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75

## 76 **Introduction**

77 Glioblastoma is the most common malignant primary brain tumor in adults with a dismal  
78 prognosis (1). The first-line treatment in patients below 70 years of age includes surgical  
79 resection as feasible, radiotherapy and concomitant and maintenance chemotherapy  
80 with temozolomide (TMZ), an alkylating agent that induces DNA damage (2, 3). In  
81 addition to these treatment modalities, several promising immunotherapeutic  
82 approaches against glioblastoma are currently being evaluated (4, 5). These efforts are  
83 supported by the observation that glioma cells express molecules that allow for an  
84 interaction with cells of the immune system such as major histocompatibility complex  
85 (MHC) class I and class II molecules (6) as well as MHC class I-like ligands which bind  
86 to the activating immune cell receptor natural-killer group 2 member D (NKG2D) (7). In  
87 humans, NKG2D ligands (NKG2DL) comprise the MHC class I-related chains (MIC) A  
88 and B (MICA, MICB) and the UL16 binding proteins (ULBP) 1–6 (8). These ligands are  
89 expressed on human glioma cells *in vitro* (9) and *in vivo* (10) as well as on glioma-  
90 initiating cells (GIC), a subpopulation of cells with stem cell properties (11, 12). In mice,  
91 NKG2DL comprise the retinoic acid early inducible-1 (RAE-1) proteins, members of the  
92 H60 family (H60a, H60b, H60c) and the murine UL16-binding protein like transcript-1  
93 (MULT-1) which are also expressed by mouse glioma cells (9, 13). All NKG2DL bind to  
94 the NKG2D receptor which is one of the major activating receptors on natural killer (NK)  
95 cells (8). In addition to NK cells, this receptor is constitutively expressed on NKT cells,  
96  $\alpha\beta$  CD8 T cells and  $\gamma\delta$  T cells (8, 14). Furthermore, its expression is induced on CD4 T  
97 cells by tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-15 (15, 16). However, various  
98 glioma-derived humoral and cellular immunosuppressive mechanisms preclude an  
99 efficient anti-tumor immune response, including the expression of transforming growth  
100 factor (TGF)- $\beta$  (17), prostaglandin E2 (PGE2) (18), IL-10 (19), growth and differentiation

101 factor (GDF)-15 (20), lectin-like transcript 1 (LLT1) (21), indoleamine 2,3-dioxygenase  
102 (IDO) (22), programmed death ligand 1 (PD-L1) (23), as well as the presence of  
103 immunosuppressive regulatory T cells (Tregs) (24) and M2-polarized microglia (25).  
104 Enhancing the immunogenicity of glioma cells may be achieved either by inhibition of  
105 these immunosuppressive mechanisms (26) or by promoting immune activating signals  
106 such as the NKG2DL (27). Since various cellular stress stimuli including malignant  
107 transformation of cells or DNA damage can induce NKG2DL (8), we explored whether  
108 TMZ or irradiation (IR) as part of the standard treatment for glioblastoma increase  
109 NKG2DL levels on glioma cells and whether this promotes their immunogenicity. We  
110 also defined the molecular mechanisms underlying the TMZ- and IR-induced NKG2DL  
111 expression in glioma cells. Finally, we investigated the significance of the NKG2D  
112 system for the survival benefit gained with TMZ and IR in several immunocompetent  
113 mouse glioma models.

114

## 115 **Material and Methods**

### 116 *Cells and materials*

117 The human glioma cell lines LN-18 and LN-229 were kindly provided by Dr. N. de  
118 Tribolet (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). LN-229-R  
119 cells were generated by repetitive exposure to TMZ resulting in a shift of the EC<sub>50</sub> (28).  
120 Generation of LNT-229\_MGMT and LNT-229\_neo (29) and LN-18\_shMGMT and LN-  
121 18\_puro cells (30) has been described. SMA-560 glioma cells were obtained from Dr. D.  
122 Bigner (Duke University Medical Center, Durham, North Carolina, USA) and GL-261  
123 were obtained from the National Cancer Institute (Frederick, Maryland, USA). SMA-  
124 560\_Turbo650 and GL-261\_NirFP were created by lentiviral transduction of GL-261 and  
125 SMA-560 cells with plasmids encoding near-infrared fluorescent proteins Turbo650 and  
126 NirFP (Evrogen, Moscow, Russia) and selection by fluorescence-activated cell sorting  
127 (FACS). Adherent cell lines were maintained in Dulbecco's Modified Eagle Medium  
128 (DMEM, Invitrogen, Basel, Switzerland), containing 2 mM L-glutamine (Gibco Life  
129 Technologies, Paisley, UK), and 10% fetal calf serum (FCS, Biochrom KG, Zug,  
130 Switzerland). The GIC cell lines S-24 and ZH-305 were generated from human  
131 glioblastoma patient specimens (31). After tumor removal, tissue was dissociated using  
132 a papain system (Worthington, New Jersey, USA) and a gentleMACS™ Dissociator  
133 (Miltenyi Biotec, Bergisch Gladbach, Germany). These cells were then maintained as  
134 suspension cultures in Neurobasal Medium with B-27 supplement (20 µl/ml) and  
135 Glutamax (10 µl/ml) from Invitrogen and fibroblast growth factor (FGF)-2, epidermal  
136 growth factor (EGF) (20 ng/ml each; Peprotech, Rocky Hill, Pennsylvania, USA) and  
137 heparin (32 IE/ml; Ratiopharm, Ulm, Germany). All cell lines were routinely tested for  
138 Mycoplasma using PCR (last test in december 2016). For all experiments described  
139 herein, the adherent cells were allowed to attach over a 24 h period. Subsequently, the



140 experiments were carried out in serum-free medium. KU-60019 (Selleckchem, Houston,  
141 Texas, USA) is a potent and specific ataxia-telangiectasia mutated (ATM) inhibitor,  
142 concentrations < 1.5  $\mu$ M ensure specificity for ATM. TMZ, kindly provided by Schering-  
143 Plough (Kenilworth, New Jersey, USA), was prepared in stock solutions (100 mM) in  
144 dimethylsulfoxide (DMSO). N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU) was  
145 kindly provided by Medac (Wedel, Germany). Cells were irradiated using a cobalt-60  
146 source (Sulzer, Winterthur, Switzerland) and for different fractionations the  
147 approximative biological effective dose and dose per fraction according to the linear  
148 quadratic model (32) were determined using the R package 'DVHmetrics' ([https://cran.r-](https://cran.r-project.org/web/packages/DVHmetrics/index.html)  
149 [project.org/web/packages/DVHmetrics/index.html](https://cran.r-project.org/web/packages/DVHmetrics/index.html)) under the assumption of an  $\alpha/\beta$  ratio  
150 of 10 for human glioma cell lines. Thiazolyl blue tetrazolium bromide (MTT) was  
151 obtained from AxonLAB (Baden, Switzerland).

152

### 153 *Antibodies and flow cytometry*

154 The following monoclonal antibodies (mAbs) were used for the assessment of cell  
155 surface expression of MICA, MICB, ULBP2, ULBP3, RAE-1, MULT-1, H60 or blocking  
156 of NKG2D: MICA (AMO1, mouse IgG1), MICB (BMO1, mouse IgG1), ULBP2 (BUMO1,  
157 mouse IgG1), ULBP3 (CUMO3, mouse IgG1). Their generation has been described  
158 (33). RAE-1\_FITC and MULT-1\_PE and blocking anti-human NKG2D (clone 149810)  
159 were obtained from R&D Systems Europe (Abingdon, UK). H60\_PerCP was obtained  
160 from Novus Biologicals (Littleton, Colorado, USA). Blocking but not depleting anti-  
161 mouse NKG2D (clone C7) was obtained from eBioscience (San Diego, California,  
162 USA). As controls, we used isotype-matched antibodies from Sigma-Aldrich (Steinheim,  
163 Germany). The PE-conjugated goat anti-mouse IgG from Dako (Baar, Switzerland) was  
164 used as secondary antibody where appropriate. Cells were detached with Accutase

165 (Life technologies), preincubated in phosphate-buffered saline (PBS) with 2% FCS, and  
166 stained with specific mAbs (10 µg/ml) or matched mouse Ig isotype for 30 min on ice,  
167 followed by incubation with PE-conjugated secondary antibody for 30 min where  
168 appropriate. After washing, flow cytometric analyses were performed using a BD  
169 FACSVerse Analyzer (BD, Allschwil, Switzerland). In case of intracellular staining for  
170 ATM<sup>ser1981</sup> Fix/Perm Buffer Set from BioLegend (San Diego, California, USA) was used.  
171 For flow cytometric assessment of tumor-infiltrating lymphocytes, live/dead staining with  
172 FVS 510, anti-CD3\_ PerCP-Cy5.5, anti-CD4\_FITC, anti-CD8\_APC-H7, anti-NKp46\_PE,  
173 anti-IFN-γ\_APC and anti-TCRγ/δ\_ BV421 from BioLegend (San Diego, California, USA)  
174 was used. Specific fluorescence indexes (SFI) were calculated by dividing median  
175 fluorescence obtained with the specific antibody by median fluorescence obtained with  
176 isotype control antibody. For *in vivo* experiments fluorescence intensity was expressed  
177 as median fluorescence intensity. Data was analyzed with FlowJo software (Tree Star,  
178 Stanford, California, USA).

179

### 180 *Immune cell cytotoxicity assay*

181 We used a flow cytometry-based cytotoxicity assay to determine immune-mediated  
182 glioma cell lysis (34). Specific lysis was expressed as percentage of cell death of the  
183 PKH-26<sup>+</sup> labeled targets. Percentage of target cell lysis was corrected for spontaneous  
184 background lysis by subtracting the percentage of dead cells in control samples (targets  
185 alone) from the percentage of dead cells within the test samples. As effector cells, we  
186 used either splenocytes isolated from mice, human NK cells isolated from PBMC by  
187 negative selection using NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach,  
188 Germany) or NKL cells obtained from M.J. Robertson (Indiana University School of

189 Medicine, Indianapolis, Indiana, USA). For blocking experiments, NKL cells were  
190 preincubated for 2 h at 4°C with anti-NKG2D or IgG1 isotype control, and the antibody  
191 was also present during the co-incubation of target and effector cells. All experiments  
192 were done in triplicates.

193

#### 194 *Real-time PCR*

195 Total RNA was isolated using the NucleoSpin RNA II system from Macherey-Nagel  
196 (Düren, Germany) and cDNA was prepared using the iScript cDNA Synthesis Kit from  
197 Bio-Rad Laboratories AG (Cressier, France). For real-time PCR, gene expression was  
198 measured in an ABI Prism 7000 Sequence Detection System (Applied Biosystems,  
199 Foster City, California, USA) with SYBR Green Master Mix (Thermo Fisher Scientific  
200 (Waltham, Massachusetts, USA) and primers (Microsynth AG, Balgach Switzerland) at  
201 optimized concentrations. Primers for MICA, MICB, ULBP2 and ULBP3 have been  
202 published (35). Primers used to detect murine NKG2DL were RAE-1 forward 5'-  
203 TTTGGGAGCACAACCACAGAT-3', reverse 5'-TAAAGTTGGCGGGCTGAAAGA-3',  
204 MULT-1 forward 5'-CTGCCAGTAACAAGGTCCTTTC-3', reverse 5'-  
205 GCTGTTCCTATGAGCACCAATG-3', H60a forward 5'-  
206 CTGAGCTATCTGGGGACCATAC-3', and reverse 5'-AGTCTTTCCATTCACTGAGCAC-  
207 3'. As reference gene, we used human HPRT1: forward 5'-  
208 TGAGGATTTGGAAAGGGTGT-3', reverse 5'-GAGCACACAGAGGGCTACAA-3' and  
209 mouse HPRT1: forward 5'- TTGCTGACCTGCTGGATTAC-3', reverse 5'-  
210 TTTATGTCCCCGTTGACTG-3' respectively. The conditions were 40 cycles at  
211 95°C/15 s and 60°C/1 min. Standard curves were generated for each gene. Relative  
212 quantification of gene expression was determined by comparison of threshold values.

213 All results were normalized to HPRT1 and calculated with the  $\Delta$ CTT method for relative  
214 quantification.

215

#### 216 *Determination of cytotoxicity, acute cytostatic or clonogenic effects*

217 For determination of cytotoxicity,  $5 \times 10^3$  cells were seeded per well in 96-well plates,  
218 allowed to attach for 24 h (adherent cells) and irradiated or exposed to TMZ, CCNU or  
219 staurosporine as indicated for 72 h in serum-free medium. Percentage of living cells  
220 was determined by flow cytometry after live/dead staining with Zombie Aqua™ Fixable  
221 Viability Kit (BioLegend, San Diego, California, USA). For acute growth inhibition  
222 assays, we used the same experimental setting but either crystal violet staining (for  
223 adherent cells) or MTT (for suspension cells) as read-out. Clonogenic survival assays  
224 were performed by seeding  $10^2$  cells per well in 96-well plates. After 24 h, the cells were  
225 irradiated or exposed to TMZ, CCNU, or staurosporine as indicated for 24 h in serum-  
226 free medium, followed by observation for 20 days. As read-out methods, we used again  
227 either crystal violet staining or MTT.

228

#### 229 *Immunoblot analyses*

230 For the detection of proteins in cell lysates, cells were lysed and processed as  
231 previously described (28). Thirty  $\mu$ g of protein were used per lane and visualization of  
232 protein bands was accomplished using horseradish peroxidase (HRP)-coupled  
233 secondary antibodies (Sigma-Aldrich) and enhanced chemiluminescence  
234 (Pierce/Thermo Fisher, Madison, Wisconsin, USA).

235

236 *Immunofluorescence*

237 Cells were cultured in chamber slides with polystyrene-treated glass (BD Biosciences),  
238 fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma-  
239 Aldrich). Blocking with 3% FCS was followed by incubation with anti-ATM protein kinase  
240 pS1981 monoclonal antibody (Rockland, Gilbertsville, Pennsylvania, USA) (diluted  
241 1:100) overnight at 4°C. Donkey anti-mouse IgG Alexa Fluor 488-labeled secondary  
242 antibody (Life technologies, Carlsbad, California, USA) was used at 1:200. Slides were  
243 mounted in Vectashield Mounting Media with DAPI (Burlingame, California, USA) and  
244 images were acquired by using a Leica TCS SP5 confocal microscope.

245

246 *Mice and animal experiments*

247 All experiments were done in accordance with the guidelines of the Swiss federal law on  
248 animal protection and they were approved by the cantonal veterinary office. C57BL/6  
249 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). VM/Dk  
250 mice were bred in pathogen-free facilities at the University of Zurich. NKG2D<sup>-/-</sup> mice  
251 have been previously described (36) and were kindly provided by D. H. Busch (Munich,  
252 Germany). Mice of 6 to 12 weeks of age were used in all experiments in groups of 7-10  
253 mice. For intracranial tumor implantation SMA-560 cells ( $5 \times 10^3$ ) or GL-261 cells ( $2 \times$   
254  $10^4$ ) were stereotactically implanted into the right striatum at day 0. Mice were observed  
255 daily and sacrificed as indicated or in the survival experiments when developing  
256 neurologic symptoms. If indicated, local cranial radiotherapy with a single dose of 12 Gy  
257 was performed at day 10 after tumor implantation using a Gulmay 200 kV X-ray unit at 1  
258 Gy/min at room temperature. If indicated, mice received TMZ (10 mg/kg/day) per oral  
259 gavage from day 7-11 after tumor implantation. MRI was performed with a 4.7 T small  
260 animal magnetic resonance imager (Pharmascan; Bruker Biospin, Ettlingen, Germany)

261 at day 13 after tumor implantation. Coronal T2-weighted images were acquired using  
262 Paravision 6.0 (Bruker BioSpin). Mean +/- SD of the tumor volume in mm<sup>3</sup> from 5  
263 mice/group were determined by the formula (length x width x depth)/2.

