DISSERTATION

Untersuchung des Einflusses von KDM1A auf die Neuroblastom-spezifische CAR-T-Zelltherapie

Analyzing the influence of KDM1A on neuroblastoma-specific CAR T cell therapy

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von

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List of abbreviations

CAR – chimeric antigen receptor

DNA – desoxyribonucleic acid

E:T – Effector to target Ratio

EGFRt – truncated epidermal growth factor receptor

FACS – fluorescence-activated cell sorting

FCS – Fetal calf serum

gRNA – guide RNA

IFNG – Interferon gamma

KO – knockout

RNA – ribonucleic acid

1 Abstract

Vormerkung: Diese Dissertation fasst meine Arbeit zum Thema „Untersuchung des Einflusses von KDM1A auf die Neuroblastom-spezifische CAR-T-Zelltherapie" als Publikationspromotion zusammen. Der Text der vorliegenden Zusammenfassung basiert zu großen Anteilen auf der Originalpublikation „Inhibiting Lysine Demethylase 1A improves L1CAM-specific CAR T cell therapy against neuroblastoma by unleashing antigen-independent killing via the FAS-FASL axis", für die ich das Manuskript erstellt habe.

1.1 Kurzfassung Deutsch

Das Neuroblastom ist der häufigste bösartige extrakranielle solide Tumor bei Kindern und entsteht aus der sympathoadrenalen Linie der Neuralleiste. Die Behandlung refraktärer oder rezidivierter Neuroblastome bleibt eine große Herausforderung in der pädiatrischen Onkologie. Ein innovativer immuntherapeutischer Ansatz verwendet chimäre Antigenrezeptor (CAR)-T-Zellen, um auf Antigene abzuzielen, die spezifisch und stark auf Neuroblastomzellen exprimiert werden, wie z. B. L1CAM. Eine große Herausforderung ist jedoch das Entkommen Antigen-negativer Tumorzellen, die ein Wiederauftreten des Tumors verursachen. Enzyme, die an der epigenetischen Genregulation beteiligt sind, wie das Histon-demethylierende Enzym, Lysin-spezifische Demethylase 1 (KDM1A, früher bekannt als LSD1), sind dafür bekannt, dass sie die Expression verschiedener Anti-Tumor-Gennetzwerke unterdrücken. Die Reexpression dieser Gene durch KDM1A-Hemmung könnte die Wirksamkeit einer CAR-T-Zelltherapie steigern. Daher könnte die Kombination der KDM1A-Hemmung mit der CAR-T-Zelltherapie eine neue Strategie zur besseren Bekämpfung dieser bösartigen Erkrankung bieten. In dieser Studie wurden Veränderungen in der Genexpression in etablierten Neuroblastom-Zelllinien identifiziert, die durch KDM1A-Suppression verursacht werden und die zur Verbesserung der CAR-T-Zelltherapie gegen das Neuroblastom genutzt werden könnten. Zuerst wurde das Niveau der KDM1A-Expression in Neuroblastom-Zelllinien sowie in primären T-Zellen bestimmt. Neuroblastom-Zelllinien mit unterschiedlichen KDM1A-Expressionsniveaus wurden titrierten Mengen an KDM1A-Inhibitor (SP-2509) ausgesetzt und IC50-Werte wurden durch Analyse des Zellüberlebens bestimmt. Ich konnte zeigen, dass KDM1A in allen analysierten Neuroblastom-Zelllinien stark exprimiert wurde. Im Gegensatz dazu war die KDM1A-Expression in primären T-Zellen signifikant geringer, aber immer noch nachweisbar. Wichtig ist, dass die CAR-T-Zellen im Vergleich zu den Tumorzellen empfindlicher auf die KDM1A-Inhibitorbehandlung reagieren, was auf die Notwendigkeit einer sequenziellen und nicht gleichzeitigen Behandlungsstrategie für Experimente hinweist, in denen CAR-T-Zellen und KDM1A-Inhibitoren kombiniert werden. Als ich die ausgewählten Neuroblastom-Zelllinien mit SP-2509 in ihren jeweiligen IC50-Konzentrationen behandelte, zeigte sich eine erhöhte FAS-Zelloberflächenexpression in streng TP53-abhängiger Weise. FAS-Hochregulierung sensibilisierte Neuroblastomzellen für FAS-FASL-abhängige Abtötung und verstärkte L1CAM-gerichtete CAR-T-Zelltherapie gegen Antigen-arme oder sogar Antigen-negative Tumorzellen in vitro. Das verbesserte therapeutische Ansprechen wurde aufgehoben, wenn die FAS-FASL-Wechselwirkung mit einem antagonistischen FAS-Antikörper unterbunden wurde. Meine Ergebnisse zeigen, dass die KDM1A-Hemmung einen Antigen-unabhängigen Abtötungsmechanismus der Tumorzellen über die FAS-FASL-Achse auslöst. Dies könnte Tumorzellvarianten, die die Antigenexpression unter

CAR-T-Zelltherapie teilweise oder vollständig unterdrücken, dennoch für eine CAR-T-Zelltherapie in Frage kommen.

1.2 Abstract englisch

Neuroblastoma, a malignant extracranial solid tumor, is a common childhood cancer that is difficult to treat when refractory or relapsed. An innovative immunotherapeutic approach involves using chimeric antigen receptor (CAR) T cells to target neuroblastoma cells expressing high levels of specific antigens like L1CAM. However, the emergence of antigen-negative tumor cells poses a major challenge leading to tumor recurrence. KDM1A, an enzyme involved in epigenetic gene regulation, has been found to suppress anti-tumor gene networks. Inhibiting KDM1A may reactivate these genes and enhance the effectiveness of CAR T cell therapy. Therefore, combining KDM1A inhibition with CAR T cell therapy is a promising strategy to combat neuroblastoma. The study aimed to identify changes in gene expression caused by KDM1A suppression that could improve CAR T cell therapy against neuroblastoma. The level of KDM1A expression was determined in neuroblastoma cell lines and primary T cells. Neuroblastoma cell lines with different levels of KDM1A expression were treated with varying concentrations of KDM1A inhibitor, and the IC50 values were calculated by analyzing cell survival. The findings revealed that KDM1A was highly expressed in all neuroblastoma cell lines but expressed at a significantly lower level in primary T cells. Importantly, CAR T cells were more sensitive to the KDM1A inhibitor treatment compared to the tumor cells, indicating the need for a sequential treatment strategy for future experiments combining CAR T cells and inhibitors. Upon treatment with SP-2509 at their respective IC50 concentrations, the selected neuroblastoma cell lines exhibited increased FAS cell-surface expression in a TP53-dependent manner. This upregulation of FAS sensitized neuroblastoma cells to FAS-FASL-dependent killing and boosted the effectiveness of L1CAM-directed CAR T cell therapy against tumor cells with little or no antigen expression in vitro. However, the improved therapeutic response was negated when the FAS-FASL interaction was disrupted with an antagonistic FAS antibody. These findings demonstrate that KDM1A inhibition triggers an antigen-independent mechanism of killing via the FAS-FASL axis, rendering tumor cell variants that partly or completely suppress antigen expression vulnerable to CAR T cell therapy.

2 Introduction

2.1 The childhood malignancy neuroblastoma

Although cancer in children and adolescents is rare, it remains one of the leading causes of death for this age group around the world (1). Among the most common cancers diagnosed in children are brain and other central nervous system cancers. Neuroblastoma is a prevalent solid tumor outside the cranial cavity in children, which originates from the sympathoadrenal lineage of the neural crest. Primary neuroblastoma tumors are found along the entire sympathetic trunk or originating in the adrenal medulla (2). According to the variable tumor localization, neuroblastoma can present with specific symptoms, such as Horner's syndrome with ptosis, miosis and enophthalmos and anhidrosis when localized around the upper sympathetic trunk or with pain, deficits, and paraplegic symptoms in lower-lying localization with displacement and compression of the spinal cord. Due to catecholamine secretion symptoms like tachycardia, hypertension and flushing may be present. Upon the first

diagnosis, around 50% of the patients already have metastases in the liver, skin, or bone marrow, sometimes with infiltration and formation of bone metastases. Infiltration of the orbit occurs often and the resulting periorbital ecchymoses are found as a typical sign of neuroblastoma (3). The risk stratification and staging of neuroblastoma is carried out according to International Neuroblastoma Risk Group (INRG). Classification is based on parameters such as age of the patient, tumor histology, imaging stage, chromosomal ploidy and presence of chromosomal abnormalities or oncogene amplification (4,5).
Disease etiopathology and clinical course are incredibly heterogeneous, with >95% survival in children with very low-risk disease (stages 1, 2 and 4s), who receive little or no therapy, and >50% mortality in children with high-risk disease (stage 4) despite intensive multimodal therapy. Around 50% of all cases are currently classified at diagnosis as high-risk for relapse. For the therapy of high-risk tumors, a multimodal therapy approach consisting of intensive radiochemotherapy with subsequent autologous stem cell transplantation is chosen. Although polychemotherapy provokes a good initial response, these tumors frequently relapse due to minimal residual disease (MRD) with dissemination of a few resistant tumor cells. Most relapsed neuroblastomas are chemoresistant, giving conventional chemotherapy little chance to be effective (5). The treatment of relapsed or refractory neuroblastoma is a significant obstacle in the field of pediatric oncology, and there is an urgent demand for novel therapeutic approaches.

2.2 Immunotherapy as a new approach to treat neuroblastoma

To date, common cancer treatment regimens lack specificity by targeting not only cancer cells but also healthy cells throughout the body, causing several side effects, some of which may remain permanently. The enduring adverse effects and subsequent development of secondary cancers can profoundly affect the quality of life, particularly for children (6).
Immunotherapy is an individualized treatment that triggers the patient`s immune system to specifically recognize and attack cancer cells. One successful implementation of such therapy was the use of Anti-GD2 antibodies against neuroblastoma, leading to a 10% increase in the overall survival of high-risk patients (7). In addition to antibody therapy, an alternative pioneering immunotherapeutic strategy involves targeting tumors through adoptive T cell therapy, which manipulates the immune system to deploy effector mechanisms against metastatic and treatment-resistant tumor cells. This method employs chimeric antigen receptors (CARs) to specifically target tumor-associated antigens. The initial design of CARs joined an antibody-derived single chain fragment variable (scFv) to the CD3ζ intracellular signaling domain of the T- cell receptor through hinge and transmembrane domains. “Second-generation” CARs incorporate an additional domain, CD28 or 4-1BB, to supply a costimulatory signal, which improved replicative capacity and persistence of modified T cells. Two costimulatory domains, a combination of CD27, CD28, 4-1BB, ICOS, or OX40, make up “third-generation” CARs (8).

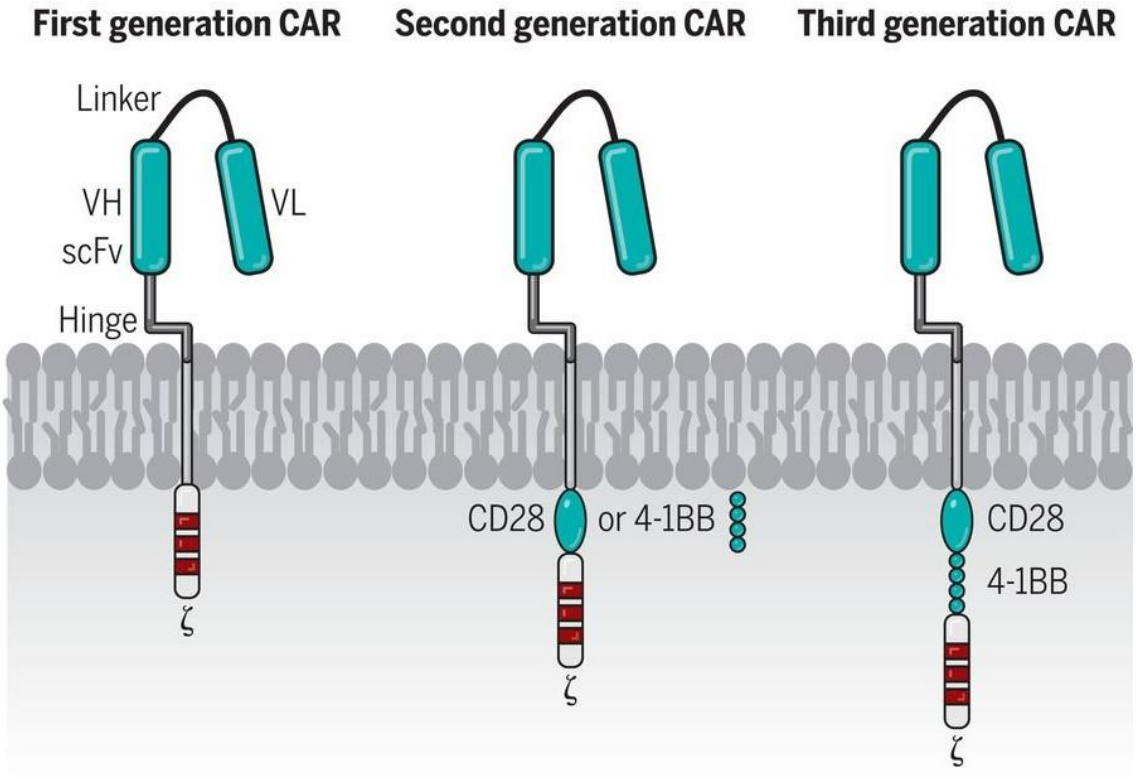


Figure 1: Schematic structure of the chimeric antigen receptor modified from June et al (8). Constructs of 1st generation contain only one intracellular stimulatory domain, usually CD3zeta. 2nd and 3rd generation constructs contain one and two additional costimulatory intracellular domains, respectively. VH: heavy chain variable region; VL: light chain variable region; scFv: single chain fragment variable.