264 For *in vivo* blockade of NKG2D signaling, mice were injected i.p. with 100 ug of the  
265 blocking but not depleting anti-NKG2D antibody (clone C7) (37) or with isotype control  
266 in PBS. Antibodies were given either one day before and one day after tumor  
267 implantation or at day 6 and 7 after tumor implantation and were re-injected every 7  
268 days until the mice were sacrificed. Time of antibody administration is indicated in the  
269 figure legends.

270 Isolation of orthotopic tumor cells was performed on day twelve after tumor implantation.  
271 Brains were harvested after transcardial perfusion with ice-cold PBS to remove all  
272 circulating leukocytes from the CNS. Tumor cells were separated from myelin and red  
273 blood cells using a Percoll gradient suspension (Sigma-Aldrich). Cells were washed with  
274 PBS and stained with Zombie Aqua™ Fixable Viability Kit and fluoro-conjugated  
275 antibodies specific to indicated cell surface markers for flow cytometry.

276

#### 277 *Tissue microarray of patient samples*

278 Studies were approved by the Institutional Review Board (KEK-StV-Nr.19/08) and  
279 informed consent was received prior to inclusion to the study. Twenty-one pairs of  
280 primary (before chemoradiation) and recurrent glioblastoma (variable timepoints after  
281 chemoradiation) specimens from patients who underwent brain tumor resection  
282 between 2000 and 2014 at the Department of Neurosurgery, University Hospital Zurich  
283 (Zurich, Switzerland) were collected. Immunohistochemistry was performed as  
284 described (31) using anti-MICA, anti-MICB, anti-ULBP2, or anti-ULBP3 antibodies from  
285 Sino Biological (Lucerna-Chem AG, Luzern, Switzerland) or anti-programmed death-

286 ligand 1 (PD-L1) from Cell Signaling Technology (Danvers, Massachusetts, USA).  
287 Images were analyzed in an unsupervised and blinded fashion using TMARKER, a  
288 software toolkit for histopathological staining estimation (38).

289

### 290 *Statistical analysis*

291 Data are presented as means +/- SD. Experiments were repeated at least three times, if  
292 not indicated differently. Viability and acute and clonogenic cell growth studies were  
293 performed at least in triplicates. Statistical analyses were performed in GraphPad Prism  
294 (La Jolla, CA, USA) using multiple two-tailed Student's t-tests and correction for multiple  
295 comparisons using the Holm-Sidak method. For analysis of tissue microarray data, we  
296 used Wilcoxon matched-pairs signed rank test. For analysis of heterogeneity of  
297 immunohistochemically stained NKG2DL, we calculated the intraclass correlation  
298 coefficient (ICC) (39) as a statistical measure to assess staining variation for 2 tissue  
299 cores from each tumor sample by using the R-package 'ICC' ( [https://cran.r-](https://cran.r-project.org/web/packages/ICC/index.html)  
300 [project.org/web/packages/ICC/index.html](https://cran.r-project.org/web/packages/ICC/index.html)). Kaplan Meier survival analysis was  
301 performed to assess survival differences among the treatment groups and p values  
302 were calculated with Gehan-Breslow-Wilcoxon test. Throughout all figures, significance  
303 was concluded at \*p < 0.05 and \*\*p < 0.01.

304

305 **Results**

306

307 ***TMZ induces NKG2DL expression in glioma cells independent from cytotoxic and***  
308 ***growth inhibitory effects***

309 Exposure of glioma cells to TMZ has growth inhibitory as well as cytotoxic effects. To  
310 define the sensitivity of LN-18 and LN-229 cells to TMZ, we treated these cells with a  
311 broad range of TMZ concentrations and determined cell death, acute growth inhibition  
312 and clonogenic cell survival (Fig. 1A). Since NKG2DL are up-regulated in response to  
313 various stress stimuli, we explored in a next step whether TMZ induces the expression  
314 of NKG2DL in these cells. We observed an induction of several NKG2DL on mRNA and  
315 protein cell surface level over a wide concentration range (Fig. 1A, Suppl. Fig 1A-B)  
316 including low concentrations with minor cytotoxic and growth inhibitory effects as well as  
317 clinically relevant concentrations around plasma levels of 30-80  $\mu$ M of TMZ that are  
318 achieved in human patients (40). To evaluate the effect on other activating immune cell  
319 receptor ligand systems, we assessed CD112 and CD155 as ligands of the human  
320 DNAX accessory molecule-1 (DNAM-1, CD226) activating immune cell receptor. In  
321 contrast to NKG2DL, the cell surface expression of CD112 and CD155 was unaffected  
322 by TMZ (Suppl. Fig. 1C). Next, we examined the effect of TMZ on NKG2DL expression  
323 in GIC, a subpopulation of glioma cells with stem-like properties which are associated  
324 with resistance to chemotherapy and irradiation (41). S-24 cells were relatively resistant  
325 to TMZ with an  $EC_{50}$  value of 267  $\mu$ M in clonogenic survival assays whereas ZH-305  
326 cells were more sensitive with an  $EC_{50}$  of 7.3  $\mu$ M (Fig. 1B). TMZ induced several  
327 NKG2DL on mRNA and cell surface protein levels in both GIC lines. Again, there was  
328 no induction of DNAM-1 ligands (Suppl. Fig. 1D). Furthermore, we determined the  
329 expression of NKG2DL on mouse glioma cells and their induction by TMZ. GL-261 and



330 SMA-560 cells differed in their sensitivity to TMZ. The EC<sub>50</sub> for clonogenic cell survival  
331 was ≈ 50 μM for GL-261 and >500 μM for SMA-560 (Fig. 1C). Similar to human cells,  
332 exposure to TMZ resulted in an up-regulation of NKG2DL in both murine glioma cell  
333 models. H60a is not expressed in C57BL/6 mice and the syngeneic GL-261 cells (42)  
334 and was therefore not detected in this cell line.

335 To corroborate our findings that the upregulation of NKG2DL is not a general response  
336 pattern of glioma cells to cell death induction but rather a specific response to alkylating  
337 chemotherapy, we exposed LN-18 and LN-229 cells to different concentrations of  
338 staurosporine. Despite its strong effect on glioma cell viability, none of the NKG2DL was  
339 up-regulated by staurosporine (Suppl. Fig. 1E). However, CCNU, another alkylating  
340 agent commonly used in patients with recurrent glioblastoma (43) also induced  
341 NKG2DL already at low concentrations, close to those typically achieved in the plasma  
342 of patients (3.4-3.8 μM) (44) (Suppl. Fig. 1F).

343

344 ***Irradiation induces NKG2DL in human and mouse glioma cells independent from***  
345 ***cytotoxic and cytostatic effects***

346 Since radiotherapy belongs to the standard of care for glioma patients, we also  
347 assessed the effect of IR on NKG2DL expression in different glioma models. LN-18  
348 cells were more sensitive to irradiation than LN-229 cells with an EC<sub>50</sub> value of 4 Gy vs.  
349 11 Gy in clonogenic survival assays. In both cell lines, IR induced the expression of  
350 several NKG2DL mRNA and cell surface protein (Fig. 2A). The induction of NKG2DL  
351 cell surface expression following IR was also confirmed when different fractionation  
352 schemes were applied (Suppl. Fig. 2A). Consistent with the TMZ data, there was no  
353 induction of DNAM-1 ligands upon irradiation (Suppl. Fig. 2B). In S-24 and ZH-305 GIC,  
354 irradiation had minor cytotoxic effects with an EC<sub>50</sub> value of > 20 Gy but clear effects on

355 clonogenic survival with  $EC_{50}$  values  $\approx$  5 Gy. A clinically relevant single fraction in the  
356 range of 2-4 Gy increased NKG2DL mRNA and cell surface protein levels (Fig. 2B).  
357 We also confirmed the irradiation-mediated induction of NKG2DL in GL-261 and SMA-  
358 560 mouse glioma cells. In both cell lines, irradiation upregulated NKG2DL on mRNA as  
359 well as on cell surface protein level (Fig. 2C).

360

361 ***TMZ- but not irradiation-mediated NKG2DL induction is modulated by MGMT and***  
362 ***both depend on ATM signaling***

363 *MGMT* promoter methylation predicts benefit from alkylating chemotherapy with TMZ in  
364 glioblastoma. To explore whether the TMZ-mediated induction of NKG2DL is influenced  
365 by MGMT, we used sub-cell lines of LN-18 with a stably silenced *MGMT* gene (30) or  
366 LNT-229 cells that stably overexpress *MGMT* (29). The modulation of *MGMT*  
367 expression affected the sensitivity to TMZ (Fig. 3A), but not to IR (Suppl. Fig. 3A).  
368 Furthermore, *MGMT* expression significantly decreased TMZ-mediated NKG2DL  
369 induction. This was demonstrated by an increased NKG2DL induction upon shRNA-  
370 mediated *MGMT* silencing in LN-18 glioma cells that naturally express *MGMT* and a  
371 diminished NKG2DL induction in *MGMT*-overexpressing LNT-229 cells compared to  
372 *MGMT*-deficient wild-type LN-229 cells (Fig. 3B). The IR-mediated upregulation of  
373 NKG2DL was unaffected by the MGMT status (Suppl. Fig. 3B). Glioma cells can also  
374 acquire resistance to TMZ independent from MGMT expression. Mechanistically, this is  
375 linked, amongst others, to the down-regulation of DNA mismatch-repair proteins (28).  
376 Because this acquired resistance is a challenge in clinical practice that needs  
377 alternative treatment options, we assessed the induction of potentially immune-  
378 activating NKG2DL in a glioma cell line with acquired TMZ resistance (28). Also in these

379 cells, TMZ or IR induced the cell surface protein level of NKG2DL. The same effect was  
380 observed after IR (Suppl. Fig. 3C, D).

381 To elucidate the molecular mechanisms mediating the treatment-induced NKG2DL  
382 induction in glioma cells, we assessed the ATM pathway as part of the DNA damage  
383 response to genotoxic stress induced by TMZ (45). In LN-229 and S-24 cells, we  
384 detected an increase of active phospho-ATM<sup>Ser1981</sup> upon exposure to TMZ (Fig. 3C).  
385 Inhibition of ATM using RNA interference (Suppl. Fig. 3E) or KU-60019, a specific ATM  
386 inhibitor that inhibited ATM at 1.25  $\mu$ M with little toxicity (Suppl. Fig. 3F), abrogated the  
387 TMZ-induced up-regulation of MICA and MICB in LN-229, S-24 cells (Fig. 3D). We  
388 confirmed this also for ZH-305 cells (Suppl. Fig. 3G). Furthermore, we observed this  
389 ATM-dependency also for irradiation-mediated NKG2DL induction (Fig. 3D, Suppl. Fig.  
390 3G).

391

392 ***Exposure to TMZ and IR promote glioma cell immunogenicity in a NKG2D-***  
393 ***dependent manner***

394 To investigate functional effects of the TMZ- or RT-induced NKG2DL induction, we  
395 performed cytotoxicity assays using polyclonal human NK cells or NKL cells (46) as  
396 immune effectors. Pre-exposure of LN-229 or S-24 cells to TMZ resulted in an  
397 enhanced immune cell-mediated cytotoxicity (Fig. 4A, Suppl. Fig. 4A). In contrast,  
398 exposure of *MGMT*-overexpressing LNT-229 cells to TMZ at the same concentrations  
399 did not enhance immune-cell mediated cytotoxicity (Suppl. Fig. 4B). Pre-incubation of  
400 effector cells with blocking but not depleting anti-NKG2D antibodies abrogated the TMZ-  
401 induced glioma cell susceptibility to immune cell killing (Fig. 4A). Similarly, LN-229 or S-  
402 24 cells that were pre-irradiated with 2 Gy were more susceptible to immune cell-  
403 mediated cytotoxicity in a NKG2D-dependent manner (Fig. 4B).

404

405 ***NKG2DL levels are increased in vivo in syngeneic glioma models following***  
406 ***treatment with TMZ or IR as well as in human glioblastoma following***  
407 ***radiochemotherapy***

408 To study the effect of TMZ and irradiation on glioma-associated NKG2DL *in vivo*, we  
409 generated GL-261\_niRP and SMA-560\_TurboFP650 mouse glioma cells, which stably  
410 express near-infrared fluorescent proteins and which are syngeneic to C57BL/6 or  
411 VM/Dk mice. This allowed for the detection of these cells by flow cytometry (Fig. 5A)  
412 and the specific assessment of NKG2DL protein levels on the cell surface *ex vivo*. After  
413 orthotopic tumor cell injection, we treated mice either with a single dose of local IR at  
414 day 10 or with TMZ per oral gavage for 5 consecutive days starting at day 7 after tumor  
415 cell inoculation. At day 12, mice were euthanized and the tumors explanted. TMZ and  
416 irradiation led to an up-regulation of NKG2DL in both orthotopic murine glioma cell  
417 models with a more pronounced effect in the SMA-560 model (Fig. 5A).

418 To study the effect of chemo- and radiotherapy on glioma-associated NKG2DL in  
419 human glioblastoma patients, we created a tissue microarray (TMA) encompassing 21  
420 paired formalin-fixed samples of human glioblastoma specimens obtained before and  
421 after treatment with TMZ and/or radiotherapy. From 9 of these paired samples, we could  
422 also isolate RNA. Compared to basal expression, we detected increased levels of  
423 several NKG2DL on mRNA as well as on cell surface protein level after treatment with  
424 TMZ or IR or both (Fig. 5B). Based on 2 cores from each tumor, we found a  
425 heterogeneous expression of NKG2DL within tumors, particularly for ULBP2 and  
426 ULBP3 (Suppl. Fig. 5A). We did not observe correlations between NKG2DL and survival  
427 or NKG2DL and the immunosuppressive ligand PD-L1 in this small patient population.

428 There were also no significant differences in PD-L1 expression between primary and  
429 recurrent human glioblastoma samples (Suppl. Fig. 5B-D).

430

431 ***The NKG2D system contributes to the therapeutic effects of TMZ and irradiation***  
432 ***in glioma***

433 Finally, we asked whether the NKG2D system plays any role for the survival benefit  
434 gained from TMZ or irradiation in murine glioma models. We inhibited the NKG2D  
435 system in fully immune-competent, orthotopic SMA-560 glioma-bearing mice by  
436 repetitive intraperitoneal injections of a blocking but not depleting anti-NKG2D antibody  
437 (37). Its biological activity reflecting target inhibition was verified by decreased *ex vivo*  
438 cytotoxicity of SMA-560 cells upon TMZ exposure or irradiation by immune effector cells  
439 isolated from anti-NKG2D-treated mice (Suppl. Fig. 6A). At the treatment schedules  
440 used, either IR or TMZ prolonged survival, but this effect was more prominent for IR.  
441 Administration of the anti-NKG2D antibody abrogated the survival benefit conferred by  
442 TMZ and attenuated the IR-mediated survival benefit in SMA-560 glioma-bearing mice  
443 (Fig. 6A). This NKG2D-dependent effect of TMZ or IR in SMA-560 glioma-bearing mice  
444 was also present when the anti-NKG2D antibody was administered at day 6 and 7 post  
445 tumor implantation when tumors had already been established (Suppl. Fig. 6B). To  
446 confirm the importance of an intact NKG2D system for the efficacy of TMZ and IR in  
447 glioma in a second syngeneic setting, we used NKG2D knockout  
448 (NKG2D<sup>-/-</sup>) mice, as an even more robust model. These mice were treated with the  
449 same regimen of TMZ or IR. In addition, we also included the combination of both  
450 treatments, reflecting the current standard of care for human glioblastoma patients.  
451 There was no difference in median survival of glioma-bearing NKG2D<sup>-/-</sup> or NKG2D-intact  
452 mice when no treatment was administered. TMZ or irradiation prolonged the median

453 survival of GL-261 tumor-bearing mice and the combination of both therapies further  
454 increased the survival (Fig. 6B). However, the survival gain conferred by TMZ,  
455 irradiation or the combination of both was significantly reduced in NKG2D<sup>-/-</sup> mice (Fig.  
456 6B). The survival data were corroborated by MRI. At day 6 post tumor implantation we  
457 could not clearly delineate the tumor due to superimposing post-surgery alterations, but  
458 at day 13 post tumor implantation, we observed reduced activity of the anti-tumor  
459 treatments with regard to tumor growth in NKG2D-deficient mice (Fig. 6C, Suppl. Fig.  
460 6C). Finally, we analyzed tumor-infiltrating immune cells. TMZ alone significantly  
461 reduced NK and CD4 T cells, and IR as well as the combination of TMZ and IR reduced  
462 NK cells within the tumor microenvironment (Fig. 6D). There was no difference in the  
463 composition of tumor-infiltrating immune cells in NKG2D<sup>-/-</sup> *versus* NKG2D-intact mice.  
464 However, the activation status of infiltrating immune cells, which did not differ in  
465 untreated NKG2D<sup>-/-</sup> or NKG2D-intact mice, was impaired in NKG2D<sup>-/-</sup> mice upon  
466 treatment. NK cells as well as CD4 and CD8 T cells produced more IFN- $\gamma$  in NKG2D-  
467 intact mice following treatment with TMZ, IR or the combination of TMZ and IR, and this  
468 induction was attenuated in NKG2D<sup>-/-</sup> mice. In NKG2D-intact mice,  $\gamma\delta$  T cells produced  
469 more IFN- $\gamma$  upon treatment with TMZ, IR or the combination of TMZ and IR compared to  
470 NKG2D<sup>-/-</sup> mice. In NKG2D<sup>-/-</sup> mice, we observed more IFN- $\gamma$  production in  $\gamma\delta$  T cells only  
471 upon IR (Fig. 6D, Suppl. Fig. 6D-F).

472

473

474 **Discussion**

475

476 TMZ chemotherapy and radiotherapy constitute the standard treatment modalities in  
477 patients with newly diagnosed glioblastoma (43). The anti-glioma effects of TMZ and IR  
478 comprise different molecular mechanisms such as induction of cell cycle arrest,  
479 senescence and apoptosis (47, 48). Furthermore, there is increasing evidence that cell  
480 death upon exposure to TMZ or IR can promote anti-tumor immune responses by  
481 releasing tumor-associated antigens or damage-associated molecular pattern  
482 molecules such as calreticulin, adenosine triphosphate or high-mobility group box 1  
483 protein (49-51). In addition to these soluble and potentially immune-stimulating  
484 molecules, glioma cells express membrane-bound ligands to the activating immune cell  
485 receptor NKG2D which basically enables target cell killing without prior sensitization and  
486 irrespective of MHC restriction.

487 We observed an up-regulation of several NKG2DL on mRNA and protein level upon  
488 exposure to TMZ, CCNU or IR in several mouse and human glioma cells including  
489 stem-like cells (Fig. 1 and 2, Suppl. Fig. 1 and 2). NKG2DL induction by TMZ has  
490 previously been reported in four other human glioma cell lines (52). We found that the  
491 NKG2DL induction was independent from cytotoxic or growth inhibitory effects and was  
492 achieved at clinically relevant concentrations of chemotherapeutic agents and low  
493 doses of IR. Furthermore, we confirmed the up-regulation of NKG2DL on glioma cells  
494 upon treatment with TMZ or IR *in vivo* in two orthotopic mouse glioma models (Fig. 5A).  
495 The use of fluorescently labeled glioma cells excluded contaminating signals from  
496 immune cells which could also express NKG2DL (53). These findings were further  
497 corroborated by an analysis of paired samples of human glioblastoma tissue specimens  
498 obtained from patients during initial surgery and at tumor recurrence following radio-

499 and/or chemotherapy. The increased NKG2DL expression levels after alkylating  
500 chemotherapy or radiotherapy support our *in vitro* data as well as findings from the  
501 mouse studies (Fig. 5B). Changes in glioma cell NKG2DL levels may be confounded by  
502 other factors than treatment such as passenger mutations which occur during the  
503 course of the disease (54, 55). Despite these limitations, our data strongly suggest that  
504 NKG2DL expression levels are increased following radiochemotherapy. Together with  
505 the observation that NKG2DL can also be induced in TMZ-resistant cells (Suppl. Fig. 3C  
506 and D), this provides a rationale to investigate NKG2D-targeting therapies (27) also at  
507 tumor recurrence.

508 We demonstrate that the up-regulation of NKG2DL upon TMZ or IR requires ATM (Fig.  
509 3D, Suppl. Fig. 3G) which supports the concept that the DNA damage response is one  
510 stimulus for the induction of NKG2DL (56). Consequently, ATM inhibitors may  
511 potentially counteract NKG2D-dependent anti-tumor immune effects. This needs to be  
512 considered in future trials evaluating the activity of such ATM inhibitors as  
513 radiosensitizers (57).

514 Although the net effect of NKG2D ligand induction is of rather small magnitude, it has  
515 important functional consequences. The induction of NKG2DL by TMZ or IR enhanced  
516 the immunogenicity of glioma cells including GIC and rendered the cells more  
517 susceptible to immune-mediated cytotoxicity (Fig. 4, Suppl. Fig. 4A). Chemotherapy and  
518 radiotherapy have various effects on tumor cells and the microenvironment comprising  
519 both immune-stimulatory and immune-suppressive mechanisms. Our study indicates  
520 that treatment-associated NKG2DL induction constitutes a relevant immune-stimulatory  
521 mechanism because inhibition of NKG2D signaling abrogated the enhanced cytotoxicity.  
522 Furthermore, tumor-infiltrating NK, CD4, CD8 T cells and to some extent also  $\gamma\delta$  T cells



523 produced more IFN- $\gamma$  in a NKG2D-dependent manner upon treatment with TMZ and/or  
524 irradiation (Fig. 6D). This emphasizes the relevance of the NKG2D-mediated immune-  
525 stimulatory mechanism of TMZ and IR and adds another relevant mechanism to the  
526 concept of immunogenic cell death.

527 We did not only demonstrate that TMZ- and radiotherapy-mediated NKG2DL induction  
528 can be used as a strategy to render glioma cells more immunogenic but also that the full  
529 efficacy of TMZ and IR against glioblastoma depends on an intact NKG2D system. The  
530 survival benefit gained with these treatment modalities was diminished upon blockade  
531 of NKG2D signaling with an inhibitory but non-depleting antibody or in NKG2D knockout  
532 mice (Fig. 6A and B, Suppl. Fig. 6A-C). Inhibition or deficiency of NKG2D in mice did  
533 not result in a significant survival difference without additional treatment, suggesting that  
534 the basal expression levels of NKG2DL are too low to promote a relevant immune  
535 response (7, 10, 26, 34, 58).

536 The NKG2DL induction upon TMZ treatment or IR could provide a rationale for future  
537 studies investigating the synergistic application of these conventional treatment  
538 modalities with other NKG2D-based immunotherapeutic strategies (27). So far, one  
539 phase I study has used pure NK cells for adoptive immunotherapy in patients with  
540 recurrent malignant gliomas (59). However, no concomitant treatment with TMZ or IR  
541 was administered and additive or synergistic effects to this adoptive cell therapy need to  
542 be explored in future clinical trials.

543 In summary, the present dataset demonstrates the relevance of a so far unrecognized  
544 mechanism mediating anti-tumor effects of TMZ and IR that is likely to be clinically  
545 relevant. Based on our findings, further studies evaluating the combination of  
546 radiochemotherapy with additional NKG2D-based immunotherapeutic strategies should  
547 be considered for the treatment of glioblastoma.

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785 **Figure legends**

786

787 **Fig. 1. TMZ induces NKG2DL in human and mouse glioma cells including human**  
788 **GIC independent from cell death and growth inhibition.** A LN-18 or LN-229 glioma  
789 cells were exposed to different concentrations of TMZ or DMSO control. Viability was  
790 assessed by live/dead staining at 72 h (black dotted line), cytostatic effects were  
791 detected by crystal violet staining at 72 h and 20 d (grey dashed and straight lines) (left  
792 panels). Transcripts for MICA, MICB, ULBP2 or ULBP3 were assessed by real-time  
793 PCR after 48 h (middle panels). Data represent mean values  $\pm$  SD from 3 independent  
794 experiments (\* $p < 0.05$ ; \*\* $p < 0.01$ ). NKG2DL protein levels at the cell surface were  
795 determined by flow cytometry following exposure to TMZ or DMSO control for 72 h (right  
796 panels). Data are presented as SFI and mean values  $\pm$  SD from 3 independent  
797 experiments are shown (\* $p < 0.05$ ; \*\* $p < 0.01$ ). Grey areas represent TMZ plasma  
798 levels achieved in patients. B, C. S-24 or ZH-305 glioma-initiating cell lines (B) and GL-  
799 261 or SMA-560 mouse glioma cells (C) were treated as indicated and human or murine  
800 NKG2DL were analysed as in (A).

801

802 **Fig. 2. IR induces NKG2DL in human and mouse glioma cells independent from**  
803 **cell death and cell growth inhibition.** A. LN-18 or LN-229 glioma cells were irradiated  
804 with different doses of gamma irradiation. Viability was assessed by live/dead staining  
805 (black dotted line), cytostatic effects were detected by crystal violet staining (grey  
806 dashed and straight lines) (left panels). Transcripts (MICA, MICB, ULBP2 or ULBP3)  
807 were assessed by real-time PCR after 48 h (middle). Data represent mean values  $\pm$  SD  
808 from independent experiments (\* $p < 0.05$ ; \*\* $p < 0.01$ ). NKG2DL protein levels at the cell

809 surface were determined by flow cytometry 72 h after IR (right). Data are presented as  
810 SFI and mean values  $\pm$  SD from 3 independent experiments are shown (\*p < 0.05; \*\*p <  
811 0.01). B, C. S-24 or ZH-305 glioma-initiating cell lines (B) and GL-261 or SMA-560  
812 mouse glioma cells (C) were irradiated as indicated and human or mouse NKG2DL  
813 were assessed as in (A).

814

815 **Fig. 3. NKG2DL induction is modulated by MGMT and depends on ATM.** A. Whole  
816 cell lysates of LN-18\_control or LN18\_shMGMT cells and LN-229\_control or LN-  
817 229\_MGMT were assessed by immunoblot for MGMT protein levels. Beta-actin was  
818 used as a control. Acute cytostatic and clonogenic effects after exposure to TMZ were  
819 determined by crystal violet staining at the indicated time points. B. The cells were  
820 exposed to TMZ and cell surface expression of MICA and MICB was determined after  
821 72 h by flow cytometry. Data are presented as SFI and mean  $\pm$  SD of 3 independent  
822 experiments is shown (\*p < 0.05; \*\*p < 0.01). C. LN-229 (left) or S-24 (right) cells were  
823 treated with KU-60019 or DMSO 4 h prior to TMZ exposure. Immunofluorescence  
824 images were acquired following pATM<sup>Ser1981</sup> staining (red). Nuclei are stained with  
825 DAPI (blue). D. LN-229 (left) or S-24 (right) were exposed to TMZ (upper row) or IR  
826 (lower panel) after ATM inhibition using KU-60019 or siRNA-mediated gene silencing.  
827 MICA and MICB cell surface expression were determined by flow cytometry. Data are  
828 presented as SFI and mean values  $\pm$  SD from 2 independent experiments are shown  
829 (\*p < 0.05; \*\*p < 0.01).

830

831



832 **Fig. 4. Exposure to TMZ or IR promotes glioma cell immunogenicity in a**  
833 **NKG2D-dependent manner.** A. Upper panel: LN-229 (left) or S-24 (right) cells, pre-  
834 exposed to TMZ (grey line) or DMSO control (black line) for 48 h, were used as  
835 target cells in a 3 h immune cell lysis assays using polyclonal NK (for LN-229) or  
836 NKL (for S-24) effector cells at various effector : target (E:T) ratios. Following TMZ  
837 treatment, viable glioma cells were counted before co-incubation with effector cells  
838 and immune-mediated cytotoxicity was corrected for spontaneous background lysis.  
839 Lower panel: NKL cells were pre-incubated with anti-NKG2D antibody or isotype  
840 control and subsequently used as effector cells in lysis assays with LN-229 or S-24  
841 glioma cells, either pre-exposed to TMZ or DMSO control, at an E:T ratio of 20:1. B.  
842 LN-229 or S-24 cells were irradiated with 2 Gy prior to use as target cells in 3 h lysis  
843 assays. The experimental setup was the same as in (A). In all figures mean +/- SD  
844 of triplicates from 1 representative out of 2 independent experiments is shown (\*p <  
845 0.05; \*\*p < 0.01).

846  
847 **Fig. 5. TMZ and IR induce NKG2DL *in vivo* in syngeneic glioma models and**  
848 **human glioblastoma patients have increased tumor-associated NKG2DL after**  
849 **radiochemotherapy.** A. Orthotopic tumor-bearing mice (SMA-560\_TurboFP in  
850 VM/Dk mice (left) or GL-261\_niRP in C57BL/6 mice (right)) received a single dose  
851 of local irradiation (12 Gy) on day 10 or TMZ (10 mg/kg/day) per oral gavage from  
852 day 7-11 after tumor implantation. Mice were sacrificed on day 12, tumors were  
853 dissociated and cells analyzed for NKG2DL cell surface expression by flow  
854 cytometry. Tumor cells were gated in the dot plot diagrams based on the fluorescent  
855 signal. Histograms represent mean fluorescence intensity of RAE-1, MULT-1 and

856 H60 on these cells. The diagrams summarize results of 5 mice per group. Data are  
857 presented as mean fluorescence intensity  $\pm$  SD (\*p < 0.05; \*\*p < 0.01). B. NKG2DL  
858 were assessed on mRNA (upper panel) and surface protein level (lower panel) in  
859 matched pairs of human primary and recurrent tumors. Positive cell surface staining  
860 events were quantified in an unsupervised fashion with the TMARKER toolkit. (\*p <  
861 0.05; ns = non-significant).