The choice of co-stimulatory domain is in this respect important because it affects the CAR T cells ability to proliferate, persist, as well as their efficacy against tumor cells (9). Substantial for a successful CAR T cell therapy apart from the CAR construct is also the choice of the targeted tumor-associated antigen. Ideally, tumor antigens should be homogenously and strongly expressed on the surface of every tumor cell, but not on the surface of healthy cells to prevent on-target off-tumor toxicity (10). Specifically for neuroblastoma, CARs have been generated to target strongly expressed antigens such as GD2 (11) and L1CAM. Künkele et al (12) demonstrated that L1CAM is expressed on human neuroblastoma cells and that a CAR directed against L1CAM does not induce acute on-target off-tumor toxicity in a robust non-human primate model. Furthermore, L1CAM-targeting CAR T cells generated from T cells acquired from 4 heavily pretreated neuroblastoma patients exhibited potent antitumor efficacy in vitro and in vivo. Given the preclinical success of this product, a phase I trial (NCT02311621, https:clinicaltrials.gov) evaluating safety in children with relapsed or refractory neuroblastoma was initiated and is ongoing at Seattle Children's Hospital. To date, 25 children have been registered and no dose-limiting toxicities have been detected, but CAR T cell efficacy appears to be limited (manuscript in preparation). There are several obstacles to overcome in order for CAR T cell therapy to effectively treat neuroblastoma, including limited T cell survival, inadequate or insufficient expression of tumor-specific targets, and an immune-suppressed tumor microenvironment, among other challenges (11).

The most successful CAR T cells, to date, are those targeting CD19, which have shown remarkably durable remissions in adults and pediatric patients with B-ALL (13). CD19 is a cell-surface antigen found on most B-lineage lymphomas and leukemia. Since CD19 is physiologically only expressed on B cells, depletion of normal B cells by CD19 CAR T cells can be easily managed by antibody substitution. Due to the impressive results of the therapy, the US Food and Drug Administration (FDA) approved the University of Pennsylvania/Novartis CART19 product (KymriahTM / tisagenlecleucel, formerly CTL019) for the treatment of children and young adults with relapsed or refractory ALL. Furthermore, five different CAR T cell therapies have been approved for the treatment of adults with leukemia, certain kinds of relapsed or refractory B cell lymphoma, as well as multiple myeloma (14). Although CAR T cell therapy has achieved remarkable success, some patients still face relapses. This is because the tumor exhibits heterogeneous CD19 expression, which causes therapy-resistant tumor variants to emerge and grow. These variants either lose or decrease the expression of the CD19 antigen (15). This hurdle becomes more relevant in solid tumors, which per se show an even greater target antigen expression heterogeneity, often leading to therapy resistance. The successes of CAR T cell therapy against solid tumors have, therefore, been very modest (16). Finding new ways to overcome antigen heterogeneity might increase the CAR T cell efficacy against solid tumors.

2.3 Role of FAS-FASL pathway in CAR T cell efficacy

The main mechanisms by which cytotoxic T cells induce death in target cells are through either the exocytosis of cytotoxic granules that contain perforin and granzymes or via the induction of apoptosis through the FAS-FASL pathway (Figure 2). It has been demonstrated that FAS cell surface expression on the tumor site can be induced in the presence of proinflammatory cytokines produced by CAR T cells, such as IFNG, while, the upregulation of its binding partner, FASL, occurs upon T cell activation (17). While the primary mechanism of CAR T cells killing target cells is via the perforin and granzyme pathway, it has been shown that blocking FASL in malignant T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoma lines significantly reduces the cytotoxicity caused by CD5-specific CAR T cells. This underscores the significance of the FAS-FASL mechanism (17,18). In contrast to the perforin and granzyme pathway that relies on target antigen expression on each tumor cell, the FAS-FASL pathway does not require such expression on every single cell for inducing cell death. As a result, monoclonal antibodies that function as a FAS agonist have been created to activate tumor cell killing via the FAS-FASL axis. Nevertheless, this approach has been hindered by systemic toxicities caused by FAS expression in normal tissues and lower antibody efficacy (100-fold less) when compared to FASL expressed on T cells. Therefore, further development of this method has been limited (19). Unlike the use of FAS agonist monoclonal antibodies, the FASL-mediated killing mechanism used by CAR T cells is tightly controlled. This is because the upregulation of FASL on CAR T cells is dependent on their activation and can only occur at the site of the tumor when they encounter the antigen (17).

Previous studies have reported that a variety of tumors tend to downregulate FAS expression on the cell surface to evade a T cell-mediated tumor lysis via the FAS-FASL axis (20,21). Known mechanisms responsible for FAS absence on the cell surface at the tumor site are transcriptional repression, promoter hypermethylation (22), histone acetylation (23) and generation of secreted soluble FAS proteins that lack the transmembrane domain via alternative mRNA splicing (24). FAS expression is also known to be positively regulated by the TP53 tumor suppressor (25) whose function is

often impaired in tumor cells either by loss-of-function mutations or epigenetic silencing (26).
Re-expression of FAS on the tumor cell surface may present a way of unleashing antigen-independent tumor eradication via CAR T cells, leading to an enhancement of their efficacy.

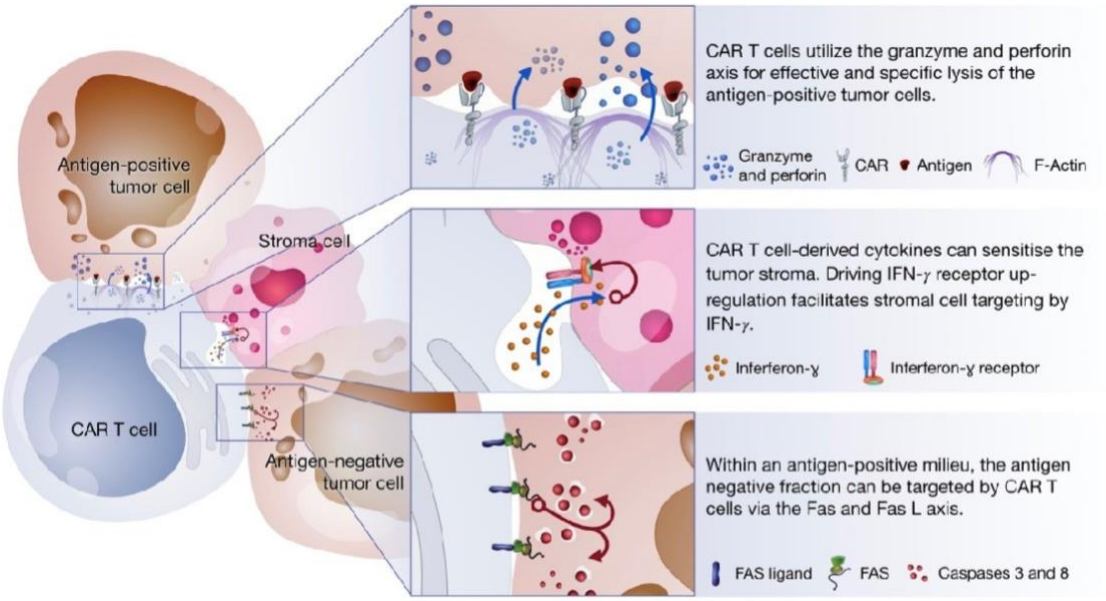


Figure 2: Killing mechanisms of CAR T cells according to Benmebarek et al (17). CAR T cells mediate tumor killing via three axes: (1) Perforin and granzyme axis: Targeting antigen positive fraction. (2) Cytokine secretion: Stromal cell sensitization. (3) Fas and FasL axis: Targeting antigen-negative fraction.

2.4 KDM1A and its link to immunotherapy

KDM1A, initially referred to as LSD1, is a histone demethylase that was the first of its kind to be discovered. It has the ability to function as both a transcriptional coactivator and a corepressor (27). Numerous studies have demonstrated that molecular features of clinically aggressive, metastasized neuroblastomas differ from those of tumors responsive to therapy. KDM1A has been found to be overexpressed in several types of cancer, including neuroblastoma, and its expression has been associated with a more aggressive disease phenotype (28). Suppressing KMD1A expression via siRNA-mediated knockdown inhibited viability in neuroblastoma cell lines in vitro, and small molecule inhibitors of KMD1A reduced neuroblastoma xenograft growth in mice.
Furthermore, Studies have demonstrated that KDM1A can inhibit the transcriptional activation of TP53 in human cancers (29). Inhibiting KDM1A could be a viable approach to restore the expression of FAS, which is regulated by TP53. Subsequent exploitation of the FAS-FASL axis might increase tumor responsiveness to CAR T cell therapy. Given this background, it is an interesting approach to test neuroblastoma-specific CAR T cells and KDM1A inhibitors combined in neuroblastoma cell lines. KDM1A inhibitors have been tested in several clinical trials in association with Ewing Sarcoma (SP-2577) ***NCT03600649***, Myelofibrosis (IMG-7289) ***NCT03136185*** and acute myeloid leukemia (tranycypromine, cytarabine, all trans retinoic acids) ***NCT02717884*** (https:clinicaltrials.gov). This strategy has not yet been tested in patients with neuroblastoma. However, there are some promising preclinical results

regarding this matter as described above. Linking together KDM1A inhibition with CAR T cell therapy may result in a synergism that could have the potential to be a potent antitumor weapon in fighting neuroblastoma.

2.5 Aims

Chimeric antigen receptor (CAR)-T cell immunotherapy alone has not yet achieved the expected therapeutic efficacy for patients with neuroblastoma. Combining different strategies may provide a way to better fight this malignancy. This study aims to test the efficacy of combining CAR T cell immunotherapy with blockade of KDM1A, which is known to be overexpressed in aggressive neuroblastomas. This project will 1) investigate the role of FAS-FASL pathway when treating neuroblastoma with L1CAM-specific CAR T cells, 2) analyze how downregulation of the histone demethylase, KDM1A (previously known as LSD1), in neuroblastoma cells affects the FAS-FASL axis, 3) investigate possible cytotoxic activity of KDM1A inhibitor treatment on CAR T cells and 4) assess the combination of neuroblastoma-specific CAR T cell therapy with inhibitors of KDM1A as a way of improving CAR T cell efficacy. These preclinical investigations will contribute to the necessary preliminary work for the design of intervention trials in patients with refractory or relapsed neuroblastoma.

3 Methods

3.1 Cell lines and cell culture

The human neuroblastoma cell lines NB-1, IMR-5/75 and SH-SY5Y were cultivated in RPMI medium (Gibco Life technologies) supplemented with 10% fetal bovine serum (Sigma) The human neuroblastoma cell line SK-N-BE(2) was cultivated in DMEM medium (Gibco Life technologies) supplemented with 10% fetal bovine serum (Sigma). Upon reaching 70 to 80 percent confluence, the cells were divided into new Petri dishes. The cell lines were authenticated by Eurofins and regularly validated by PCR to be free from mycoplasma.
T cells were cultivated in RPMI medium (Gibco Life technologies) supplemented with 10% fetal bovine serum (Sigma) and 500 µg/ml L-Glutamine (Gibco Life technologies). All cells were maintained in culture in a humidified atmosphere containing 5% CO2 at 37C.

Table 1: Cell lines used (own representation).

Cell line	ATCC Number	Culture medium	Medium additives
NB-1	CVCL_1440	RPMI	10% FCS 500 µg/ml G418
IMR-5/75	CVCL_1306	RPMI	10% FCS 500 µg/ml G418
SH-SY5Y	CRL-2266	RPMI	10% FCS 500 µg/ml G418
SK-N-BE(2)	CRL-2271	DMEM	10% FCS 500 µg/ml G418

3.2 CRISPR-Mediated Generation of L1CAM Knockout in SH-SY5Y Neuroblastoma Cells

The CRISPR/Cas9 system is an innovative technology, which enables targeted editing of selected genes. This technology is based on a genome editing system that bacteria use as an immune defense mechanism against viruses. CAS9 is an enzyme used to recognize and bind a specific CRISPR RNA sequence (crRNA). This is part of the so-called guide RNA (gRNA), which is designed to find and bind to a specific sequence in the DNA. If the target DNA contains the additionally necessary protospacer adjacent motif (PAM), the binding of the gRNA activates the endonuclease Cas9 and both strands of the target DNA are cut. During DNA repair, non-homologous end joining leads to insertion and deletion of bases, which leads to a loss-of-function mutation by shifting the reading frame (30).

Two guide RNAs targeting L1CAM exon 1 were designed using Dr. Feng Zhang's online CRISPR design tool (http://www.genome-engineering.org/crispr/?page_id=41) and synthesized with BbsI restriction overhangs. Guide RNA sequences excluding the BbsI-compatible overhangs are: RNA1, GACTGTTCCGTGATGACAGG and RNA2, CACCGCCTCGGGGATCTGGATAAGC. Respective oligonucleotides were annealed and ligated into the BbsI-digested pSpCas9(BB)-2A-Puro (PX459) V2.0 (donated by Feng Zhang to the Addgene nonprofit plasmid repository). Generated vectors were validated by sequencing before transfection into SH- SY5Y neuroblastoma cells using the Effectene transfection reagent kit (cat #301425, Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, 5×10^6 SH-SY5Y neuroblastoma cells were seeded per well into a 6-well plate 24 hours before transfection using 100 µL Effectene buffer, 1 µg plasmid DNA, 2 µL Enhancer, and 7.5 µL Effectene per well. Puromycin selection (0.5 µg puromycin/mL complete medium) was conducted 3 to 6 days after transfection. Single-cell clones were established from FACS-sorted L1CAM-negative cells. One single-cell clone with a bi-allelic L1CAM knockout confirmed by sequencing was selected and propagated for experiments.

3.3 Chemical compound treatment

Tumor cells were seeded in 10 or 15 cm dishes and treated with 0,5-3 µM of the KDM1A inhibitor SP-2509 (M60160-2s, Xcessbio) for 3 days, during which the cells were replenished with fresh compound and fresh media every 24 hours. Irradiated cells were used as a positive control for the induction of FAS. The cells were exposed to a total dose of 2 Gy at a rate of 0.848 Gy/min using a gamma irradiator (GSR D1; GSM GmbH) operating with a maximum surface dose rate of <5µSv/h at a maximum activity of 200 TBq Cs-137.
For Fas induction by IFNG (#300-02-250UG, PEPROTECH), the cells were seeded in 6-well plates and treated in triplicates with 15 ng/ml IFNG for 24 hours.
For FAS blocking experiments, the tumor cells were incubated with the anti-FAS antibody (CAT# 05-338, clone ZB4, MERCK) at a concentration of 10-500 ng/mL for 1 hour prior to co-culture with CAR T cells.