862

863 **Fig. 6. The NKG2D system contributes to the therapeutic effects of TMZ and**  
864 **IR in glioma.** A. SMA-560 tumor-bearing mice received injections of anti-NKG2D  
865 or isotype control antibody one day before and one day and then every 7 days  
866 after tumor implantation. Subsequently, the animals were treated with TMZ or  
867 solvent control from day 7-11 or a single dose of IR at day 12. Survival data are  
868 presented as Kaplan-Meier plots (left and center). Combined analysis of median  
869 survival is plotted on the right. Survival differences were compared between  
870 different treatment groups (\*p < 0.05; \*\*p < 0.01) and within a treatment group  
871 between isotype or anti-NKG2D treatment (+p < 0.05; ++p < 0.01). B-D. GL-261  
872 tumor-bearing C57BL/6 or NKG2D<sup>-/-</sup> mice were treated with IR (single local dose of  
873 12 Gy at day 10), TMZ (10 mg/kg p.o., day 7-11) or the combination of both. B.  
874 Survival data are presented as Kaplan-Meier plots (left and center). Combined  
875 analysis of median survival of the different groups is plotted on the right (\*p < 0.05;  
876 \*\*p < 0.01 between treatment groups and +p < 0.05; ++p < 0.01 within a treatment  
877 group for intact NKG2D vs. NKG2D<sup>-/-</sup>). C. T2-weighted coronal scans were  
878 acquired at day 13 after tumor implantation. Two representative scans for each  
879 group are shown (left). The white arrow marks the tumor region. Mean  $\pm$  SD of

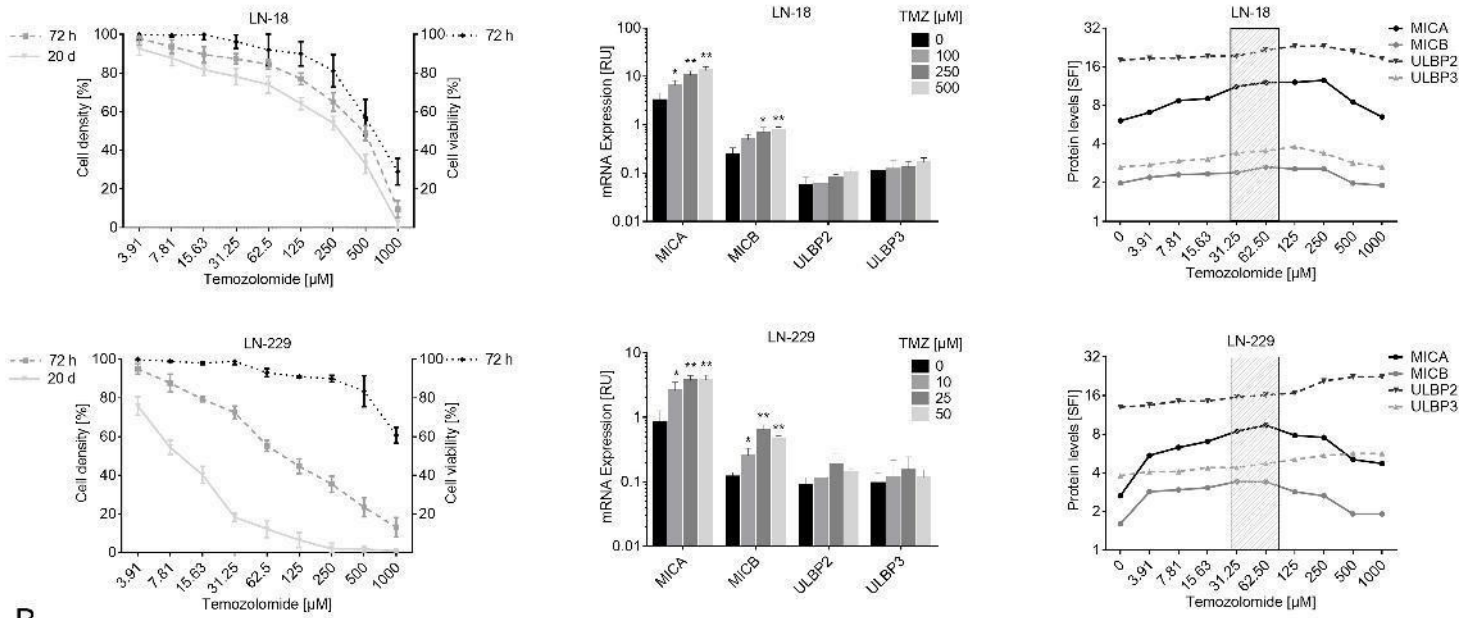
880 the tumor volume in mm<sup>3</sup> from 4 mice/group is shown (right). D. Percentage of NK,  
881 CD4, CD8, and  $\gamma\delta$  T cells (left) and the corresponding IFN- $\gamma$  secretion (right) of  
882 tumor-infiltrating lymphocytes derived from mice described in B and C were  
883 determined at day 14 after tumor implantation. Mean +/- SD from 3 mice is shown  
884 (\*p < 0.05; \*\*p < 0.01 between treatment groups and +p < 0.05; ++p < 0.01 within  
885 a treatment group for intact NKG2D vs. NKG2D<sup>-/-</sup>).

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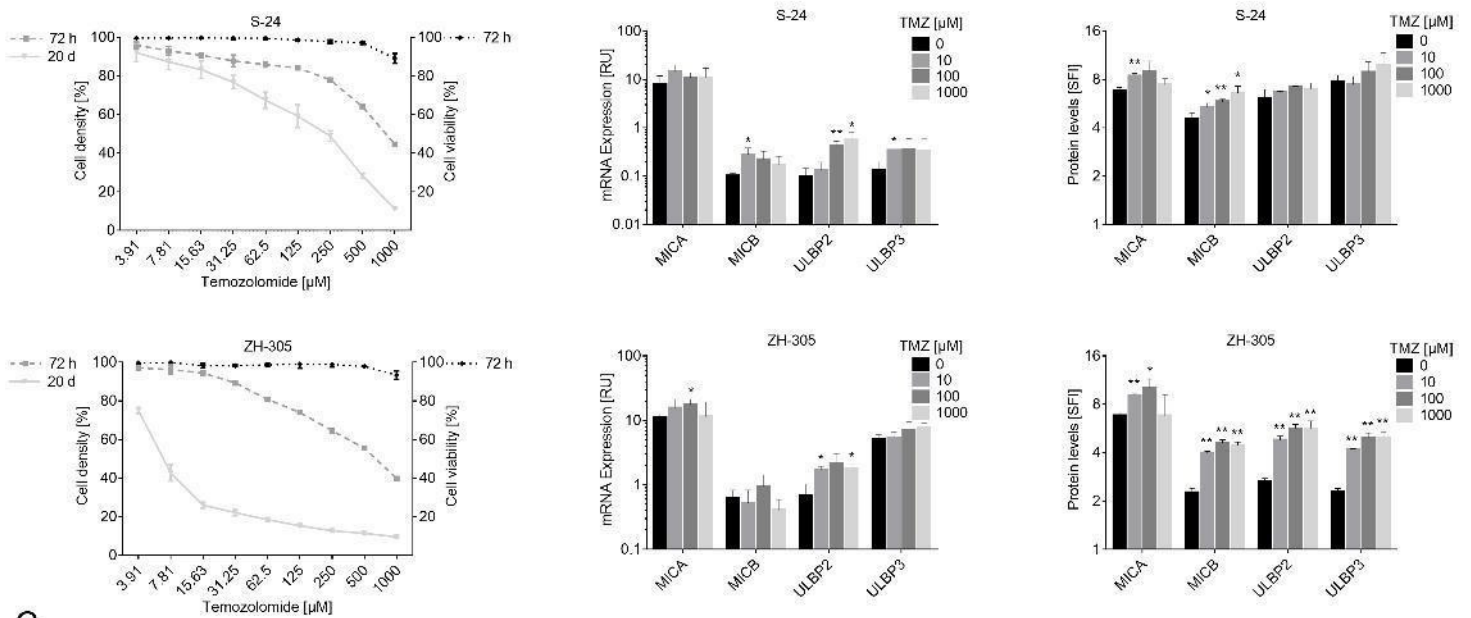
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Fig. 1

A



B



C

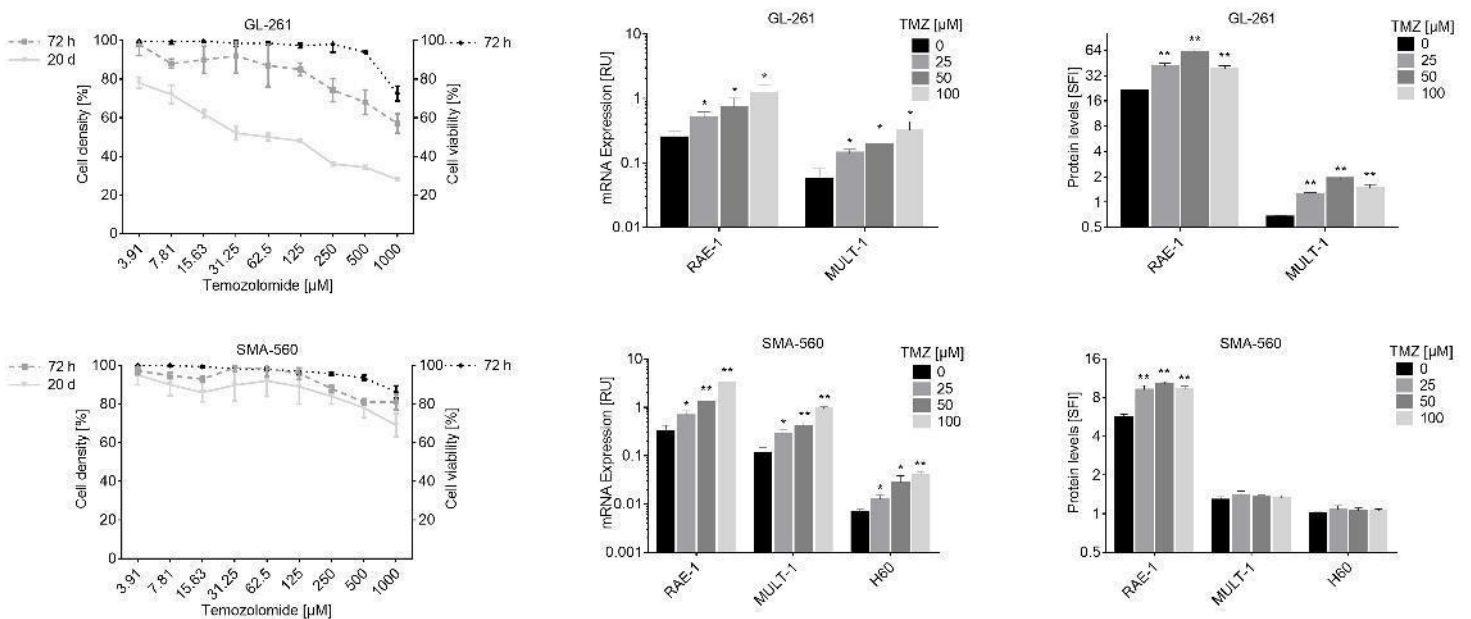
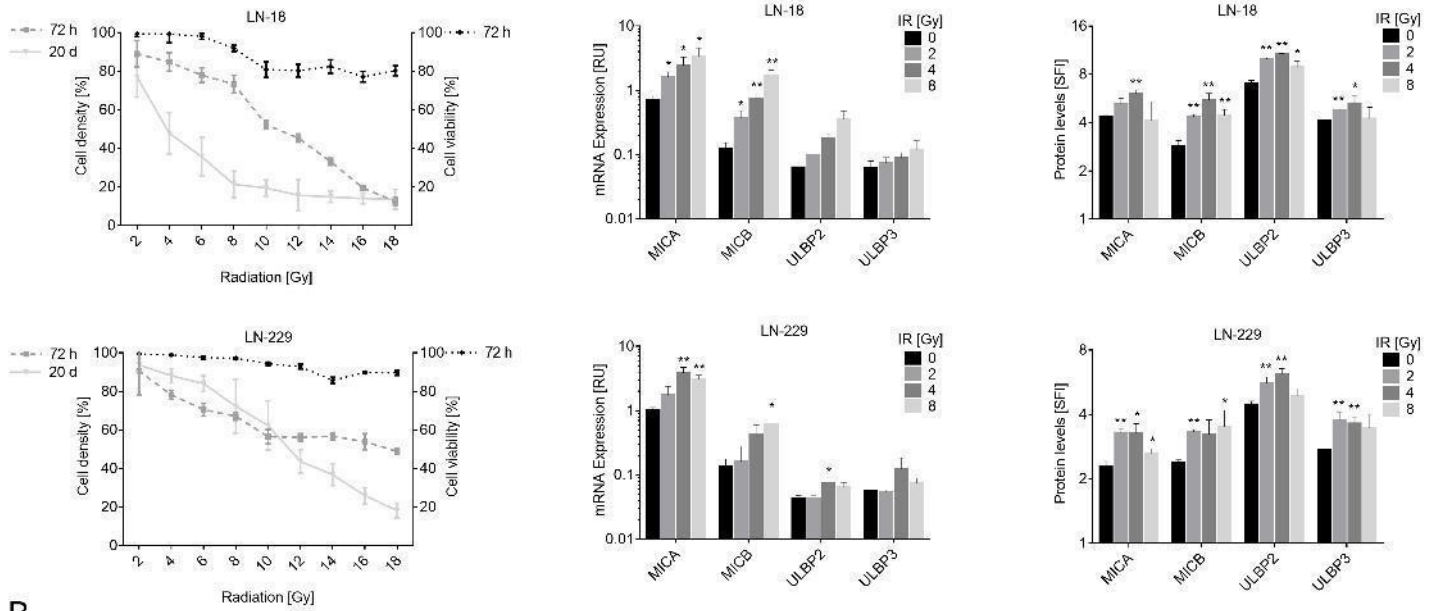
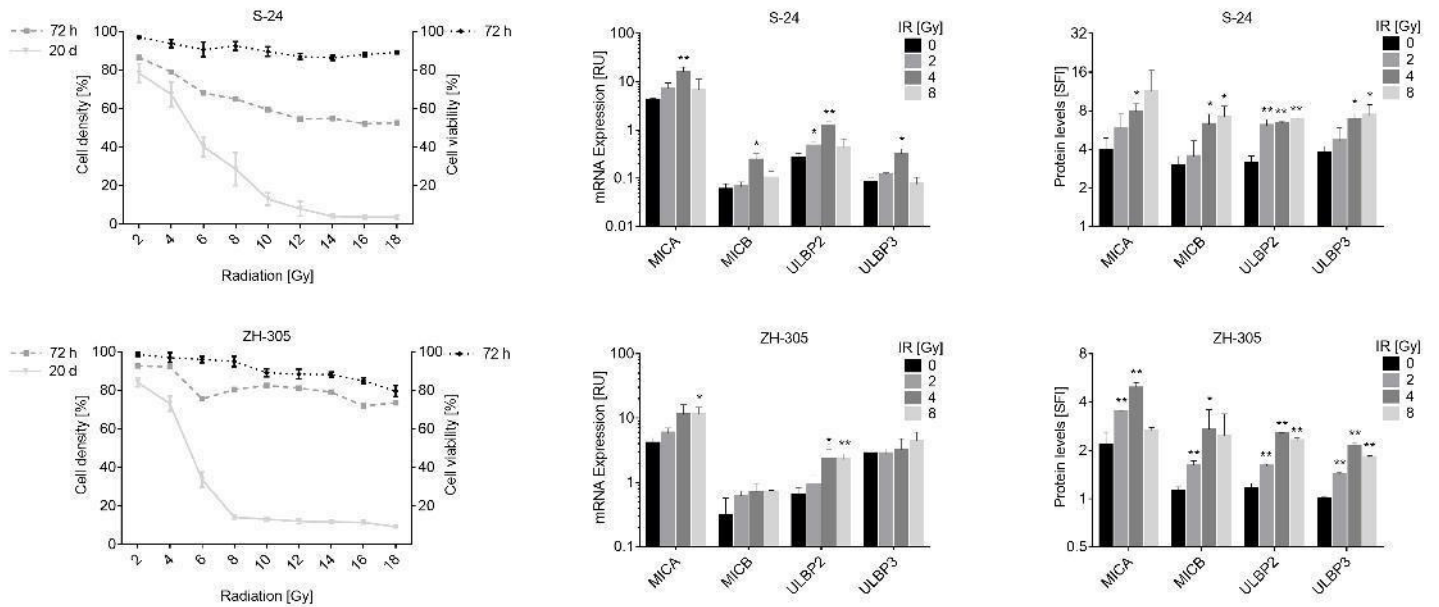


Fig. 2

A



B



C

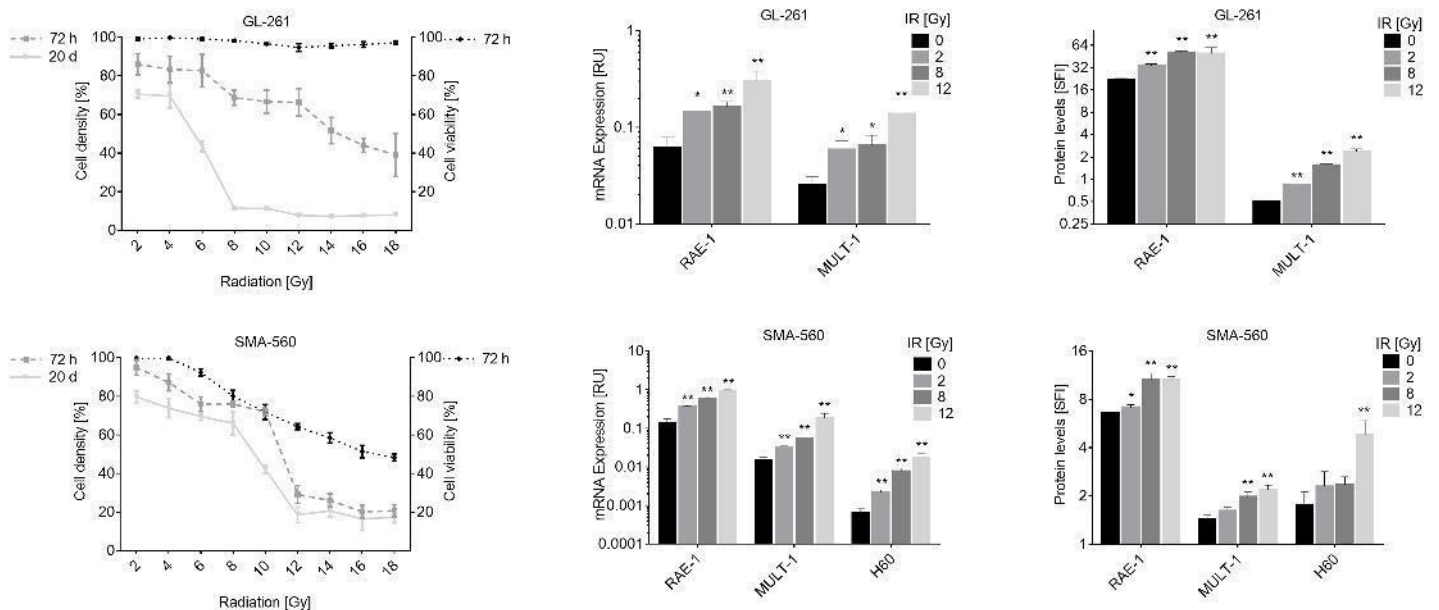
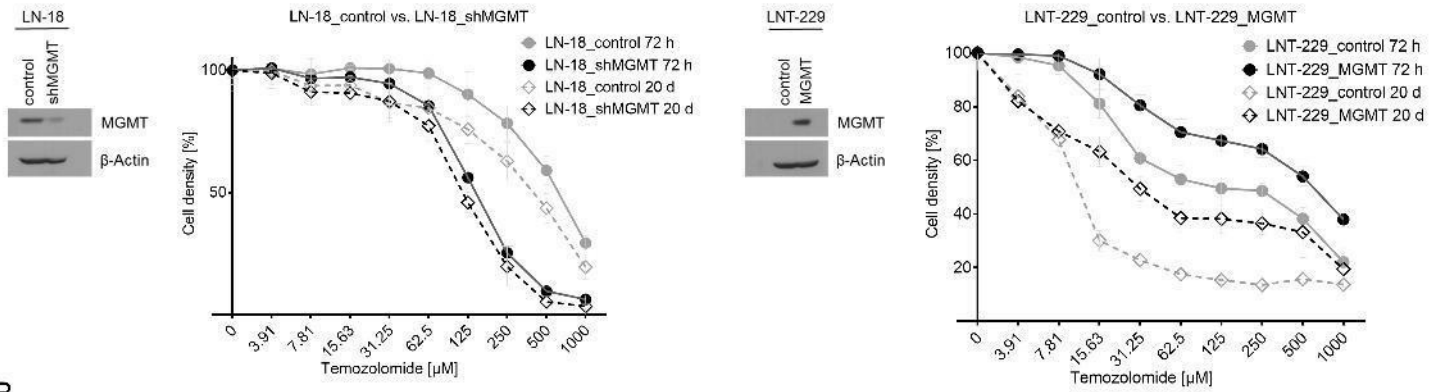
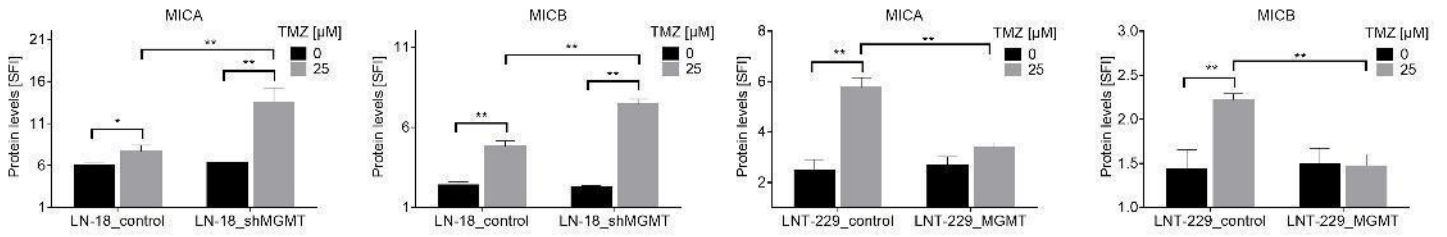


Fig. 3

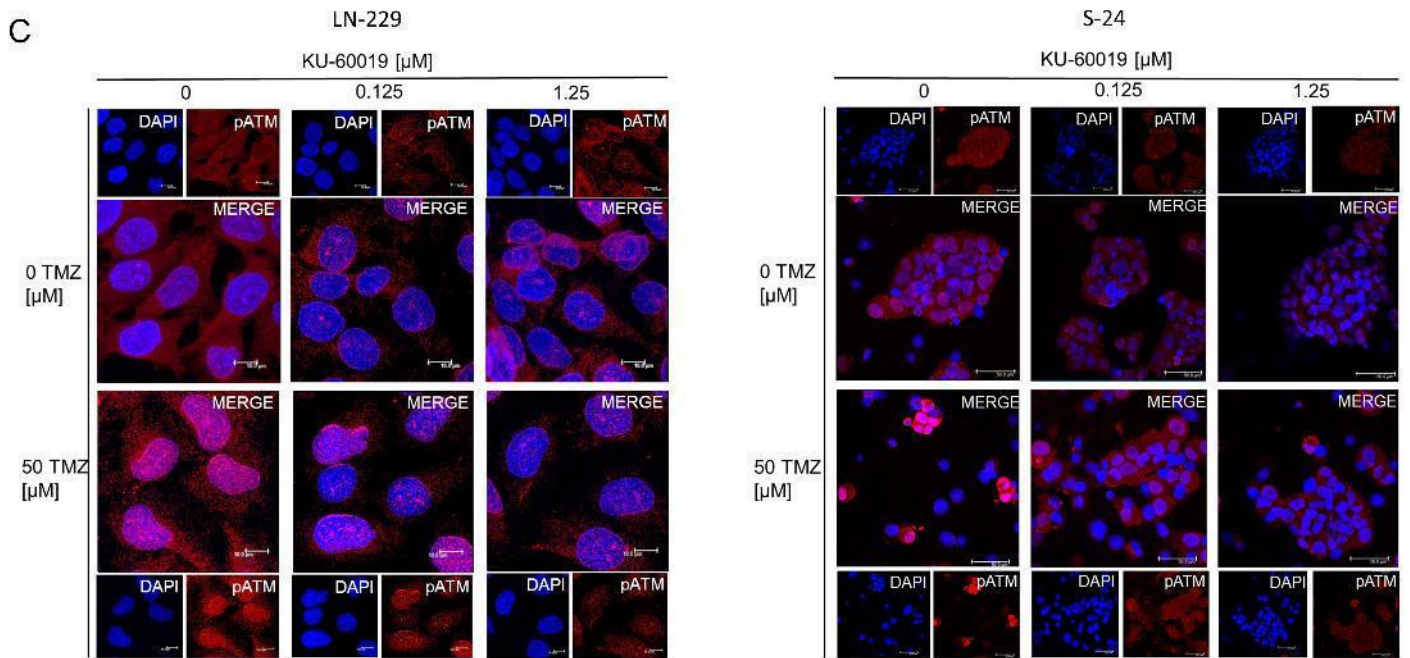
A



B



C



D

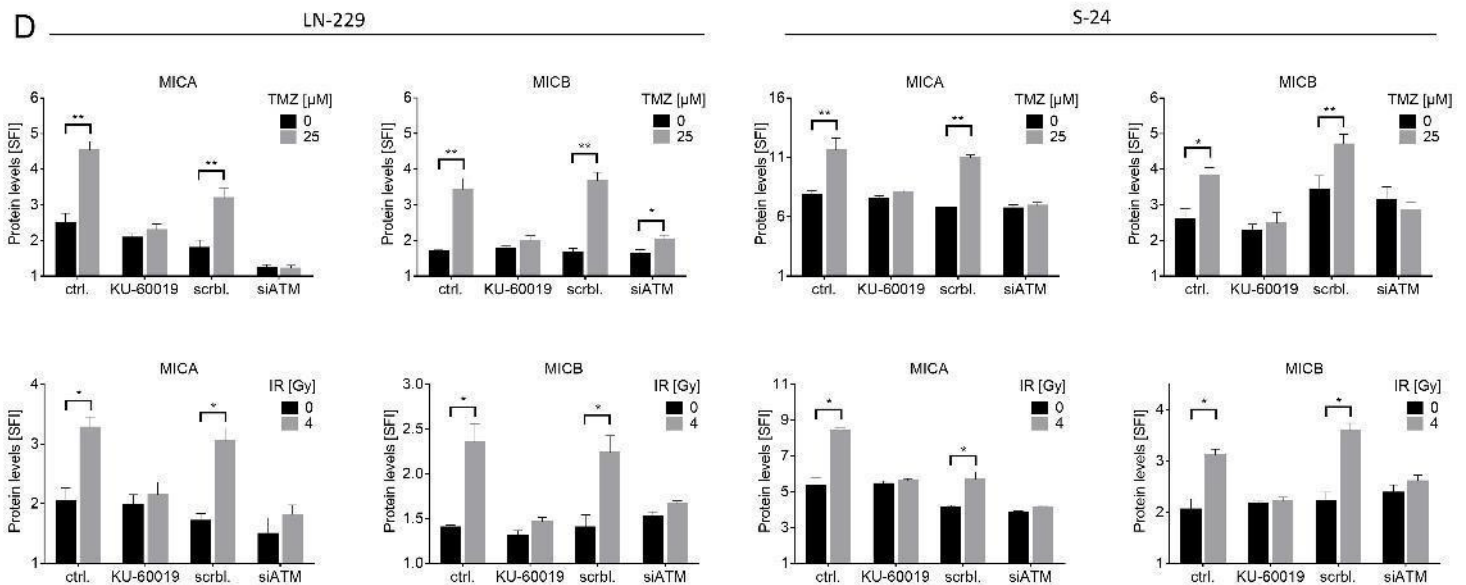
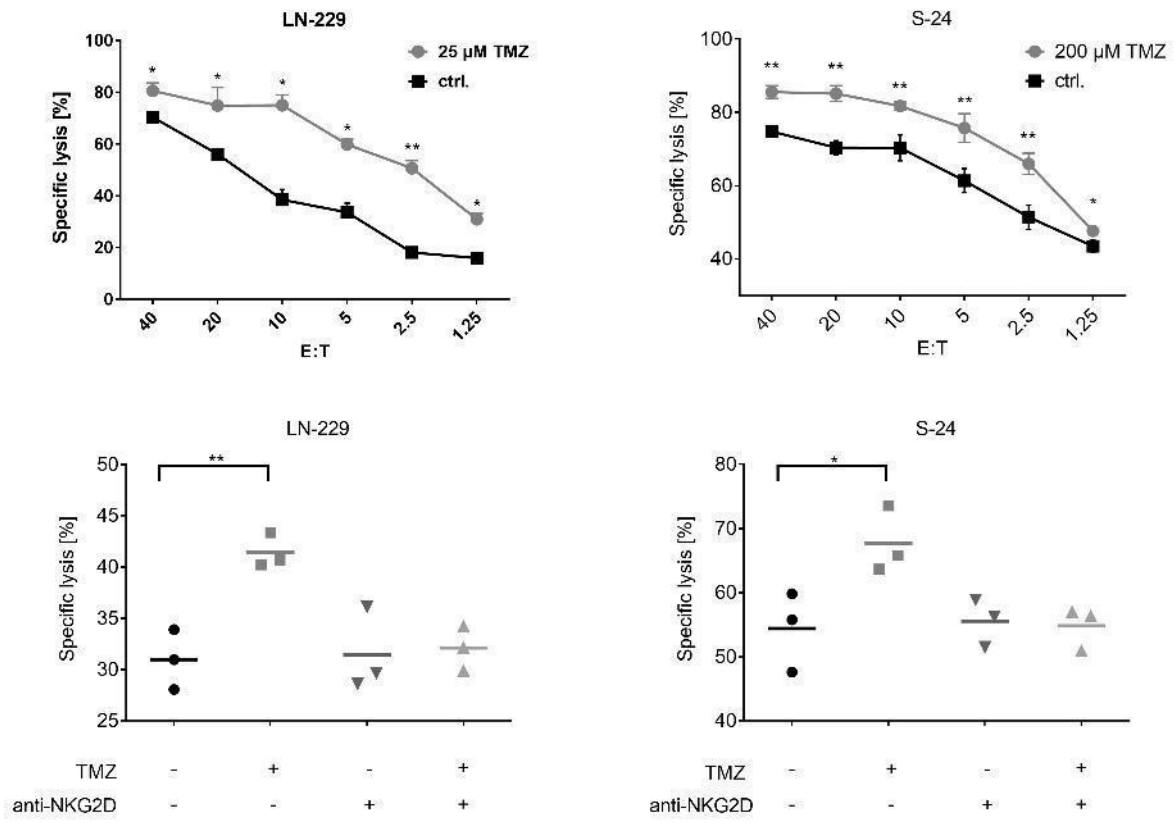