3.4 Cell titer glow

For determining IC50 of SP-2509, a total of 10,000-25,000 tumor cells were seeded in 96-well plates in triplicates. After 3 hours, different concentrations of the inhibitor (range from 0,5 to 500 µM) were added to the wells. 72 hours later, CellTiter-Glo®

Luminescent Cell Viability Assay (G9242 Promega) was performed according to the manufacturer's instructions. Viability was calculated relative to untreated tumor cells using GraphPad prism (Version 6.00).

3.5 Western blot

Tumor cells were detached by trypsin, washed twice with phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4. and lysed in radio-immunoprecipitation Assay (RIPA) buffer containing 15 mM HEPES, 150 mM NaCl, 10 mM EGTA and 2% Triton X100 and protease inhibitors (#11697498001, MilliporeSigma). Total proteins (20 µg) were mixed with sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS–PAGE, and the resolved proteins were transferred onto nitrocellulose membranes. Blots were then blocked with 5% non-fat dry milk diluted in Tris-buffered saline with Tween20 (TBST) containing 1.5 M NaCl, 0.1 M Tris Base, HCl and ddH2O for 1 hour at room temperature and incubated over night at 4 °C with primary antibodies recognizing L1CAM (UJ127.11, Thermo Fischer); KDM1A (#2139, Cell Signaling) FAS (ab82419, abcam); p53 (sc-126, Santa Cruz Biotechnology) diluted 1:1000 and GAPDH (sc-32233, Santa Cruz Biotechnology) diluted 1:5000 followed by incubation with secondary antibody diluted 1:5000 for 2 hours at room temperature. Blots were developed using the ECL kit (GE Healthcare Life Sciences, RPN2232). Fusion Fx Vilber Lourmat (PeqLab) was used to measure signal intensities.

3.6 RNA extraction and qRT-PCR

The neuroblastoma cells lines were treated with SP-2509 (0,5-3 µM) for 72 hours, and total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions (Cat No./ID: 74106). The RNA concentration and purity were determined using NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed using the reverse transcription kit (04897030001, Roche). Real-time PCR was performed with SYBR Green master mix (4913850001, Roche). Data were normalized to h28S and the difference in fold change was calculated using delta-delta-CT method. Primer sequences are provided in the Supplementary table.

3.7 Flow cytometry analysis

Cell surface expression of FAS and FASL was detected by the respective fluorophore-conjugated monoclonal antibody (#305611, DX2 and #306407, NOK-2; BioLegend). L1CAM cell surface expression was detected by the fluorophore-conjugated monoclonal antibody (#130-100-691, REA163, Miltenyi Biotec) and standardized QuantiBRITE calibration beads (#655050, BioLegend). T cell activation was assessed by fluorophore-conjugated monoclonal antibodies detecting CD8 (#344741, SK1, BioLegend) and CD137 (#309819, 4B4-1, BioLegend). Flow cytometry was performed on an LSRFortessa X-20 (BioLegend) and data were processed using FlowJo software (Tree Star Inc.). Samples were acquired with at least 10,000 alive events. Dead cells were excluded from analyses using the LIVE/DEAD^{TM} Fixable Green Dead Cell Stain Kit (cat#L23101, Thermo Fischer Scientific). Following KDM1A inhibitor pretreatment (or control condition without treatment), SH-SY5Y tumor cells were labeled with Vybrant^{TM} Dil and DiO Cell-Labeling Solution (Thermo Fischer Scientific) according to the manufacturer's instructions, mixed in a 50:50 ratio with each other, and seeded at 5×10^5 tumor cells/well of a 12-well plate. Stimulated or unstimulated untransduced

CD8+ T cells were added (E:T ratio of 1:1) after tumor cells had settled 3 h. The ratio of living KDM1A-treated to untreated tumor cells remaining in each well was flow cytometrically determined after 24 hours. Specific lysis was calculated using the formula, [1 − (Ratio untreated:treated tumor cells co-cultured with unstimulated T cells/Ratio untreated:treated tumor cells co-cultured with stimulated T cells)] × 100.

Table 2: Fluorophore- conjugated antibodies (own representation).

Fluorophore-conjugated monoclonal antibody	Fluorochrome	Manufacturer	Catalog number
L1CAM	Phycoerythrin (PE)	BioLegend	#130-100-691
CD8	Brilliant Violet 421™	BioLegend	#344741
CD137	Allophycocyanin (APC)	BioLegend	#309819
FAS	Phycoerythrin (PE)	BioLegend	#305611
FASL	Phycoerythrin (PE)	BioLegend	#306407

3.8 CAR constructs

The L1CAM-specific CE7-CARs used in this project have been described in previous publications (31).
The L1CAM-specific CE7-CAR was cloned into the SIN epHIV7 lentiviral vector plasmid. The scFvs was codon optimized and subsequently linked to a 12 (short) amino acid spacer domain from the human IgG4 hinge. The spacer domain connects the antigen-binding domain to CD28 transmembrane domain followed by the signaling module containing the either 4-1BB or CD28 endodomain and the CD3zeta cytoplasmic domain. The CAR constructs were linked downstream to a T2A self-cleaving peptide and truncated epidermal growth factor receptor (EGFRt) allowing CAR T cell detection and enrichment.

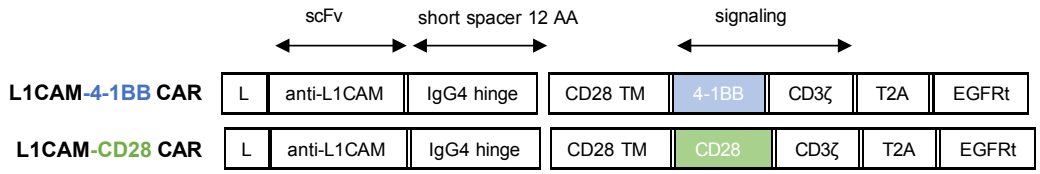


Figure 3: Schematic illustration of L1CAM-specific CAR T cell constructs 2nd generation with 4-1BB or CD28 costimulatory domain (own representation). L: Long Terminal Repeats; scFv: single chain fragment variable; TM: transmembrane; EGFRt: truncated epidermal growth factor receptor.

3.9 Generation of L1CAM-specific CAR T cells

Apheresis products were obtained from healthy donors and peripheral blood mononuclear cells were isolated using Ficoll-Plaque (GE Healthcare). $CD8^+$ T cells were obtained by positive selection using immunomagnetic microbeads (Miltenyi Biotec) and activated with anti-CD3/CD28 beads at a ratio of 1:1 (Life Technologies). On day three, activated $CD8^+$ T cells were transduced with the CAR-containing lentivirus. The $EGFRt^+$ CAR T cell subset was enriched by immunomagnetic selection with biotin-conjugated cetuximab (Bristol-Myers Squibb) and streptavidin microbeads (Miltenyi Biotec). T cells used as mock negative controls alongside CAR T cells in experiments were not lentivirally transduced. CAR and mock control T cells were cryopreserved until further use.

3.10 Rapid amplification of CAR T cells

Cryopreserved aliquots from CAR and mock transduced T cells were thawed and stimulated with irradiated PBMCs, TMLCLs and OKT3 (30 ng/mL, Miltenyi Biotec). For rapid expansion, T cells were maintained in RPMI media (Gibco Life technologies) supplemented with 10% fetal bovine serum, IL15 (0.5 ng/mL, Miltenyi Biotec) and IL-2 (50 U/mL, Novartis) according to a rapid expansion protocol (32). Functional in vitro assays were conducted between day 11 and day 16 after starting the T cell culture.

3.11 Functional assays

For cytokine release assays, $2x10^5$ T cells were seeded together with tumor cells at a 1:5 effector to target ratio (E:T). After 24 hours, conditioned media was collected and stored at -80°C until analysis of IL2 and IFNG using the OptEIA™ ELISA (BD Biosciences) according to the manufacturer's instructions. CAR T cell-induced cytotoxicity was quantified in a biophotonic luciferase assay in which the neuroblastoma cells, stably transduced with a GFP-ffLuc_epHIV7 reporter, served as tumor target cells. Target cells were co-cultured at first in triplicate with 1-3 µM of the KDM1A inhibitor and after 72 hours with mock transduced or CAR T cells. After 24 hours 0.14 mg D-luciferin (PerkinElmer Inc.)/mL medium was added to each well, and the biophotonic signal detected. The maximal biophotonic luciferase signal was defined either by untreated target cells plated alone in case of the co-culture experiments without prior inhibitor treatment or by KDM1A pre-treated target cells plated alone in case of the co-culture experiments with prior inhibitor treatment at the same densities (RLUmax, maximal relative light unit). Lysis was determined as [1-(RLUsample/RLUmax)]x100 in relation to either untreated cells or KDM1A pre-treated cells plated alone.

3.12 Statistical analyses

Differences in cytotoxic activity, cell surface marker expression and cytokine release between treatment groups and controls were analyzed using the paired or unpaired Student's T test in GraphPad prism 8 software (GraphPad Software, La Jolla). All experiments were independently repeated ($n = 3$). P values <0.05 were considered statistically significant.

4 Results

4.1 CAR T cells show a low killing efficacy upon co-culture with low antigen expressing neuroblastoma

L1CAM-specific CAR T cells equipped with either 4-1BB or CD28 costimulatory domain were generated from $CD8^+$ cells (Figure 3). By means of a blind-ended epidermal growth factor receptor (EGFRt), which is cleaved off from the CAR construct during translation, comparable CAR expression could be achieved by immunomagnetic selection for EGFRt (Figure 4).
To evaluate the effectiveness of CAR T cells in treating neuroblastoma with different levels of antigen expression., three different cell lines were selected with high, intermediate, or low surface density of the L1CAM tumor antigen with the background that limited antigen cell surface expression can offer a greater challenge for the CAR T cells and thus the effectiveness of the therapy can be better evaluated. L1CAM surface expression density was determined using flow cytometry and QuantiBRITE quantification. The NB-1 cell line expressed high levels of L1CAM, the IMR-5/75 intermediate and SH-SY5Y cell lines low antigen levels. The L1CAM negative Raji cell line was used as a negative control throughout the experiment (Figure 5A, 5B) (33).

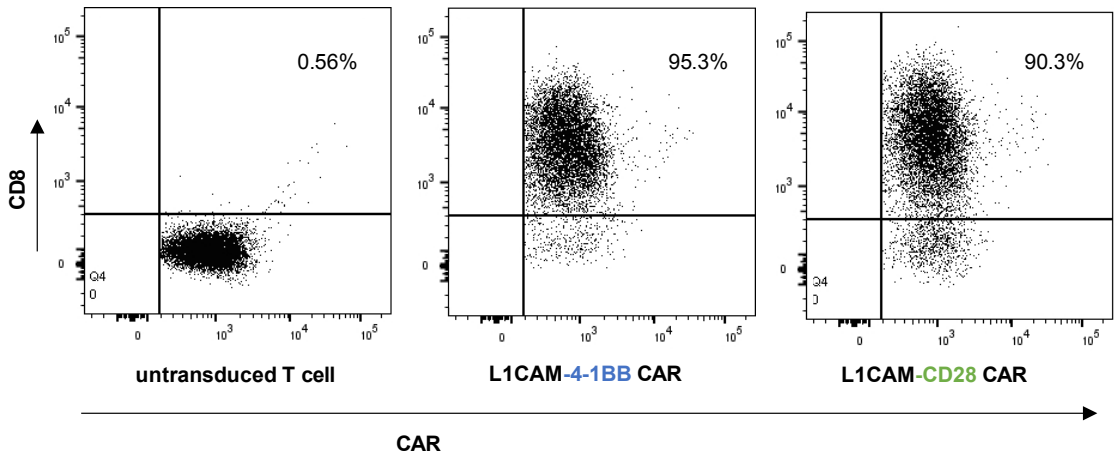


Figure 4: Transduction efficiency for CAR constructs (own representation). Transduction efficiency for CAR constructs and negative control was assessed by flow cytometry (mean ± SD, n = 3).

Both L1CAM-specific T cells equipped with either 4-1BB or CD28 costimulatory domain were co-cultured with the selected neuroblastoma reporter cells in different effector to target (E:T) ratios ranging from 10:1 to 1:10. Cytotoxicity was assessed following 24 hours of co-culture by measuring the biophotonic signal released by the remaining viable tumor cells.
The results showed that CAR T cell-induced tumor cytotoxicity was the highest when co-cultured with the antigen high expressing cell line NB-1, followed by IMR-5/75 (intermediate) and SH-SY5Y (low) for all the tested ratios, confirming that the ability of the CAR T cells to eradicate the tumor was generally dependent on target antigen expression level for either CAR construct used. I also observed that CAR T cells equipped with CD28 costimulation achieved equivalent cytotoxicity against cells expressing high or intermediate antigen levels. In direct comparison with the 4-1BB

CAR T cells, the CAR T cells equipped with CD28 costimulation performed better against intermediate or low antigen expressing neuroblastoma. CAR T cells equipped with either construct killed none of the L1CAM-negative Raji lymphoma cells serving as negative controls, confirming CAR T cell specificity for the L1CAM tumor antigen (Figure 5C) (33).
These results demonstrate that L1CAM-specific CAR T cells show good efficacy against antigen high expressing neuroblastoma cell lines in vitro, however, low antigen expression remains a significant limitation, especially at lower E:T ratio and if L1CAM-specific CARs harboring 4-1BB costimulation domain are used.