Fig. 4

A



B

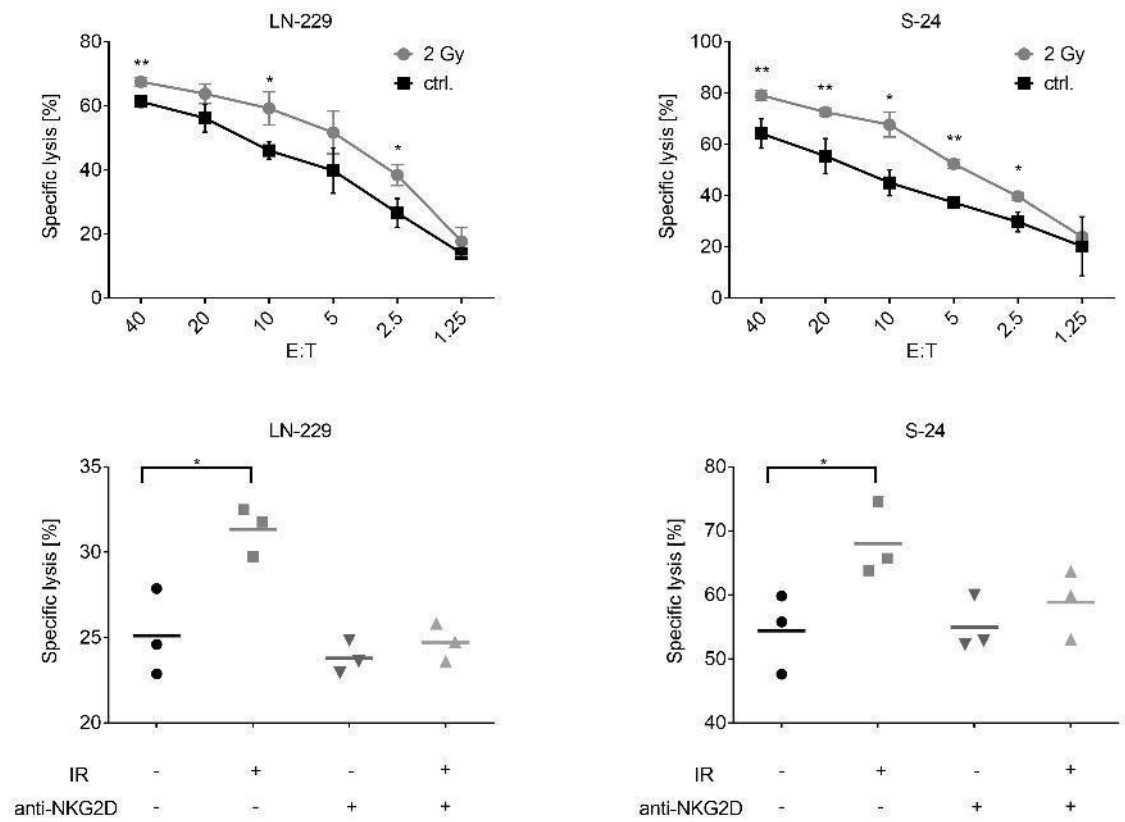


Fig. 5

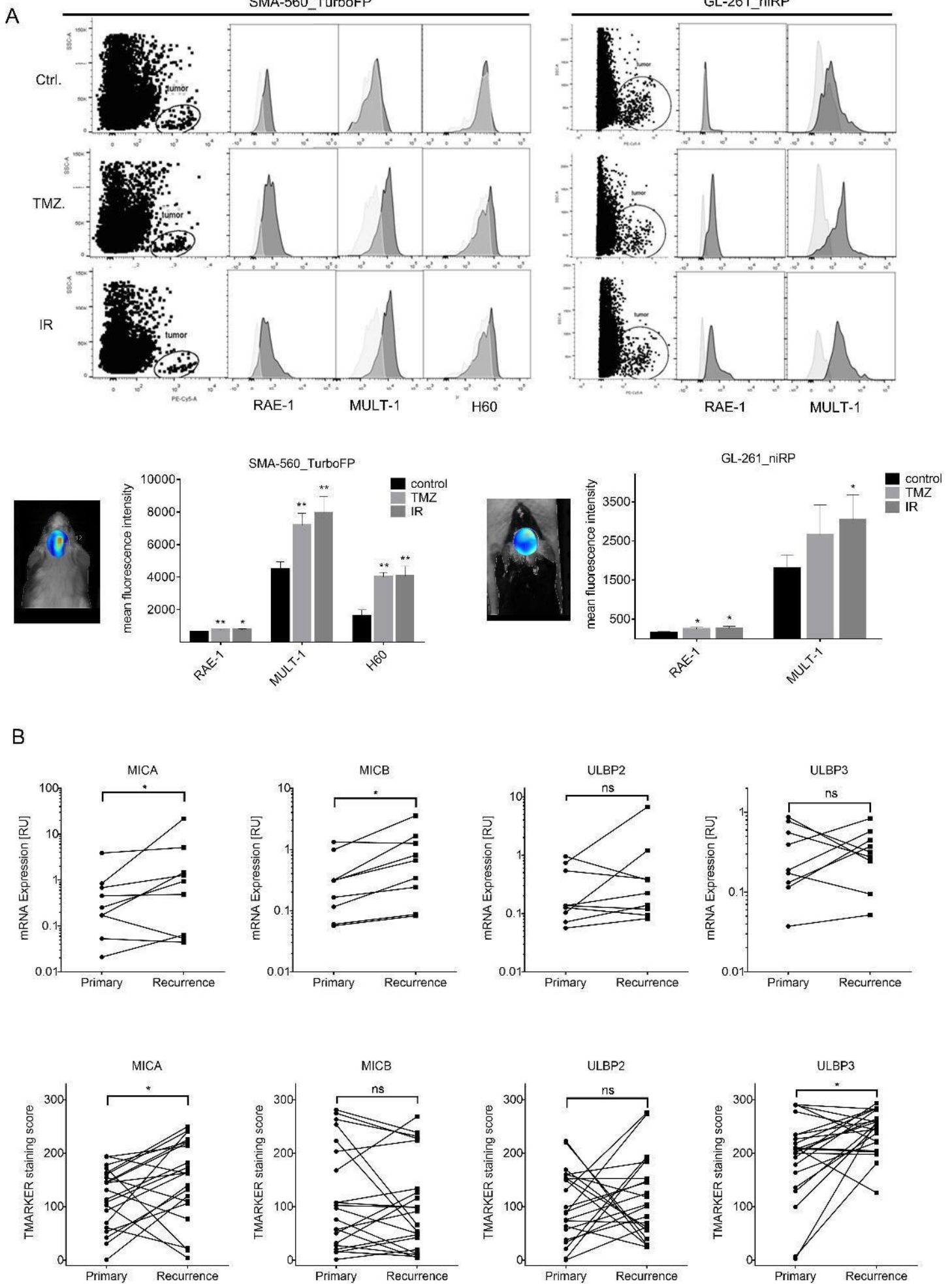
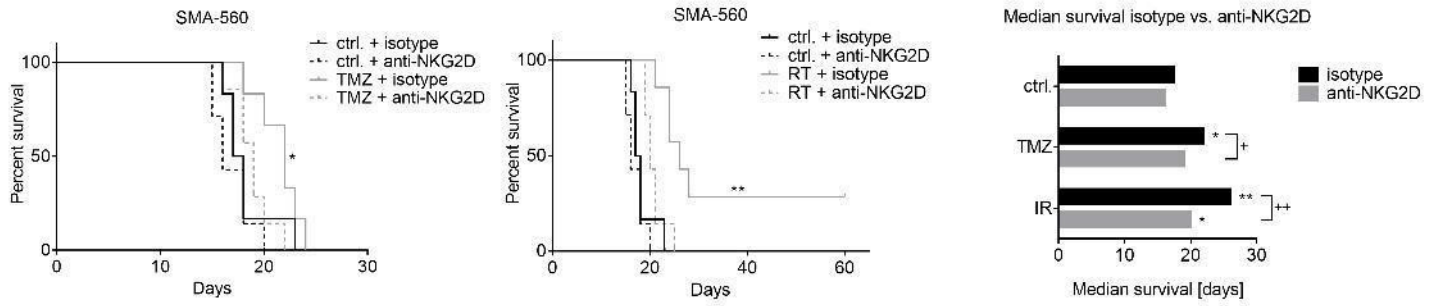


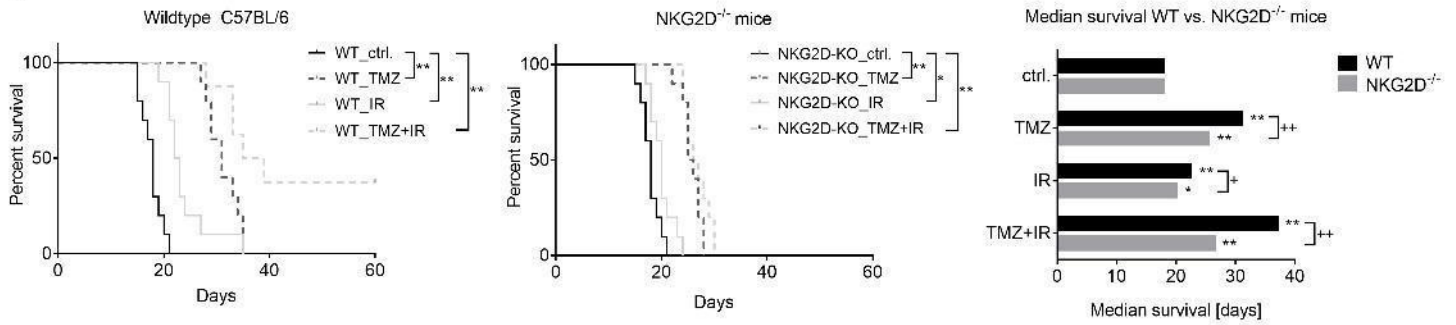


Fig. 6

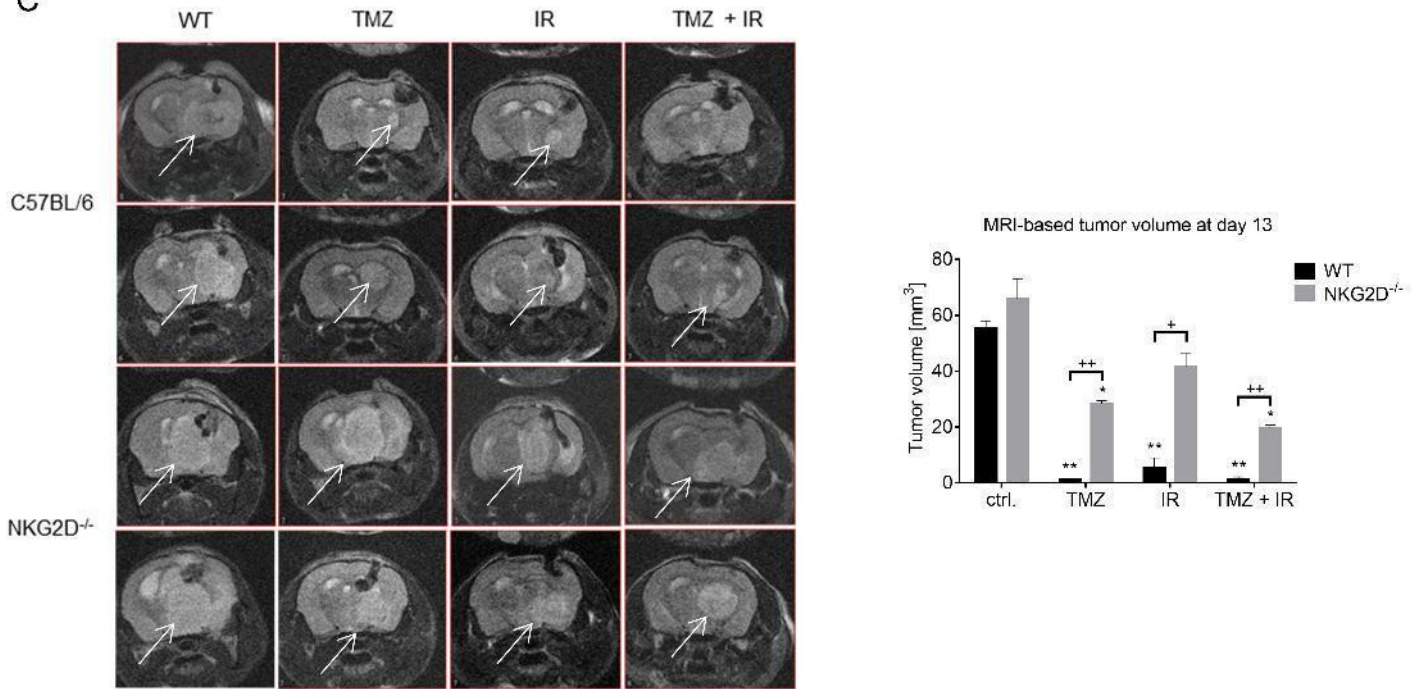
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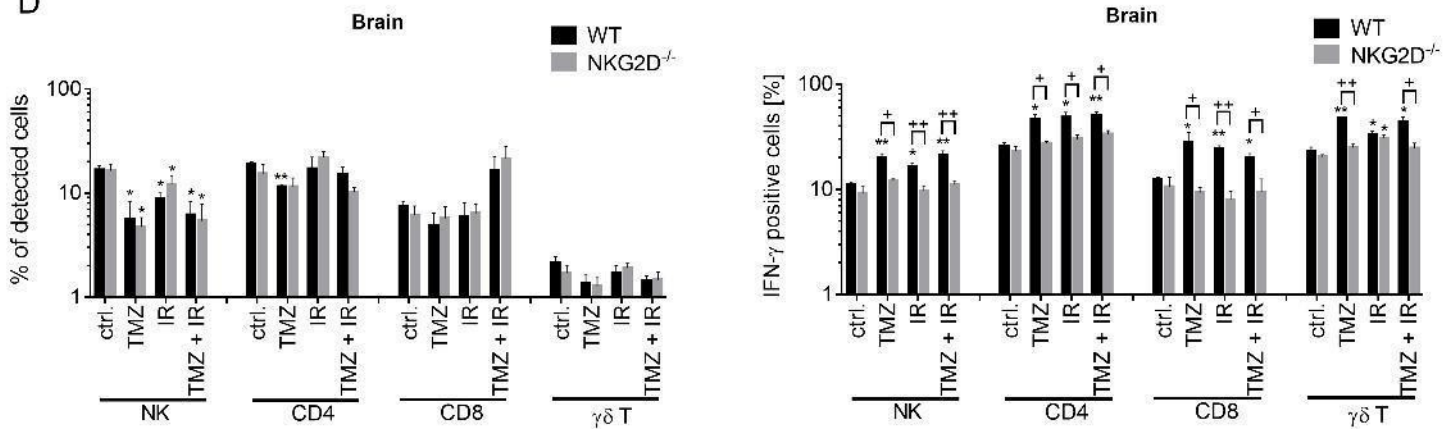
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C

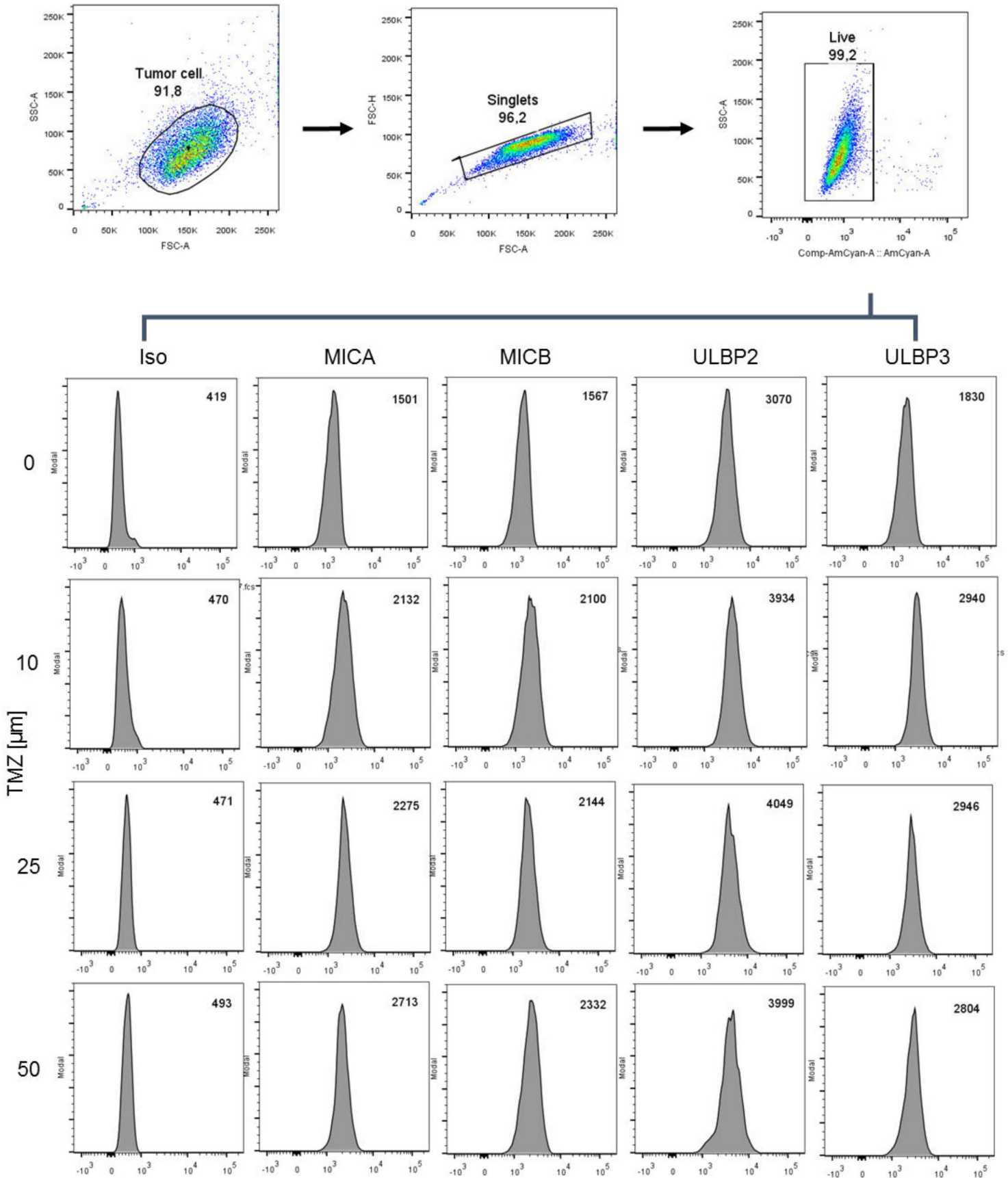


D



# Supplementary Materials:

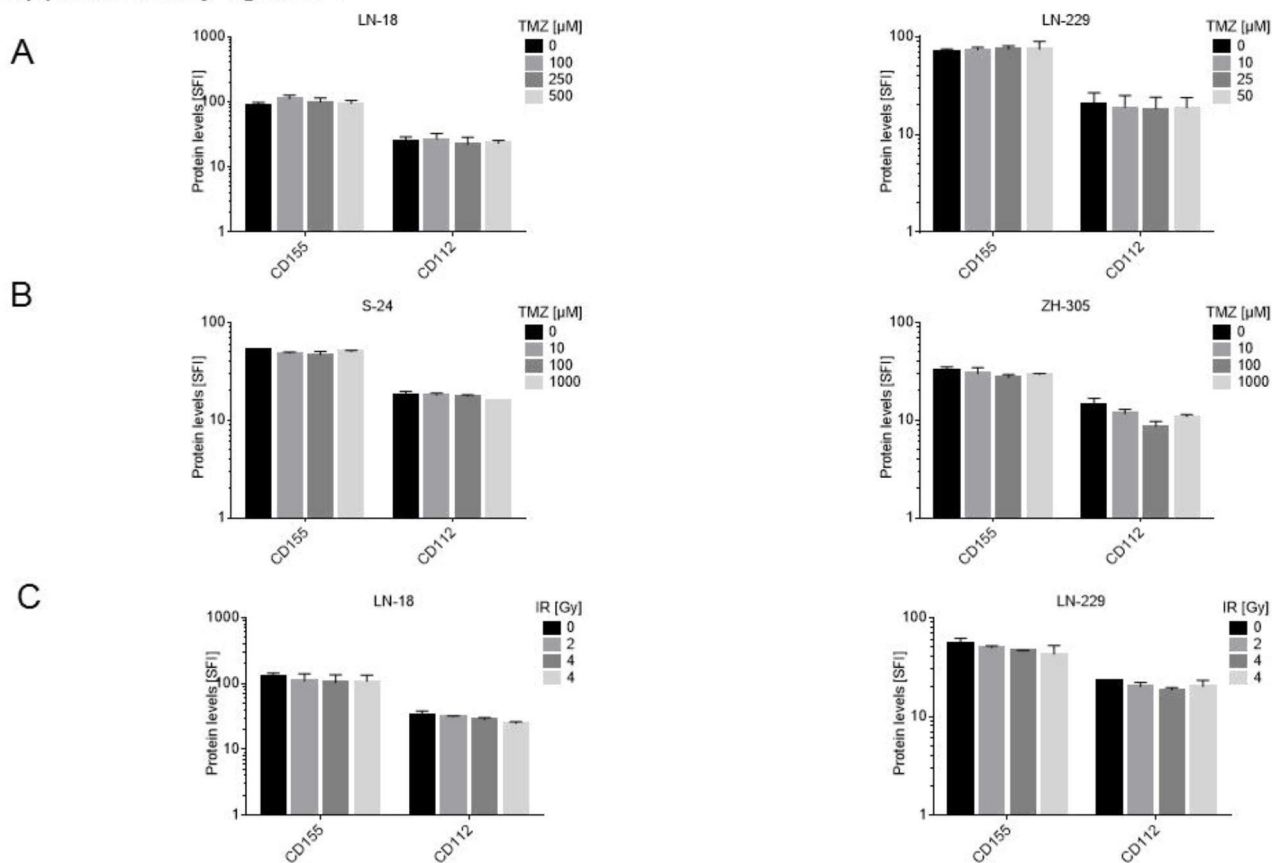
## Supplementary figure 1



**Suppl. Fig. 1. Gating strategy for detection of cell surface NKG2DL.**

Representative data for LN-229 cells 72 h after treatment with different concentrations of TMZ is shown. Within the tumor cell population, we gated on single living cells. Cell surface expression after staining with isotype control, anti-MICA, anti-MICB, anti-ULPB2, or anti-ULBP3 are displayed in histograms. Numbers in the upper right corners indicate the mean fluorescence intensity, which allows further calculation of specific fluorescence indexes (SFI) by dividing median fluorescence obtained with the specific antibody by median fluorescence obtained with isotype control antibody.

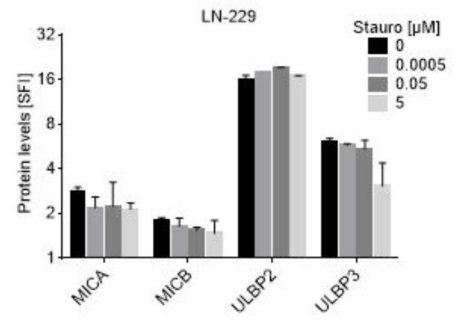
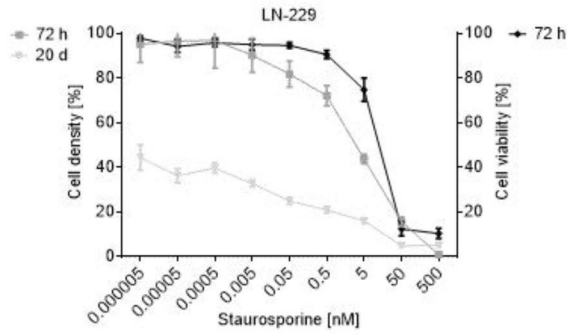
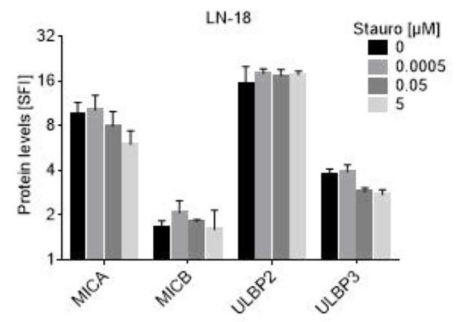
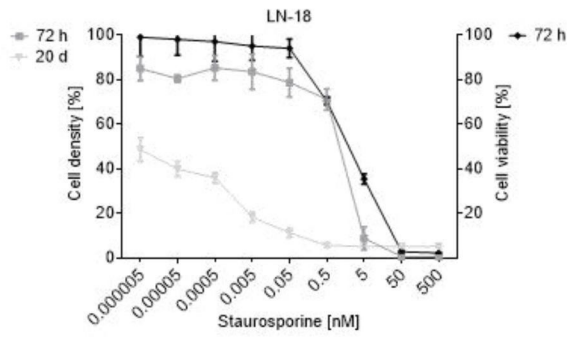
## Supplementary figure 2



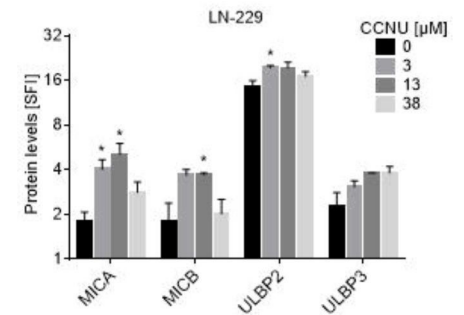
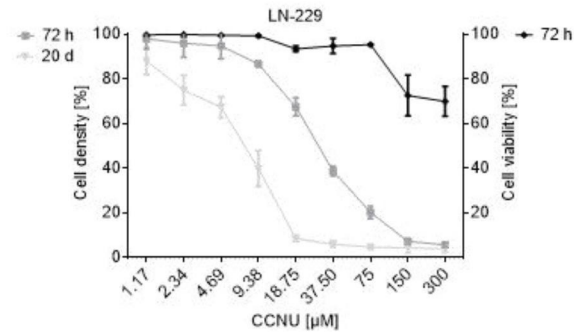
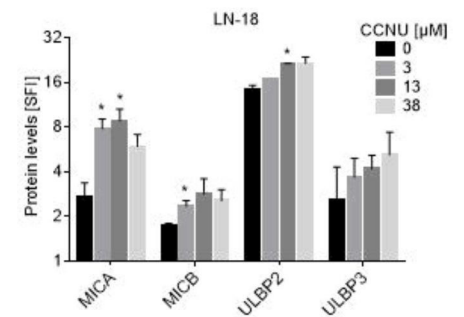
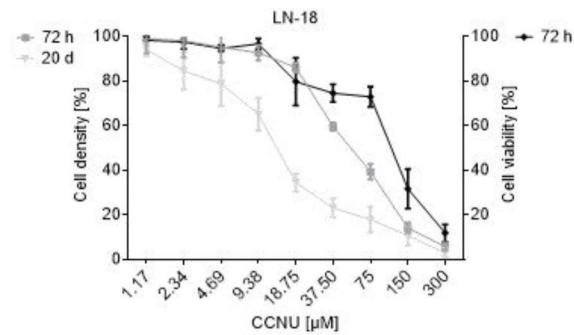
**Suppl. Fig. 2. DNAM-1 ligands are not induced upon treatment of TMZ or IR.** CD112 and CD155 protein levels at the cell surface of LN-18 or LN-229 cells (A) or S-24 or ZH-305 (B) were determined by flow cytometry 72 h after exposure to TMZ or DMSO or single irradiation of LN-18 or LN-229 cells (C). Data are shown as SFI and median $\pm$  SD from 3 independent experiments is shown.

### Supplementary figure 3

A



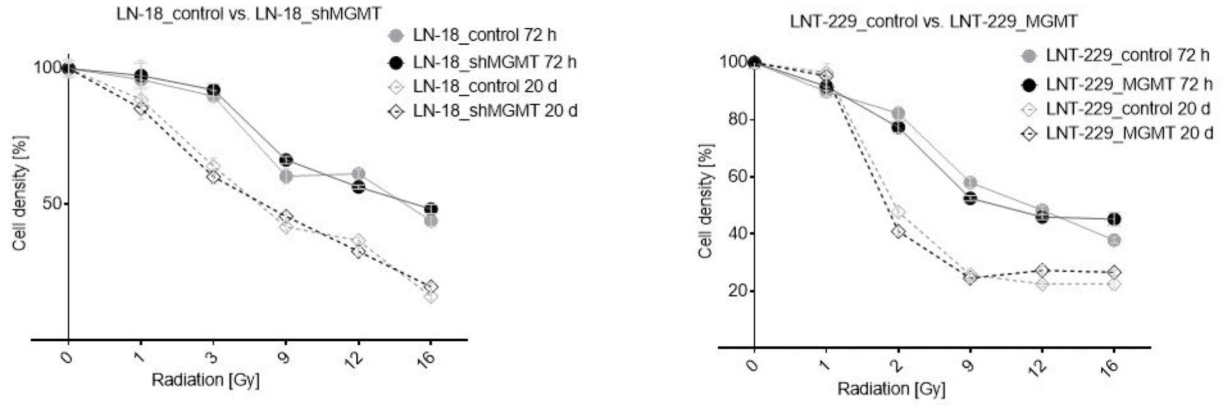
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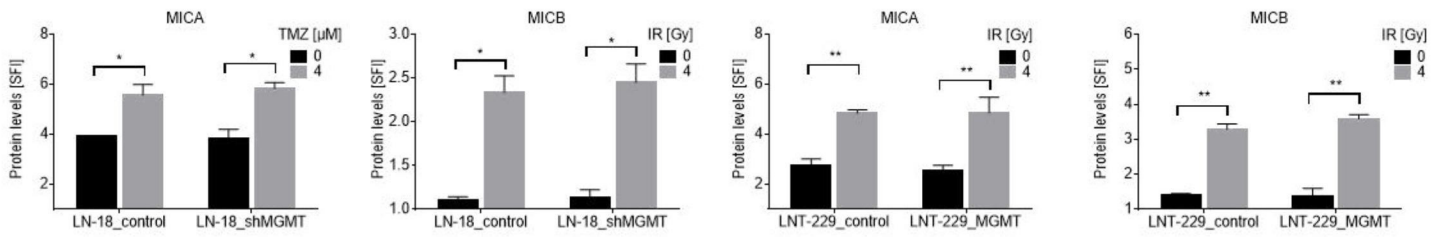
**Suppl. Fig. 3. Induction of NKG2DL is not an unspecific response to cell death induction in glioma cells.** A. LN-18 (upper panel) or LN-229 (lower panel) cells were exposed to different concentrations of staurosporine. Viability was assessed by live/dead staining at 72 h (black line), cytostatic effects were detected by crystal violet staining at 72 h and 20 d (grey lines) (left panels). NKG2DL protein levels at the cell surface were determined by flow cytometry following exposure to staurosporine or DMSO control for 72 h (right panels). Data are presented as SFI and mean values  $\pm$  SD from 2 independent experiments are shown (\* $p < 0.05$ ; \*\* $p < 0.01$ ). B. Same experimental setup as in A, but the cells were exposed to different concentrations of CCNU.