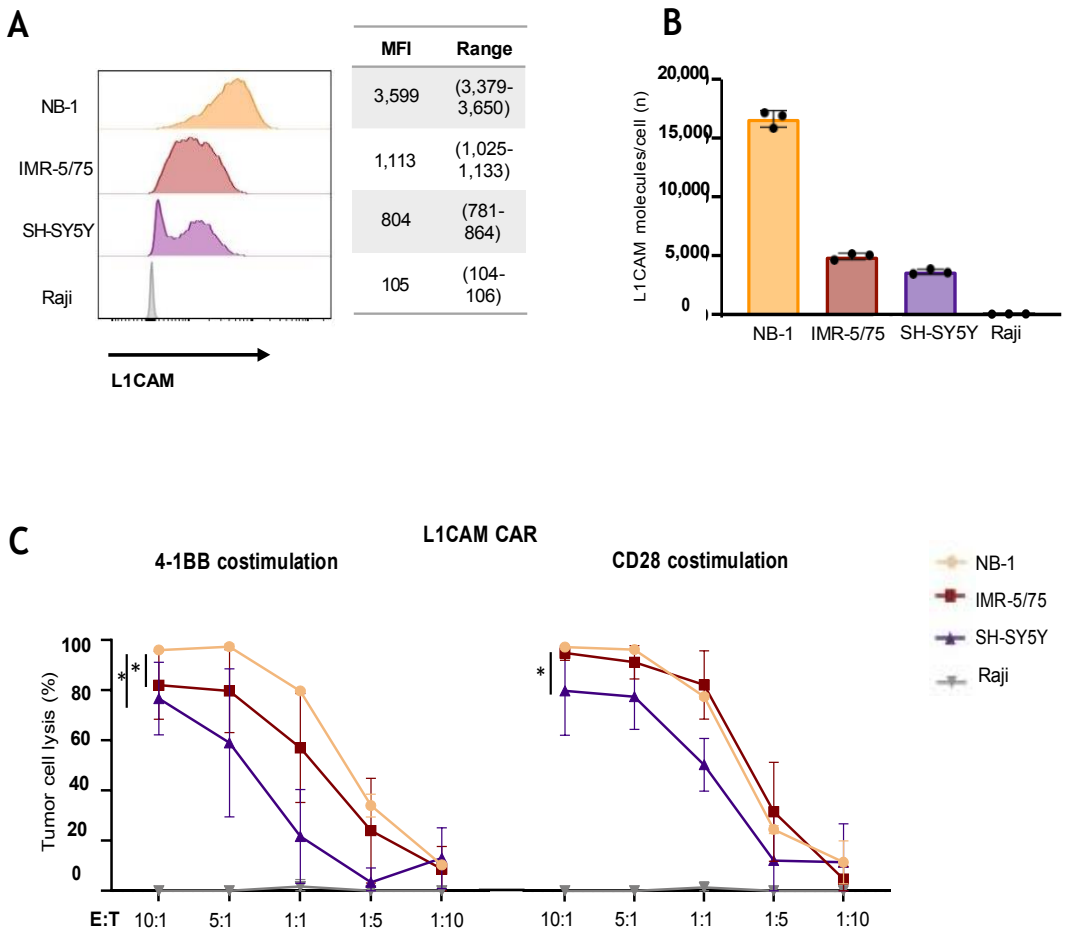


Figure 5: L1CAM-specific CAR T cells fail to eliminate antigen low expressing neuroblastoma cell lines (33). (A) Target antigen expression of selected neuroblastoma cell lines measured by flow cytometry. (B) Target antigen density of selected neuroblastoma cells assessed by standardized QuantiBRITE calibration beads (mean ± SD, n = 3). (C) CAR T cell-induced tumor cell lysis after 24 hours of co-culture with neuroblastoma cell lines in different effector:target ratios followed by luciferase-based quantification of viable cells (mean ± SD, n = 3). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (33).

4.2 FASL expression is upregulated on activated primary T cells, but FAS is not induced in low antigen expressing neuroblastoma

The utilization of FAS-FASL antigen-independent killing mechanisms by CAR T cells has been reported to enhance their effectiveness in combating cancerous growths that exhibit low levels of antigen expression (34). My aim was to further investigate whether this mechanism is present in neuroblastoma. First, I checked whether the T cells express the necessary ligand for this axis, FASL, upon activation. Primary T cells were either stimulated with 20ng/ml PMA (Sigma #P8139-1MG) and 1µg/ml Ionomycin (Sigma # I0634-1MG) for 1 hour followed by 1mg/ml Brefeldin A (BD #347688) and 500 µg/ml Monensin (Sigma #M5273-500MG) for an additional 3 hours at 37°C with 5%CO2 or were left untreated and subsequently stained with FASL fluorophore-conjugated monoclonal antibody to assess the FASL surface expression via flow cytometry. I observed that upon activation the T cells indeed upregulate the FASL on their surface (Figure 6).

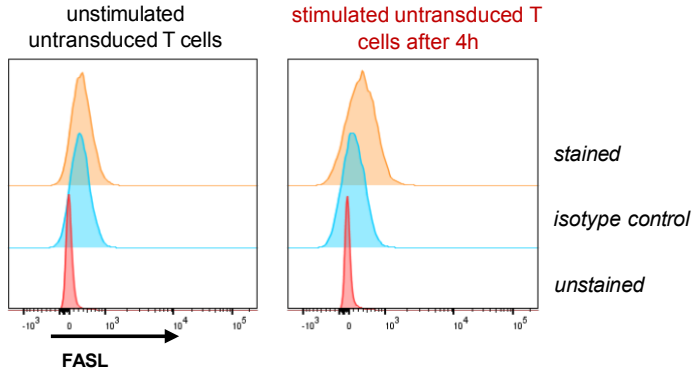


Figure 6: FASL expression is upregulated on activated primary T cells (own representation). Primary T cells were either stimulated with PMA/Ionomycin and Brefeldin A/Monensin for 4 h or were left untreated and subsequently stained with FASL fluorophore-conjugated monoclonal antibody to assess the FASL surface expression. Unstained T cells served as a control. (mean ± SD, n = 3).

Next, I checked whether neuroblastoma cell lines express the binding partner of FASL, the FAS receptor. It has been described that upon antigen-dependent activation T cells release IFNG, which upregulates the FAS expression on the tumor site (17). I therefore measured the IFNG amounts released by the CAR T cells when co-cultured with the neuroblastoma tumor cells. As expected, the CAR T cell cytokine release was dependent on the antigen density. Both constructs released significantly less IFNG when co-cultured with either intermediate or low antigen expressing neuroblastoma. The CAR equipped with CD28 costimulation showed a higher IFNG release compared to the CAR equipped with 4-1BB costimulation, which correlated with the previous observation that the first construct showed greater cytotoxicity in vitro (Figure 7A) (33). In a second step, I treated the tumor cells with IFNG concentrations ranging from 1 to 15 ng/mL, which was up to 15 times greater than the quantities of IFNG released by CAR T cells during the co-culture experiments. The untreated high antigen expressing NB-1 cell line showed per se a high FAS expression and further upregulation at an IFNG dose of 5 ng/mL. However, I observed low FAS expression and no significant upregulation after IFNG treatment on the intermediate and low antigen expressing

cells, when treated with the respective IFNG amounts released by the T cells (Figure 7B) (33). I selected these cell lines for further experiments.
These results indicated that while the FASL is expressed on the surface of the T cells upon activation, it cannot interact with its respective receptor, FAS, which is neither highly expressed nor induced by IFNG on the intermediate and low antigen expressing neuroblastoma cell lines.

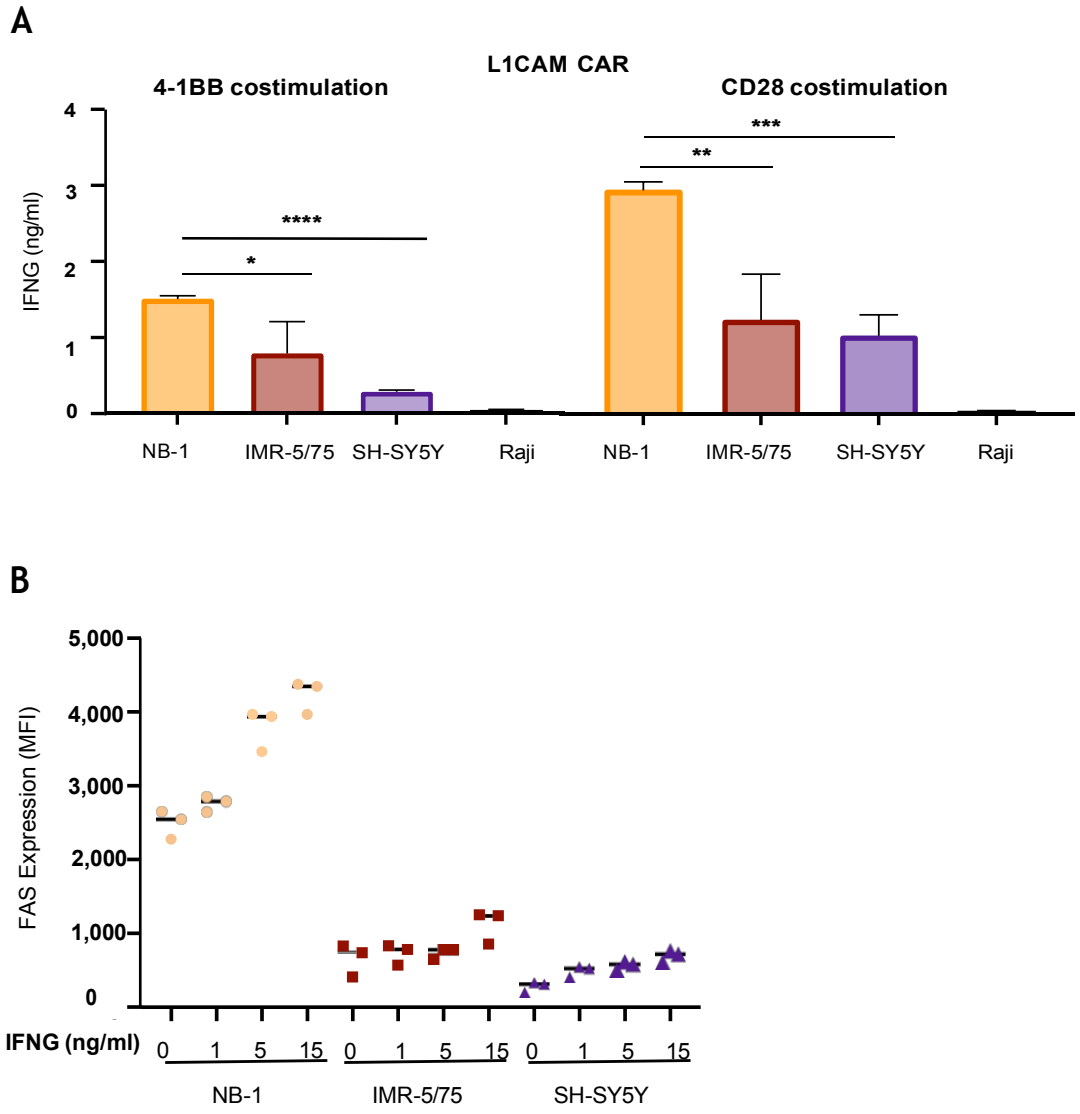


Figure 7: IFNG released by CAR T cells failed to induce FAS expression in neuroblastoma cell lines (33). (A) IFNG release by CAR T cells after 24 hours of co-culture with neuroblastoma cell lines at an effector:target of 5:1 (mean ± SD, n = 3). (B) Flow cytometrically determined FAS expression in neuroblastoma cell lines after IFNG treatment for 24 hors (mean ± SD, n = 3). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (33).

4.3 Inhibiting KDM1A upregulates FAS expression on neuroblastoma cell lines without affecting the target antigen expression

I hypothesized, that by inducing the FAS expression on neuroblastoma cells, CAR T cells could show greater antitumor potency by exploiting this antigen independent mechanism. The upregulation of FAS receptor expression is stimulated by operational

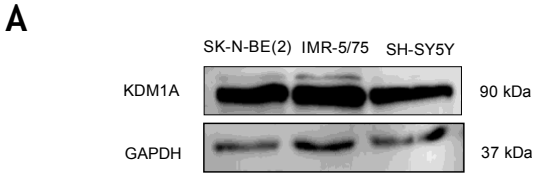
TP53 signaling, which is activated upon exposure to agents that induce DNA double-strand breaks, like irradiation. However, for this study, I was aiming for a more tumor specific approach. Irradiation was therefore used as a positive control for subsequent experiments.

KDM1A is an epigenetic regulator highly expressed in neuroblastoma cells (Figure 8A) (33) and can inhibit TP53-mediated transcriptional activity by removing the methyl group from TP53 lysine 370, which hinders DNA binding and the expression of TP53-regulated genes (29). Pearson correlation analysis in two independent neuroblastoma cohorts consisting of 47 (35) and 51 (36) tumor samples demonstrated that KDM1A and FAS expression were negatively correlated (Figure 8B) (33). To check whether blockade of KDM1A would upregulate FAS expression, among other TP53-responsive genes, I treated the two selected cell lines as well as the SK-N-BE(2), a cell line with inactive TP53, with a small molecule inhibitor of KMD1A. The optimal treatment concentration of the KDM1A small molecule inhibitor, SP-2509, was determined by assessing its IC50 in all three cell lines with doses ranging from 0.5 to 500 µM. As anticipated, the neuroblastoma cell lines with functional TP53 exhibited sensitivity to lower doses of the inhibitor (1-3 µM), while the SK-N-BE(2) cell line with inactive TP53 required higher doses (8.9 µM) to elicit cell death (Figure 8C) (33).

Treatment with 3 µM SP-2509 resulted in an upregulation of FAS expression in both cell lines with functional TP53, but not in the cell line lacking TP53 activity. Low-dose irradiation was employed as a positive control to induce TP53 activity and FAS surface levels in neuroblastoma cells. To explore the possibility of enhancing FAS levels on tumor cells further, the KDM1A inhibitor treatment was combined with low-dose irradiation (2 Gy). However, the combination therapy failed to induce higher FAS expression levels than KDM1A inhibitor treatment alone (Figure 8D) (33).

These results were also confirmed by western blot data, where I also checked for changes in TP53 as well as a second target gene, CDKN1A (also known as P21). As expected, there was no changes in TP53 responsive genes for the SK-N-BE(2) cell line. For the other two cell lines, IMR-5/75 and SH-SY5Y, I observed the upregulation of both TP53 responsive genes, FAS and CDKN1A upon either irradiation (positive control) or inhibitor treatment, indicating that functional TP53 is necessary for FAS upregulation via KDM1A inhibition. Furthermore, I checked for alterations in tumor antigen expression and observed no such changes after KDM1A inhibitor treatment for either of the selected cell lines (Figure 8E) (33).

These results show that FAS can be induced in neuroblastoma cells by KDM1A inhibition in a TP53-dependent matter.



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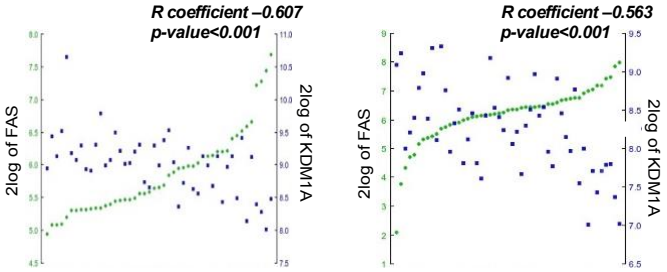

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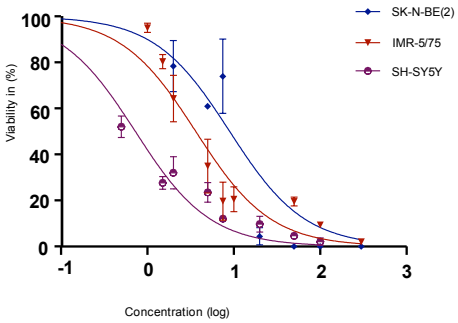

Cell line	SK-N-BE(2)	IMR-5/75	SH-SY5Y
IC50 value (µM)	8.9	3.6	0.7

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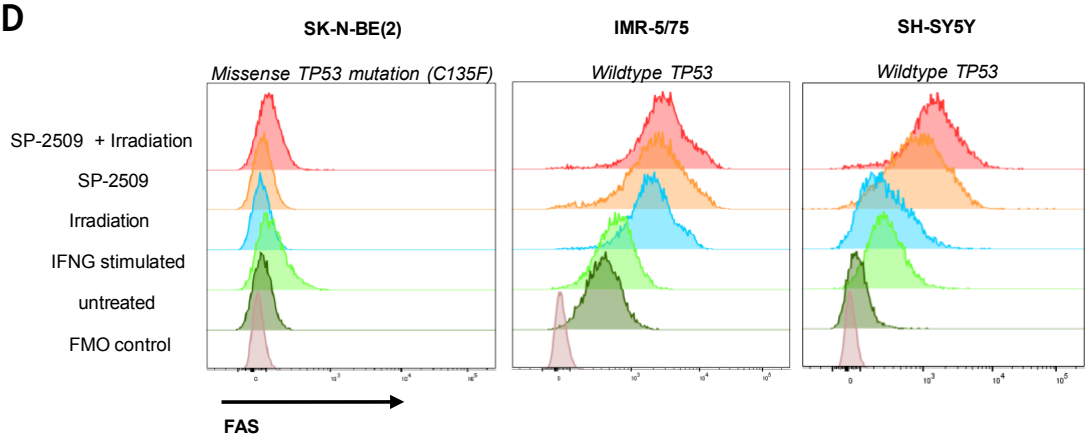

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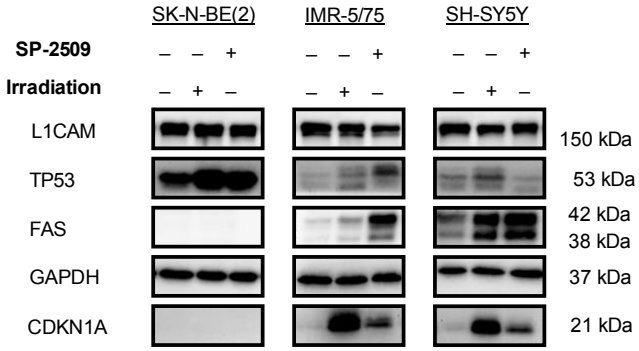
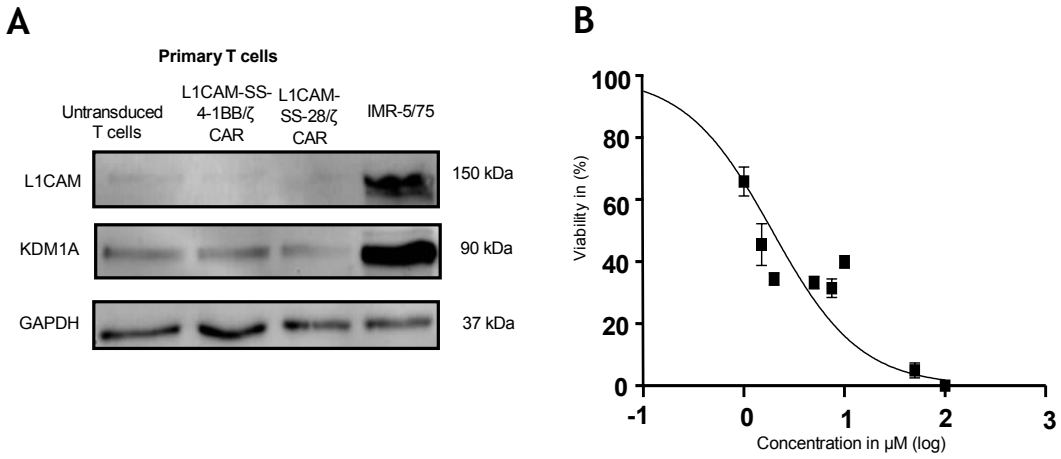


Figure 8: Drug-mediated KDM1A inhibition induces FAS expression in neuroblastoma cell lines (33). (A) Western blots showing KDM1A expression in the selected neuroblastoma cell lines. GAPDH was used as a loading control. (B) Pearson correlations between FAS and KDM1A expression across on the left side 47 samples (Khan et al, 2011) and on the right side across 51 samples (Hiyama et al, 2009) from primary neuroblastomas (source: R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl, accessed on 27 September 2021)). (C) Dose-

response curves and calculated IC50 scores for the selected neuroblastoma cell lines treated 72 hours with the KDM1A inhibitor, SP-2509. (D) Flow cytometrically determined FAS surface expression in neuroblastoma cell lines after 72 hours of treatment with 0.5 µM KDM1A inhibitor (SP-2509), irradiation with 2 Gy, or a combination of both (mean ± SD, n = 3). (E) L1CAM, TP53, FAS, and CDKN1A protein expression in neuroblastoma cells after 72 hours of treatment with 0.5 µM KDM1A inhibitor (SP-2509) or irradiation with 2 Gy. GAPDH served as loading control (33).

4.4 Primary T cells are susceptible to KDM1A inhibition

To prove my initial hypothesis that an induction of FAS could improve CAR T cell efficacy, I aimed to test a combination therapy consisting of KDM1A small molecule inhibitor and L1CAM-specific CAR T cells. Before performing this experiment, it was important to consider the effects of the KDM1A inhibitor not only on the tumor cells but also on the T cells as well. I started by assessing the expression of KDM1A on the T cells. While the expression of KDM1A was significantly lower on the T cells as compared to the IMR-5/75 cells, it was still present (Figure 9A). I continued by assessing the IC50 of the KDM1A small molecule inhibitor, SP-2509, in the T cells with doses again ranging between 0.5 and 500 µM. Interesting was, that the T cells were sensitive to lower doses of the inhibitor (1.9 – 2.5 µM) compared to the tumor cells I proceeded by investigating the effects of the inhibitor treatment on cytokine release by the CAR T cells when cocultured with the SH-SY5Y cell line for 24 hours. I observed no significant differences regarding IFNG release between SP-2509 pre-treated T cells and untreated controls for both constructs (Figure 9C). Nevertheless, due to the observed cytotoxicity of the inhibitor on the T cells (Figure 9B) I decided to continue with a sequential approach regarding further experiments.
Overall, I show here that T cells express low levels of KDM1A and are susceptible to lower doses of the KDM1A inhibitor compared to the neuroblastoma cells. Furthermore, the inhibitor treatment on the CAR T cells does not impair their effector function in this experimental setting.



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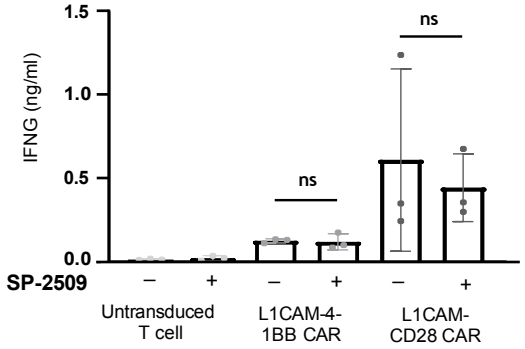
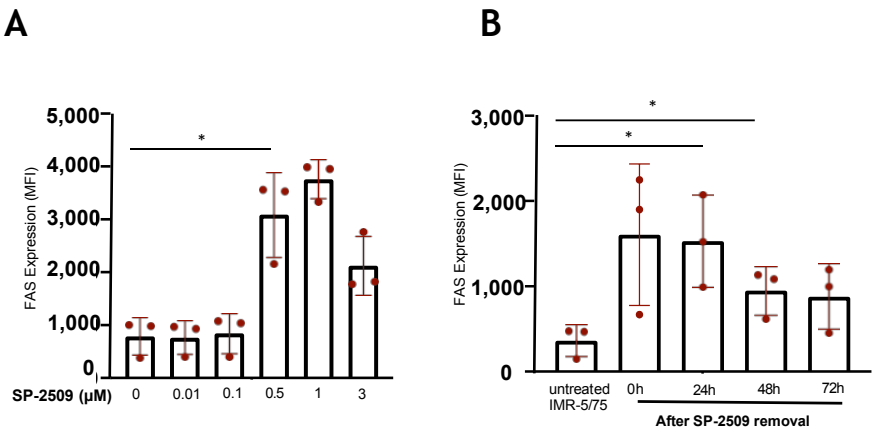


Figure 9: KDM1A inhibitor does not impair CAR T cell effector function (own representation). **(**A) KDM1A and L1CAM protein expression was determined by western blotting whole-cell extracts from primary T cells and indicated cell lines. GAPDH was used as a loading control. (B) Dose-response curve indicating calculated IC50 of primary T cells treated 72 hours with the KDM1A inhibitor, SP-2509. (C) IFNG release from untreated and KDM1A pre-treated CAR T cells after 24 hours of co-culture with tumor cells assessed by ELISA. Bars show mean ± SD from 3 biological replicates.

4.5 Combining KDM1A inhibition with CAR T cell therapy results in an increased killing of low antigen expressing neuroblastoma via the FAS-FASL axis

My aim for the subsequent experiments was the KDM1A-mediated upregulation of FAS expression on tumor cells as a way of improving the neuroblastoma-specific CAR T cell efficacy. Therefore, to limit any KDM1A inhibitor cytotoxicity, I titrated the KDM1A inhibitor by treating the neuroblastoma cell line IMR-5/75 with doses ranging from 0.01 µM to 3 µM and assessed FAS expression via flow cytometry. I observed a significant FAS upregulation at a dose as low as 0.5 µM, which I selected for subsequent experiments (Figure 10A) (33). Since I aimed for a sequential treatment regimen, it was necessary to evaluate whether the FAS upregulation on the tumor cells was stable enough for a subsequent CAR T cell treatment. The results showed a significant upregulation of FAS after KDM1A inhibitor treatment (0.5 µM) which remained stable for up to 48 hours after inhibitor removal, allowing subsequent T cell treatment to test combination therapy (Figure 10B) (33).
My experimental design consisted in the treatment of the neuroblastoma cell lines with the KDM1A inhibitor, SP-2509, for 72 hours followed by removal of the inhibitor and addition of CAR T cells. Aiming to present the CAR T cells with a challenge, I selected an E:T of 1:5, a ratio at which the two constructs did not perform well against our selected neuroblastoma cell lines (Figure 5C). The results were obtained via a bioluminescence-based killing assay after 24 hours of co-culture (Figure 10C) (33). To evaluate the impact of FAS upregulation on CAR T cell efficacy, the toxicity caused by the inhibitor alone was subtracted by utilizing neuroblastoma cells pretreated with SP-2509 alone for 72 hours as the baseline reference. The findings indicated that SP-2509 pretreatment of neuroblastoma cells resulted in a significant increase in cytotoxicity induced by L1CAM-specific CAR T cells (Figure 10D) (33). Against neuroblastoma cells with intermediate levels of the target antigen (IMR-5/75), a 3.2-fold increase in L1CAM-4-1BB CAR T cell-induced cytotoxicity and a 1.5-fold increase in L1CAM-CD28 CAR T cell-induced cytotoxicity were observed compared to CAR T cell

treatment alone. The effect was more pronounced against neuroblastoma cells with low L1CAM levels (SH-SY5Y), with a 12.6-fold increase in L1CAM-4-1BB CAR T cell-induced cytotoxicity and a 3.4-fold increase in L1CAM-CD28 CAR T cell-induced cytotoxicity. The augmented cytotoxicity induced by L1CAM-targeting CAR T cells was further increased by costimulating 4-1BB signaling over CD28 costimulation. The cytotoxicity induced by either CAR T cell construct against SK-N-BE(2) neuroblastoma cells (lacking functional TP53) remained unchanged after SP-2509 pretreatment, supporting the conclusion that TP53-dependent FAS upregulation is responsible for the enhanced efficacy of CAR T cells (Figure 10D) (33).
The author conducted experiments to confirm that the induction of FAS on the tumor cells was responsible for the increased cytotoxicity of L1CAM-specific CAR T cells. The author used an antibody that blocks FAS binding to its ligand without inducing cell death to treat IMR-5/75 neuroblastoma cells that were either untreated or pretreated with the KDM1A inhibitor. The results showed that the blockade of FAS activity in the neuroblastoma cells eliminated the increase in tumor cell lysis observed in cells pretreated with the KDM1A inhibitor, but it did not affect CAR T cell-directed killing of untreated neuroblastoma cells. This indicates that the enhanced cytotoxicity of CAR T cells was dependent on FAS induction on the tumor cells (Figure 10E) (33).
The experimental results and analysis support the hypothesis stating that KDM1A-mediated FAS upregulation at the tumor site is responsible for the enhanced efficacy of CAR T cells.