# Supplementary figure 4

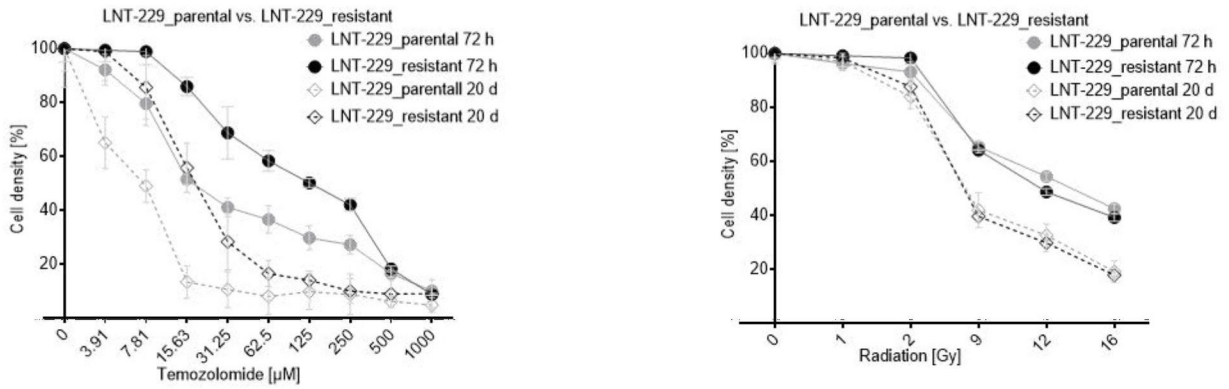
**A**



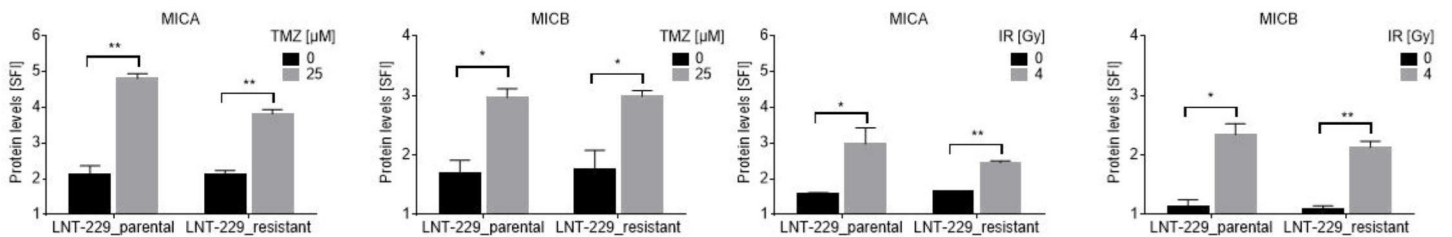
**B**



**C**



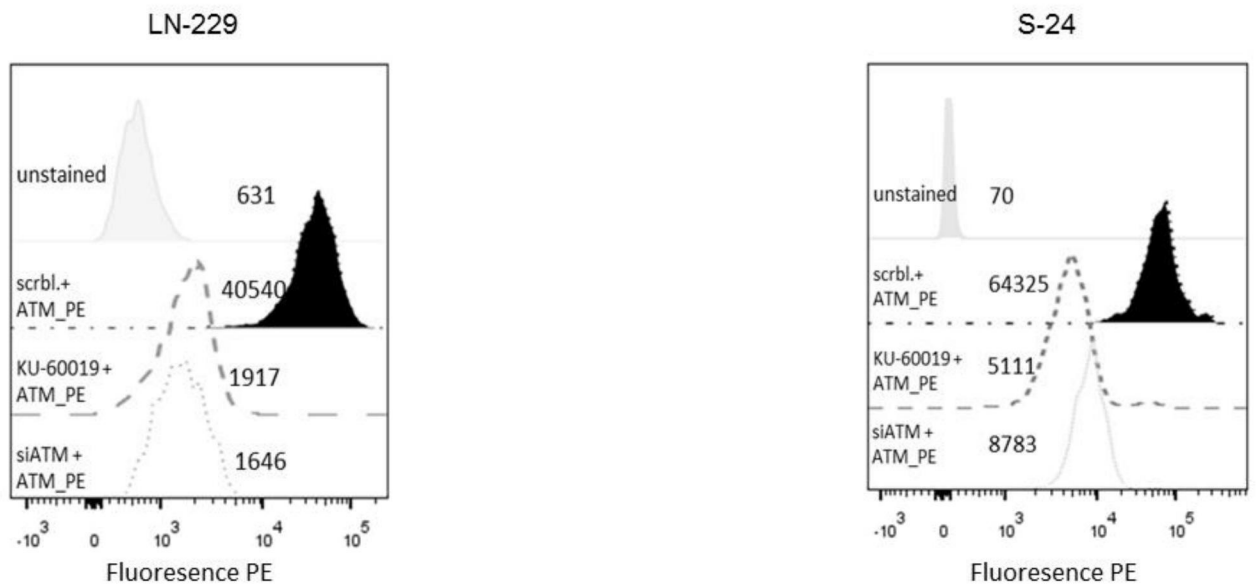
**D**



**Suppl. Fig. 4. MGMT does not affect irradiation-induced NKG2DL expression and NKG2DL can be induced in glioma cells with acquired TMZ resistance**

A. LN-18\_shMGMT and the corresponding LN-18\_control cells or LN-229\_MGMT and the corresponding LN-229\_control cells were irradiated with increasing doses. Cell density was assessed by crystal violet staining after 72 h (acute cytostatic effect) or 20 days (clonogenic cell survival), respectively. B. On the cells described in A, cell surface expression of MICA and MICB was determined after 72 h by flow cytometry. Data are presented as SFI and mean values  $\pm$  SD from 2 independent experiments are shown (\* $p < 0.05$ ; \*\* $p < 0.01$ ). C. LNT-229 cells with acquired resistance to TMZ or parental control cells were exposed to different concentrations of TMZ (upper panel, left graph) or doses of irradiation (upper panel, right graph). Cytostatic effects were detected by crystal violet staining at 72 h and 20 d. D. On the cells described in C, NKG2DL protein levels at the cell surface were determined by flow cytometry 72 h after exposure to TMZ (left graph) or irradiation (right graph). Data are presented as SFI and mean values  $\pm$  SD from 2 independent experiments are shown (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

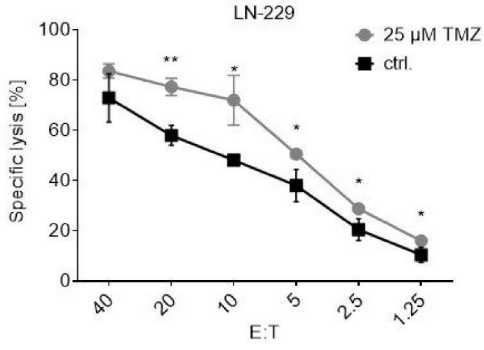
## Supplementary figure 5



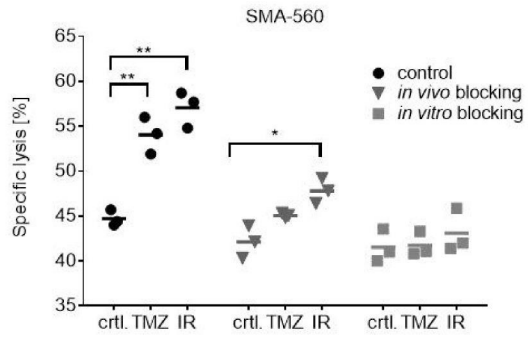
**Suppl. Fig. 5. Inhibition of ATM in LN-229 or S-24 cells.** LN-229 or S-24 cells were exposed to 1.25  $\mu$ M of KU-60019 or siRNA oligonucleotides specific for ATM or scrambled control. After 72 h, the cells were stained with anti-phospho-ATM<sup>Ser1981</sup> PE antibody and assessed by flow cytometry. Data are presented as mean fluorescence intensity (indicated by numbers).



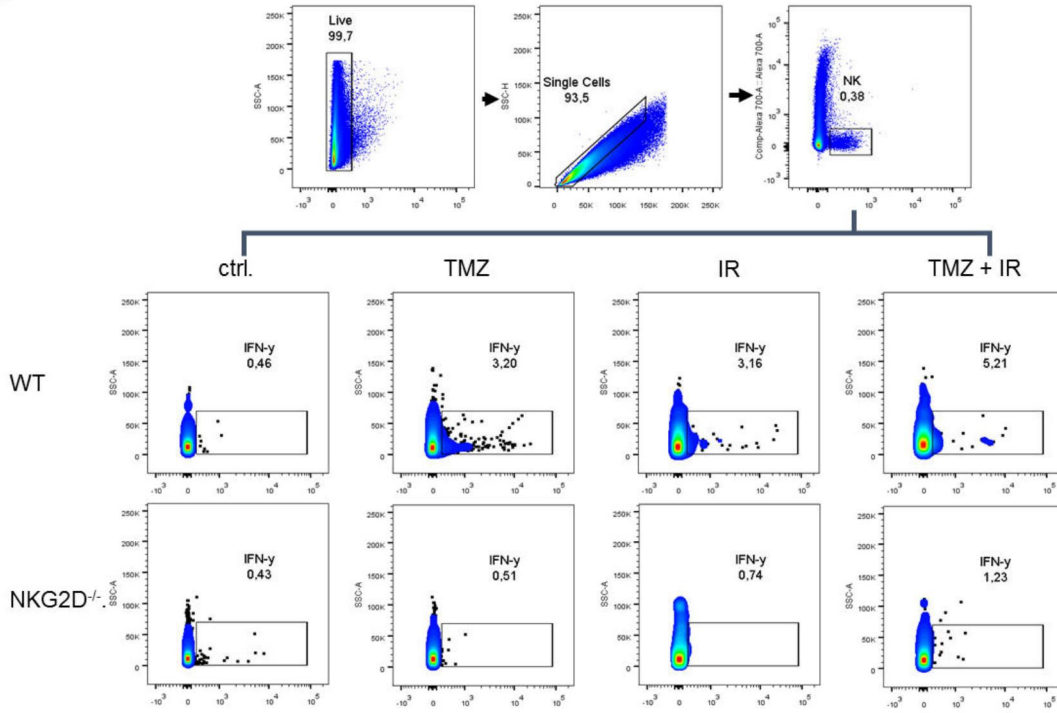
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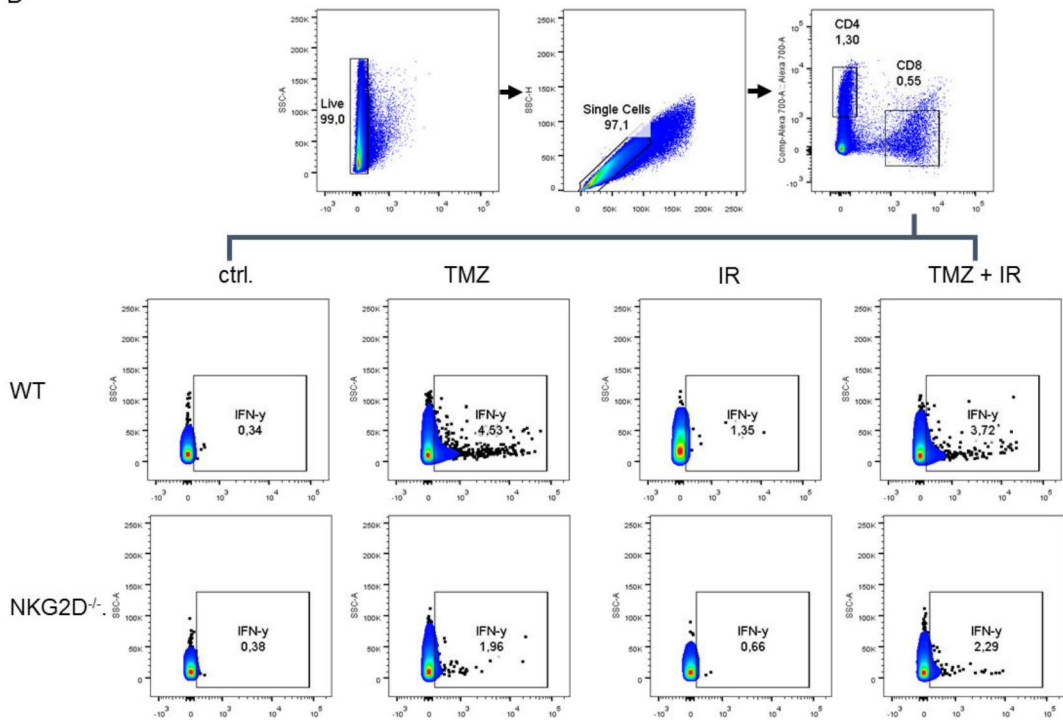
B



C



D



**Suppl. Fig. 6. Functional consequences of NKG2DL induction by TMZ or IR.** A. Exposure to TMZ promotes immune-cell mediated glioma cell lysis. LN-229 cells, pre-exposed to TMZ (grey line) or DMSO control (black line) for 48 h, were used as target cells in a 3 h lysis assays using NKL effector cells at various effector : target (E:T) ratios. B. Blocking of NKG2D signaling with an inhibitory antibody *in vivo*. SMA-560 tumor-bearing mice that were treated with vehicle ctrl. (day 7-11), TMZ (day 7-11) or local IR (10 Gy at day 10) and received injections of anti-NKG2D or isotype control antibody one day before and every 7 days after tumor implantation. At day 14, splenocytes from these mice were used as effector cells in immune cell lysis assays. Target cells were SMA-560 cells pre-treated *in vitro* with TMZ, RT or not. E:T was 20:1. As an additional control, splenocytes from non tumor-bearing, untreated mice were pre-treated *ex vivo* with blocking anti-NKG2D antibody and used as effector cells against the described target cells. C and D. Gating strategy for detection of tumor-infiltrating NK, CD4 and CD8 T cells. Fourteen days after tumor implantation, tumor-infiltrating lymphocytes were isolated after tumor dissociation and Percoll separation. NKp46<sup>+</sup>CD3<sup>-</sup> cells were determined as percentage of NK cells (C). Numbers indicate the percentage of positive cells. Furthermore, CD4 and CD8 positive cells were gated (D). Plots are representative for one out of three mice.