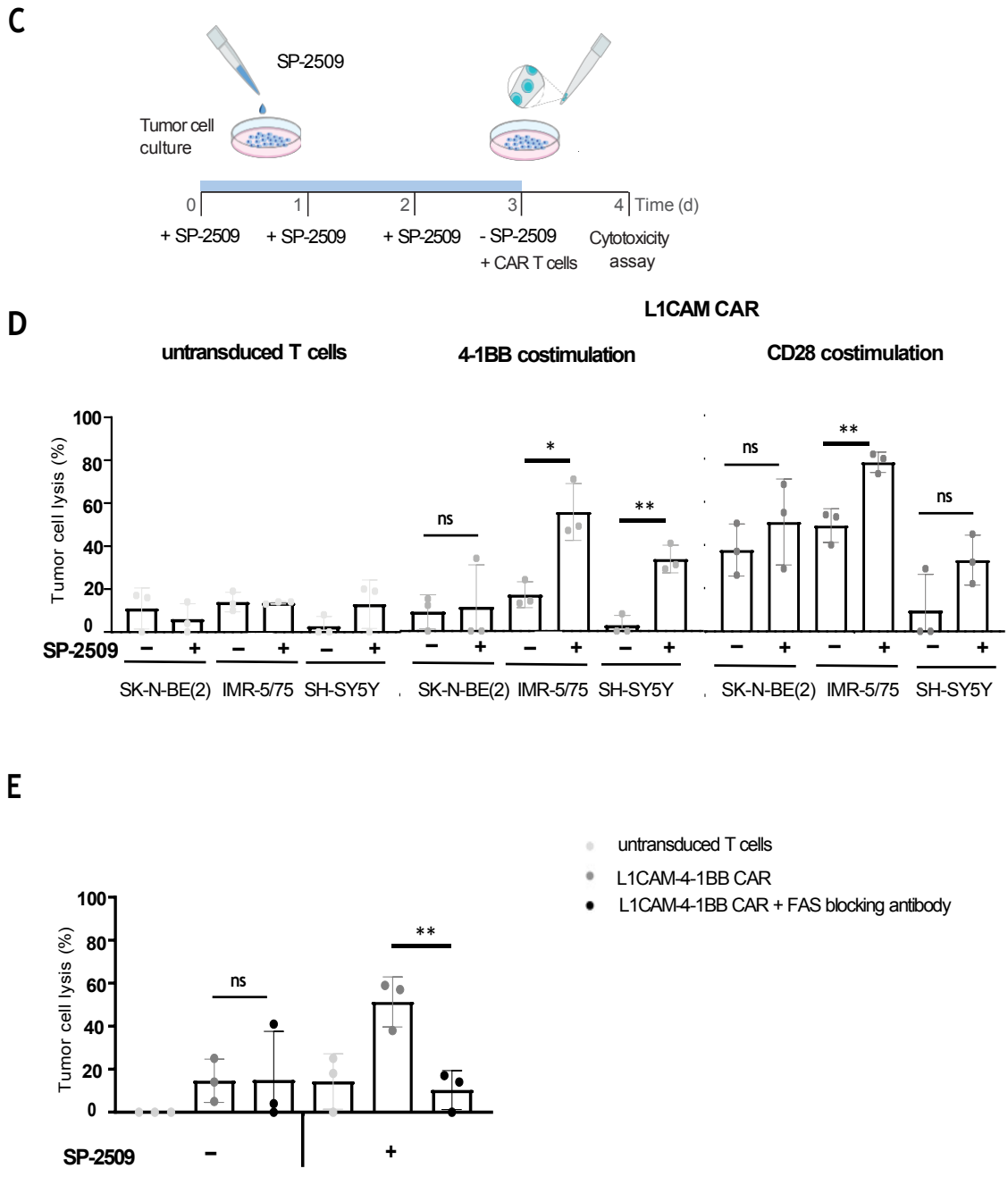


*Figure 10: Combining KDM1A inhibition with CAR T cell therapy kills neuroblastoma cells expressing low tumor antigen levels via the FAS-FASL axis (33). (*A) Flow cytometric determination of FAS expression on the surface of IMR-5/75 cells after 72 hours treatment with the indicated concentrations of the SP-2509 inhibitor. MFI=mean fluorescence intensity (B) Flow cytometric determination of FAS expression on the surface of IMR-5/75 cells after 0, 24, 48 and 72 hours removal of the inhibitor by replacing medium after washing cells once (bars show mean ± SD, n = 3). (C) Experimental timeline for the combination therapy. (D) Killing efficacy of L1CAM-specific CAR T cells after 24 hours co-culture of neuroblastoma cell lines treated with 0.5 µM SP-2509 (or untreated controls) at an effector:target ratio of 1:5 (mean ± SD, n = 3). (E) Untreated or KDM1A-pre-treated SH-SY5Y cells were incubated for 1 hour in the presence or absence of the human neutralizing anti-FAS antibody before measuring the tumor cell lysis by L1CAM-specific CAR T cells co-cultured with the tumor cells at an effector:target ratio of 1:5 for 24 hours (mean ± SD, n = 3). *p ≤ 0.5, **p ≤ 0.01 (33).

4.6 The upregulation of FAS via KDM1A inhibition enhances the CAR T cells ability to eradicate antigen-negative neuroblastoma cells via the FAS-FASL pathway

Because FAS-FASL is an antigen independent mechanism, my aim was to show that the enhanced CAR T cell efficacy observed above was not only a result of a better eradication of the antigen low expressing cells but also due to eradication of the antigen negative fraction. To test whether the FAS-FASL mechanism was sufficient to eradicate antigen negative neuroblastoma cells, I generated a SH-SY5Y cell model with a complete L1CAM knockout (SH-SY5Y-L1CAM-ko; Figure 11A) with the purpose of excluding any antigen-related killing. I excluded the Raji cell line, which is L1CAM-negative and derived from Burkitt lymphoma, from this experiment since my main objective was to demonstrate the effect on a solid tumor like neuroblastoma. My assumption was that activated T cells expressing FASL should be able to eradicate the KDM1A pretreated antigen negative cells expressing FAS on their surface. To test this hypothesis, I designed an experiment where SH-SY5Y-L1CAM-ko cells were pretreated for three days with 0.5 µM of the KDM1A inhibitor SP-2509 and then labeled the cells with the fluorescent dye DiI. For flow cytometric identification, the untreated SH-SY5Y-L1CAM-ko cells were labeled with DiO dye and co-cultured for 24 hours with stimulated and unstimulated T cells at an E:T ratio of 1:1 (Figure 11B). To determine the most effective ratio, different ratios (1:1, 5:1 and 10:1) were tested, as I hypothesized that the previously used E:T of 1:5 may not be favorable for untransduced T cells, which cannot eliminate tumor cells through L1CAM antigen recognition. Here, I present results for the experimental design using the lowest number of effector cells (1:1 ratio) that produced a distinct effect.
As anticipated, the results indicated that only the KDM1A-inhibited tumor cells were selectively eliminated by activated T cells, leading to a shift in the initial 1:1 ratio towards untreated tumor cells with a ratio of 1.6:1 in the resulting population (Figure 11C). Conversely, unstimulated T cells did not induce cytotoxicity, as evidenced by the nearly 1:1 ratio of both tumor cell populations (Figure 11D), confirming that only the FASL-expressing T cells were responsible for the observed cytotoxicity. These findings demonstrated that KDM1A inhibition was sufficient to enhance the killing ability of either pre-activated endogenous T cells or CAR T cells present in the tumor to eliminate a heterogeneous tumor cell population lacking the target antigen expression

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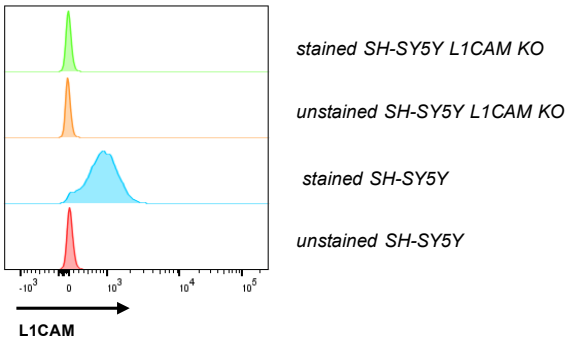

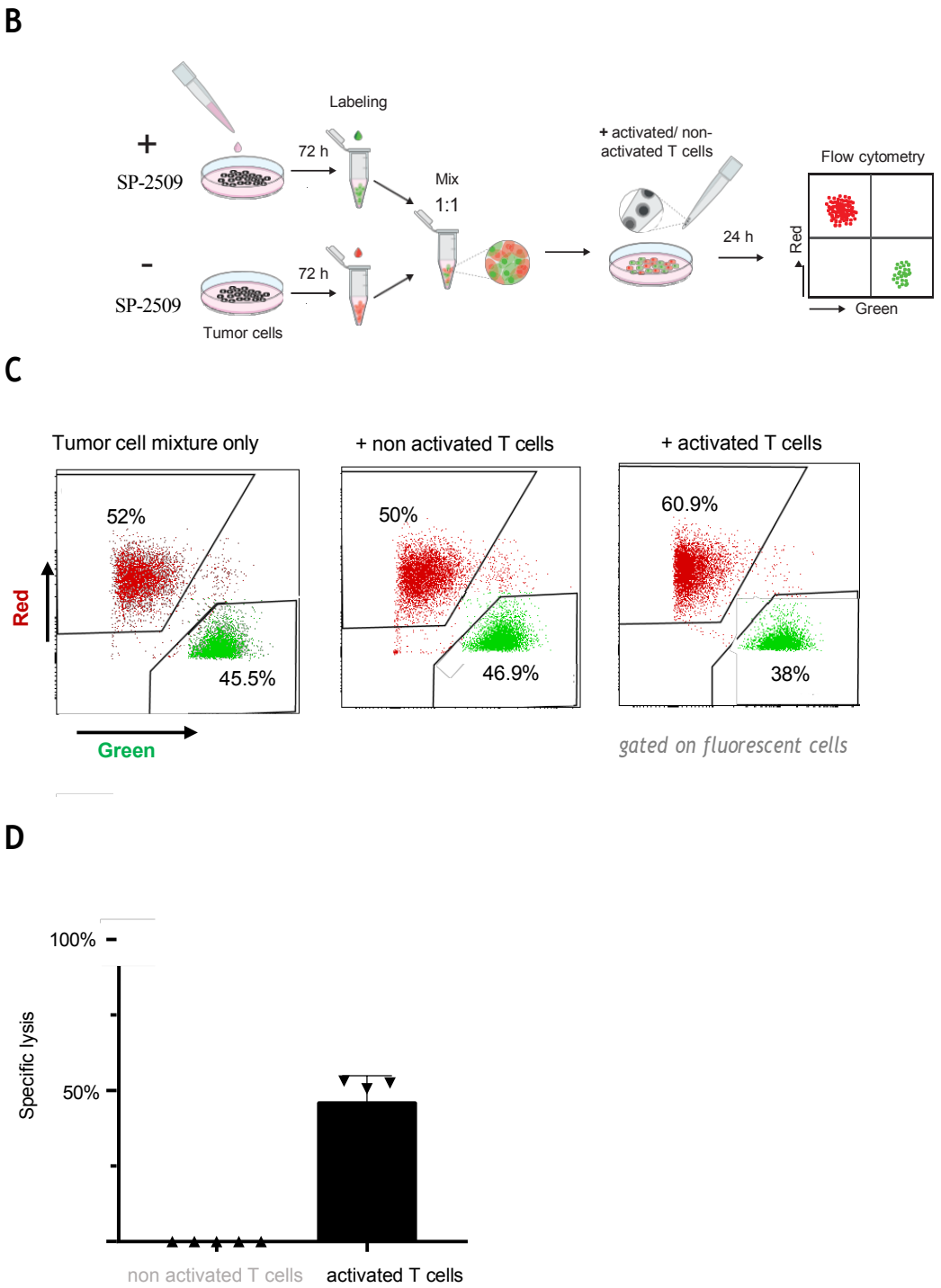

Figure 11: Combination therapy is beneficial for eradicating tumor cells lacking the tumor antigen (33). (A) Flow cytometric assessment of L1CAM surface expression after CRISPR-mediated L1CAM knockout in the SH-SY5Y neuroblastoma cell model. Shown are histogram plots of the parental SH-SY5Y neuroblastoma cell line and the knockout clone, either stained with L1CAM fluorophore-conjugated monoclonal antibody or unstained (mean ± SD, n = 3). (B) Experimental timeline for the combination therapy. (C) Killing efficacy of activated or non activated untransduced T cells after 24 hours co-culture with SH-SY5Y-L1CAM-ko cells treated with 0.5 µM SP-2509 (or untreated controls) at an effector:target ratio of 1:1. (D) Lack of tumor cells lysis of SP-2509 pre-treated SH-SY5Y-L1CAM-ko cells by non activated T cells (mean ± SD, n = 4) (33).

In summary, my findings suggest that inhibiting KDM1A activity to increase FAS expression on tumor cells enhances the effectiveness of L1CAM-specific CAR T cell

therapy against neuroblastoma with low or variable levels of target antigen expression. This is achieved by activating antigen-independent killing through the FAS-FASL pathway.

5 Discussion

5.1 Short summary of results

In this study I observed that L1CAM-specific CAR T cells showed reduced antitumor potency against antigen low expressing neuroblastoma. The antigen independent FAS-FASL mechanism was not being exploited by CAR T cells due to lack of FAS upregulation on antigen low expressing neuroblastoma upon IFNG release. My hypothesis was that activating the FAS-FASL axis might be a potential promising approach for effectively targeting neuroblastoma with low or heterogeneous antigen expression by enabling the elimination of an antigen-negative tumor subpopulation by CAR T cells.
The small molecule inhibitor SP-2509 led to a significant increase in FAS cell-surface expression in neuroblastoma cells when KDM1A was blocked, and this effect was strictly dependent on TP53. In line with my hypothesis, By upregulating FAS expression, neuroblastoma cells became more susceptible to death through the FAS-FASL axis, which allowed L1CAM-specific CAR T cells to effectively eliminate tumor cells that did not express the L1CAM antigen, resulting in an enhancement of their efficacy in vitro.

5.2 Interpretation of results

Here, I used established neuroblastoma cell lines with high, low, or heterogeneous antigen expression. I observed a significant reduction of the in vitro efficacy of L1CAM-specific CAR T cells when the tumor antigen was less strongly or heterogeneously expressed on tumor cell lines. This observation is consistent with numerous studies indicating that while CAR T cell therapy has achieved remarkable success in the treatment of hematological malignancies, it often fails to eliminate tumors that have low or heterogeneous antigen expression or that lose antigen expression during treatment. For example, Xu et al. reported that 10-20% of patients with B-cell acute lymphoblastic leukemia (B-ALL) experienced relapse after CD19-specific CAR T cell treatment due to the emergence of CD19-negative leukemic cells (15). Studies have also reported tumor escape through CD22 antigen loss in a significant proportion of patients with B-cell acute lymphoblastic leukemia (B-ALL) treated with CD22-specific CAR T cells, up to 30% (37). Ramakrishna et al. conducted a study using cell lines of acute lymphoblastic leukemia (ALL) with varying levels of CD22 expression, and found that low levels of CD22 (621 molecules/cell) not only impaired the functionality of CD22-targeting CAR T cells, but also led to decreased in vivo persistence of the CAR T cells (38).
Another finding of this study was that the CAR utilizing CD28 costimulation performed better against neuroblastoma cells expressing low antigen levels, compared with the CAR imparting 4-1BB costimulation. These differences regarding the efficacy of them CAR constructs against antigen low expressing tumors are supported by studies from Majzner et al. found that CD28-containing CARs had higher efficacy against tumors with low antigen density, possibly due to the higher signal strength of the CD28 co-stimulatory domain. The study showed that T cells expressing CD28-containing CARs

had a more rapid and robust calcium influx compared to T cells with 4-1BB-containing CARs (39).

Next, I investigated the antigen independent FAS-FASL pathway in neuroblastoma and showed that FAS in not highly expressed in antigen low expressing tumor cells analyzed in my study. Several other groups have reported that this pathway might indeed remain inactive, since FAS expression is downregulated on cells in several tumors, including gastric (40), colon (41), thyroid (42) and small cell lung (23) carcinomas, consistent with my observations in neuroblastomas. When CAR T cells recognize an antigen, they produce proinflammatory cytokines such as IFNG. This upregulates the expression of FAS on tumor cells, sensitizing them to death receptor-mediated cell death (43). Although FAS upregulation was observed in neuroblastoma cells upon exposure to high concentrations of IFNG, this may not be feasible in an in vivo setting. IFNG concentrations equivalent to those produced by T cells upon tumor cell encounter did not induce FAS upregulation in neuroblastoma cells, except for the L1CAM high-expressing NB-1 cell line, which expressed high levels of FAS protein on its cell surface even in the absence of IFNG. However, this cell line was not included in further experiments, as it was hypothesized that the FAS-FASL axis would play a secondary role in cell lines with high antigen expression. My strategy for FAS induction on antigen low expressing neuroblastoma was through the epigenetic regulator, KDM1A, which is recognized for its ability to hinder TP53-mediated transcriptional activation by keeping TP53 inactive and preventing its binding to DNA (29). Müller et al. discovered a regulatory element within the FAS gene that responds to TP53 (25). These reports support my observation, that FAS upregulation via KDM1A inhibition was TP53-dependent, since I only observed a FAS upregulation in cell lines with a functional TP53. However, for the antigen low expressing SH-SY5Y I observed an upregulation of FAS in the absence of an upregulation of TP53 when treated with the KDM1A inhibitor. I hypothesize that another additional mechanism leading to FAS and CDKN1A upregulation might be underlying this observation. Further experiments for understanding this mechanism are indispensable.

Another observation I made was that T cells were sensitive to lower doses of the KDM1A inhibitor treatment (IC50 of 1.9 – 2.5 µM). However, I observed no reduction in their effector function when treated with the inhibitor for 72 hours before starting the coculture. Given these observations, I proceeded with a sequential approach nevertheless because I aimed to exclude any additional effects of the inhibitor treatment on the T cells to test my original hypothesis regarding the FAS/FASL axis. It would of course be very interesting to evaluate the simultaneous approach in future projects, also regarding in vivo experimentation.

The combination therapy I propose here was superior compared to a neuroblastoma-specific CAR T cell therapy alone. Due to exploitation of the KDM1A mediated FAS expression, CAR T cells were able to eradicate antigen negative cells. In line with my observation, other groups have shown that ectopic FAS expression at the tumor site improves CAR T cell efficacy against embryonal carcinomas by enabling antigen-independent FASL-dependent tumor cell lysis (34). Evidence for FAS-dependent bystander killing of antigen-negative tumors by T cells was also demonstrated by Upadhyay et al. (44). The development of monoclonal antibodies that function as a FAS agonist is another strategy to induce tumor cell killing via the FAS-FASL axis. However, this approach has been limited by systemic toxicities resulting from FAS expression in normal tissues and a significantly lower efficacy of antibodies compared to FASL expression on T cells, which has hindered its further development (19). Furthermore, the FASL on the T cells is only upregulated after antigen dependent T

cell activation. The resulting cell death via the FAS/FASL axis is therefore only induced in the tumor, making it a tumor specific approach.
To my knowledge this is the first study to combine KDM1A inhibitors and CAR T cells with the purpose of unleashing FAS-FASL mediated tumor eradication. The combination therapy showed potent antitumor cytotoxicity in vitro, however, further evaluation of this strategy *in vivo* will be necessary before clinical applications.

5.3 Embedding the results into the current state of research

This study seeks to improve L1CAM-specific CAR T cell therapy in neuroblastoma by identifying new approaches. Solid tumors like neuroblastoma pose challenges to new therapeutic concepts due to the heterogeneity of tumor cells. Therefore, a multi-targeted approach to solid tumor therapy is needed. The proposed strategy aims to enhance CAR T cell potency by reviving an antigen-independent pathway through epigenetic means, thus increasing efficacy even in the absence of high levels of tumor antigen expression.

Using this strategy may offer a way to overcome tumor escape caused by intrinsic antigen heterogeneity or treatment-induced antigen loss, which are significant obstacles that impede CAR T cell therapy efficacy. Furthermore, this strategy could find applications not only against neuroblastoma but potentially against different KDM1A expressing-malignancies such as AML, breast cancer and glioblastoma among others.

5.4 Strengths and weaknesses of the study

The findings of these experiments provide important insights for the development of L1CAM-CAR T cell therapy for neuroblastoma with low antigen expression. The proposed approach may also have potential applications in other malignancies that express KDM1A.
However, these experiments were conducted in vitro using only three neuroblastoma cell lines, and more extensive research both in vitro and in vivo is required to evaluate the potential benefits and drawbacks of combining CAR T cells with KDM1A inhibitors in greater detail.

5.5 Implications for practice and/or future research

Following the results of my experiments, more questions need to be investigated and answered to translate these findings to clinical therapy. Mostly answering questions regarding the toxicity and effectiveness of this strategy *in vivo* will be necessary. KDM1A has a biological role in normal hematopoietic and neuronal stem cells (27). This must be further evaluated as inhibiting KDM1A in these normal tissues could potentially cause treatment toxicity. However, to date, SP-2509 inhibitor, which allosterically inhibits KDM1A by targeting its H3 pocket, exhibited no signs of toxicity in tumor-bearing mice treated with 15-25 mg/kg b.i.w. via IP injection for three weeks suggesting an acceptable safety profile (45). Tranylcypromine (TCP), a monoamine oxidase (MAO) inhibitor, is currently used in the clinic to treat therapy-resistant depression. It was later discovered to also weakly and irreversibly inhibit KDM1A (46). Clinical trials have shown that the administration of 40 mg of TCP was well-tolerated with an acceptable safety profile in patients suffering from relapsed or refractory AML

and myelodysplastic syndromes (45). Future investigation of this inhibitor regarding the strategy proposes here is also needed.
Another interesting aspect to be evaluated in the future would be, whether this strategy could be feasible for other KDM1A expressing tumor entities with low or heterogeneous antigen expression.

6 Conclusions

This study suggests that combining small molecule KDM1A inhibitors with CAR T cell therapy could be a promising approach to target neuroblastoma with low or variable antigen expression, as well as other solid tumors that overexpress KDM1A. In vitro experiments demonstrated that KDM1A blockade upregulated FAS expression in neuroblastoma cells with functional TP53 signaling, leading to increased sensitivity to FAS-FASL-mediated cell death and improved efficacy of L1CAM-directed CAR T cells against antigen-negative tumor cells. These findings highlight the potential of epigenetic manipulation via KDM1A inhibition to enhance CAR T cell therapy and overcome challenges posed by antigen heterogeneity in solid tumors. However, further in vivo studies are necessary to evaluate the clinical feasibility and safety of this approach.

7 Literature

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8 Eidesstattliche Versicherung und Anteilserklärung

8.1 Eidesstattliche Versicherung

„Ich, Ornela Sulejmani, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Untersuchung des Einflusses von KDM1A auf Neuroblastom-spezifische CAR-T-Zelltherapie / Analyzing the influence of KDM1A on neuroblastoma-specific CAR T cell therapy selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

8.2 Anteilserklärung an den erfolgten Publikationen

[Die Anteile an der/n jeweiligen Publikation/en sind so deutlich und detailliert zu erklären, dass es der Promotionskommission und den wissenschaftlichen Gutachtern ohne Probleme möglich ist zu erkennen, was Sie selbst dazu beigetragen haben. Wünschenswert wäre ein konkreter Bezug zur Publikation wie z. B.: „aus meiner statistischen Auswertung sind die Tabellen 1, 4, 47 und 60 entstanden.“

[Ihre Anteilserklärung binden Sie bitte mit in alle Exemplare Ihre Dissertation ein. Auf diesen eingebundenen Erklärungen brauchen nur Sie zu unterschreiben, die Unterschrift Ihres/r Erstbetreuers/in ist nicht erforderlich! Allerdings benötigen wir ein separates Exemplar für Ihre Akte. Auf diesem muss auch Ihr/e Erstbetreuer/in unterschreiben!

Wenn Sie im Standard-Track (Dr. med., Dr. med. dent., Dr. rer. medic.) eine Publikationspromotion beabsichtigen und dazu nur eine einzige Publikation vorlegen, gilt Folgendes:

Sie sind alleinige/r Erstautor/in: Dann binden Sie bitte Ihre Anteilserklärung mit in Ihre Dissertation ein. Auf dieser Version brauchen nur Sie zu unterschreiben. Ein weiteres Exemplar, auf dem Sie und Ihr/e Erstbetreuer/in unterschrieben haben, legen Sie den Unterlagen für die Akte bei.

Ornela Sulejmani hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Sulejmani O, Grunewald L, Andersch L, Schwiebert S, Klaus A, Winkler A, Astrahantseff K, Eggert A, Henssen AG, Schulte JH, Anders K, Künkele A. Inhibiting Lysine Demethylase 1A Improves L1CAM- Specific CAR T Cell Therapy by Unleashing Antigen-Independent Killing via the FAS-FASL Axis. Cancers (Basel). 2021 Oct 31;13(21):5489. doi: 10.3390/cancers13215489. PMID: 34771652

Beitrag im Einzelnen (bitte ausführlich ausführen):

Diese Studie wurde im Bereich der pädiatrischen Onkologie durchgeführt und von Cancers of the MDPI veröffentlicht. Die Arbeit erforderte die Konzeption, Durchführung und Auswertung verschiedener Experimente. Unter der Betreuung der letztautorin Annette Künkele leistete die Erstautorin einen entscheidenden Beitrag zur Gewinnung neuer wissenschaftlicher Erkenntnisse.

Der Schwerpunkt der Arbeit von Ornela Sulejmani liegt in der Etablierung und Durchführung funktioneller Assays und deren Auswertung. Innerhalb der Arbeitsgruppe konnten CAR-Konstrukte und identisches verwendet werden. CAR-T-zell sets wurden in etwa gleichen Anteilen eigenständig transduziert und standen bereits vor Beginn der Doktorarbeit zur Verfügung. Innerhalb der Arbeitsgruppe wurden CRISPR/Cas9 gRNAs und Vektoren generiert. Ornela Sulejmani etablierte die Behandlung verschiedener Neuroblastomzelllinien mit dem KDM1A-Inhibitor SP-2509, nachfolgende Funktionstests und die Auswertung dessen wurde von Ornela Sulejmani durchgeführt.

Schwerpunkte:
- Mitwirkung bei der Versuchsplanung
- T-Zell Kultur, Herstellung von CAR-T-Zellen
- Etablierung einer Behandlungsschema mit KDM1A-Inhibitoren
- Transfektion mit CRISPR/Cas9-gRNAs
- In-vitro-Experimente und funktionelle Assays einschließlich ELISA, Luciferase-basierte Assay
- Neu etablierte Panels für durchflusszytometrische Färbung
- Auswertung aller generierten experimentellen Rohdaten
- Erstellung und Erstellung der endgültigen Version des Manuskripts

cancers

MDPI

Article

Inhibiting Lysine Demethylase 1A Improves L1CAM-Specific CAR T Cell Therapy by Unleashing Antigen-Independent Killing via the FAS-FASL Axis

Ornela Sulejmani [1], Laura Grunewald [1], Lena Andersch [1], Silke Schwiebert [1], Anika Klaus [1], Annika Winkler [1], Kathy Astrahantseff [1], Angelika Eggert [1,2,3], Anton G. Henssen [1,2,3,4], Johannes H. Schulte [1,2,3], Kathleen Anders [2,3] and Annette Künkele [1,2,3,*]

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Simple Summary: Solid tumor cells can lose or heterogeneously express antigens to become resistant to chimeric antigen receptor (CAR) T cell therapy. Here, we explore whether epigenetic manipulation to unleash antigen-independent killing mechanisms can overcome this hurdle. KDM1A is overexpressed in many cancers and removes lysine methylation on histones that keeps the DNA firmly packed to selectively activate or repress gene activity, depending on the specific lysine target. KDM1A also regulates the expression of nonhistone proteins. We inhibited KDM1A in the childhood tumor, neuroblastoma, to increase FAS expression on tumor cells. The FAS receptor can be triggered to induce cell death when bound by the FAS ligand on CAR and other activated T cells present in the tumor environment, even if the tumor cells lack the target antigen. FAS upregulation via KDM1A inhibition sensitized neuroblastoma cells to FAS-FASL-mediated killing and augmented CAR T cell therapy against antigen-poor or even antigen-negative neuroblastoma.

Abstract: Chimeric antigen receptor (CAR) T cell therapy has emerged as a promising treatment strategy, however, therapeutic success against solid tumors such as neuroblastoma remains modest. Recurrence of antigen-poor tumor variants often ultimately results in treatment failure. Using antigen-independent killing mechanisms such as the FAS receptor (FAS)-FAS ligand (FASL) axis through epigenetic manipulation may be a way to counteract the escape achieved by antigen downregulation. Analysis of public RNA-sequencing data from primary neuroblastomas revealed that a particular epigenetic modifier, the histone lysine demethylase 1A (KDM1A), correlated negatively with FAS expression. KDM1A is known to interact with TP53 to repress TP53-mediated transcriptional activation of genes, including *FAS*. We showed that pharmacologically blocking KDM1A activity in neuroblastoma cells with the small molecule inhibitor, SP-2509, increased FAS cell-surface expression in a strictly TP53-dependent manner. FAS upregulation sensitized neuroblastoma cells to FAS-FASL-dependent killing and augmented L1CAM-directed CAR T cell therapy against antigen-poor or even antigen-negative tumor cells in vitro. The improved therapeutic response was abrogated when the FAS-FASL interaction was abolished with an antagonistic FAS antibody. Our results show that KDM1A inhibition unleashes an antigen-independent killing mechanism via the FAS-FASL axis to make tumor cell variants that partially or totally suppress antigen expression susceptible to CAR T cell therapy.

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1. Introduction

An innovative immunotherapeutic approach to target tumors is adoptive T cell therapy, which hijacks the immune system to direct effector mechanisms against metastatic and resistant tumor cells. Autologous T cells redirected to a specific tumor-associated antigen by introducing a chimeric antigen receptor (CAR) hold great therapeutic promise. CARs are chimeric molecules that combine an antibody-derived extracellular single-chain antigen-binding domain with intracellular signaling and costimulatory domains from the T cell [1]. The most successful CAR T cell therapy to date received FDA approval in 2017 and targets CD19, a B cell linage-specific antigen whose expression is retained in most B cell malignancies [2]. Despite the unprecedented efficacy of CD19-CAR T cell therapy, a number of patients still experience relapses due to heterogeneous CD19 expression in the tumor cells, leading to outgrowths of therapy-resistant tumor variants that have lost or downregulated CD19 expression [3]. Therapy resistance is even more likely to occur in solid tumors, which show greater heterogeneity in target antigen expression [4–7]. Major barriers to the success of CAR T cell therapy against neuroblastoma comprise suboptimal T cell persistence, a lack or poor expression of tumor-specific targets, and an immunosuppressive tumor microenvironment, among other factors [8]. New strategies to enhance potency are necessary to improve CAR T cell therapeutic success.

Cytotoxic T cells kill target cells mainly by two major pathways, either by exocytosis of cytotoxic granules that contain perforin and granzymes or by FAS-FASL-mediated induction of apoptosis. CAR T cells mainly kill tumors by the perforin and granzyme pathway [9,10]. However, blocking FASL in malignant T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoma lines has been demonstrated to substantially decrease cytotoxicity caused by CD5-specific CAR T cells, highlighting the importance of the FAS-FASL mechanism [11]. While the perforin and granzyme pathway requires target antigen expression on the cell surface of each tumor cell for its destruction, target antigen expression on each individual cell is not necessary for killing via the FAS-FASL pathway. Once initial antigen encounter has upregulated FASL on a CAR T cell, it can subsequently kill FAS-expressing tumor cells even if they lack the target antigen [1]. Promoting tumor cytotoxicity mediated by FAS-FASL might improve the therapeutic efficacy of CAR T cells directed against tumors with heterogeneous antigen expression and present a way to overcome therapeutic failure due to antigen loss.

Previous studies have reported that a variety of tumors can downregulate FAS expression on the cell surface in order to escape a T cell-mediated tumor lysis via the FAS-FASL axis [12,13]. FAS expression is known to be positively regulated by the TP53 tumor suppressor [14], whose function is often impaired in tumor cells either by loss-of-function mutations or epigenetic silencing [15]. KDM1A, a histone demethylase originally called LSD1, was the first histone demethylase discovered, and can act as both a transcriptional coactivator and corepressor [16]. Overexpression of KDM1A has been reported in a variety of cancers including neuroblastoma, where the enzyme expression correlates with more aggressive disease [17]. KDM1A has been shown to repress transcriptional activation mediated by TP53 in human cancers [18]. Targeting KDM1A could, therefore, provide a feasible strategy to re-express the TP53-responsive gene, *FAS*, and subsequently increase tumor responsiveness to CAR T cell therapy.

Neuroblastoma is the most common malignant extracranial solid tumor in children, arising from the sympathoadrenal lineage of the neural crest. Disease etiopathology and clinical course are heterogeneous ranging from spontaneous regression to very aggressive tumors that poorly respond to multimodal therapy, classified as high-risk disease. Mortality in children with high-risk disease remains over 50%, and is mainly due to primary

or acquired resistance to standard chemotherapy [19]. Treating patients with refractory or relapsed neuroblastoma remains a great challenge in pediatric oncology and new therapeutic approaches are urgently needed. Our group developed CAR T cells targeting the glycosylated CE7 epitope of L1CAM (formerly CD171) [20], which is specifically expressed on tumor cells [20,21]. Children suffering from primary refractory or relapsed neuroblastoma were treated with L1CAM-targeting CAR T cells in an ongoing clinical phase I trial (NCT02311621, https: clinicaltrials.gov, accessed on 27 September 2021).

Here, we investigated whether pharmacologically inhibiting KDM1A can cause TP53-mediated FAS re-expression on neuroblastoma cells to enable antigen-independent killing via the FAS-FASL axis by our L1CAM-specific CAR T cells. Our aim was to epigenetically manipulate neuroblastoma cells to overcome the barrier presented by heterogeneous target antigen expression by unleashing antigen-independent CAR T cell-mediated killing.

2. Results

2.1. IFNG Release by CAR T Cells Does Not Induce FAS Expression in Neuroblastoma Cell Lines with Intermediate or Low Antigen Expression Levels

To test the efficacy of L1CAM-specific CAR T cells against neuroblastoma cells expressing varying antigen levels, we first quantified L1CAM expression in different neuroblastoma cell lines using flow cytometry and standardized QuantiBRITE calibration beads. We selected cell lines with high (NB-1) or low, and heterogeneous (IMR-5/75, SH-SY5Y) antigen expression (Figure 1A,B) for in vitro assessment of CAR T cell-dependent cytotoxicity and cytokine release. Selected neuroblastoma cell lines were transduced with a GFP-firefly luciferase reporter plasmid to quantify viable tumor cells in a luciferase-based reporter assay. L1CAM-specific second-generation CAR T cells [20] expressing CARs harboring either CD28 (L1CAM-CD28 CAR) or 41BB (L1CAM-4-1BB CAR) as costimulatory domain (Figure S1A) and enriched for homogenous levels of EGFRt expression by cetuximab immunomagnetic positive selection (Figure S1B) were cocultured with the selected neuroblastoma reporter cells in different effector to target (E:T) ratios ranging from 10:1 to 1:10. Cytotoxicity was assessed following 24 h of coculture by measuring the biophotonic signal released by the remaining viable tumor cells. CAR T cell-induced tumor cytotoxicity was generally dependent on target antigen expression level for either CAR construct used, except that CAR T cells equipped with CD28 costimulation achieved equivalent cytotoxicity against cells expressing high or intermediate antigen levels (Figure 1C). CAR T cells equipped with either construct killed none of the L1CAM-negative Raji lymphoma cells serving as negative controls. Cytokine (IFNG) release from CAR T cells after coculture with the three selected neuroblastoma cell lines was quantified by ELISA. IFNG release was proportional to antigen levels expressed on the neuroblastoma cells, independent of the costimulatory domain used (Figure 1D). However, CAR T cells using CD28 costimulation released on average twice as much IFNG than CAR T cells using 4-1BB costimulation. No IFNG was released by CAR T cells exposed to the L1CAM-negative Raji cells, confirming CAR T cell specificity for the L1CAM tumor antigen. Our results demonstrated that low antigen expression impairs CAR T cell efficacy against neuroblastoma cells.

Exploitation of antigen-independent killing mechanisms by CAR T cells has been described as a way to improve anticancer efficacy against tumors with low antigen expression [1]. IFNG release by CAR T cells sensitizes tumor cells to antigen-independent cytotoxicity by upregulating expression of death receptors such as the FAS receptor (Figure 1E). We tested whether IFNG treatment could upregulate FAS expression on our selected neuroblastoma cell lines. Cultured neuroblastoma cells were exposed to IFNG concentrations ranging from 1 to 15 ng/mL, which was up to 15 times higher than IFNG amounts released by the CAR T cells in coculture experiments. FAS expression on the neuroblastoma cell surface was flow cytometrically assessed after 24 h of IFNG treatment. Interestingly, an IFNG-mediated increase in FAS expression only occurred in NB-1 neuroblastoma cells, which already expressed high FAS levels before IFNG treatment (Figure 1F). The other two neuroblastoma cell lines, with the lower levels of L1CAM target expression, exhibited only very low FAS levels on the cell surface that were only slightly upregulated by IFNG

treatment regardless of concentration. These results indicate that low FAS induction on neuroblastoma cells via IFNG hinders exploitation of the antigen-independent FAS-FASL pathway by L1CAM-specific CAR T cells.

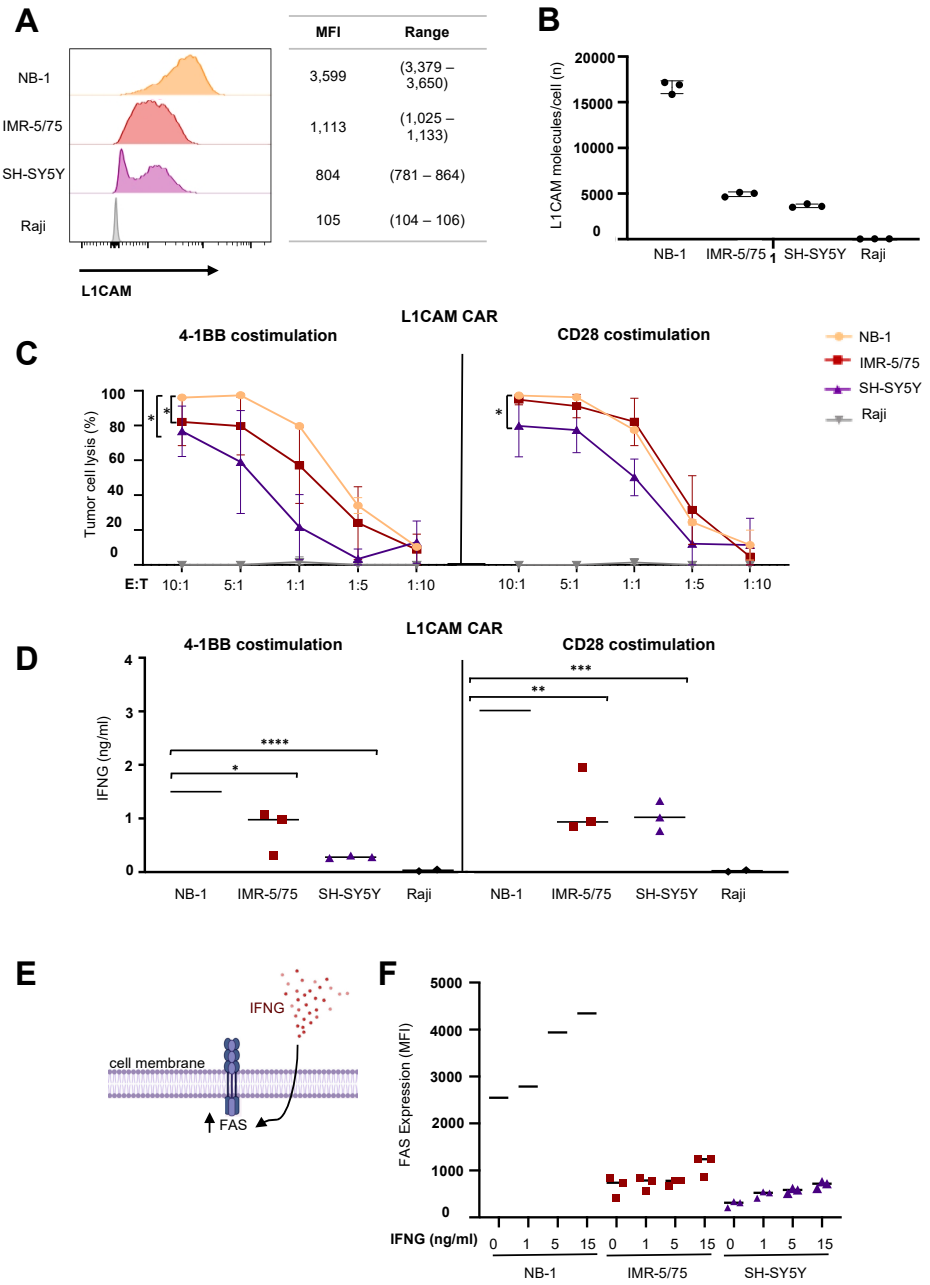
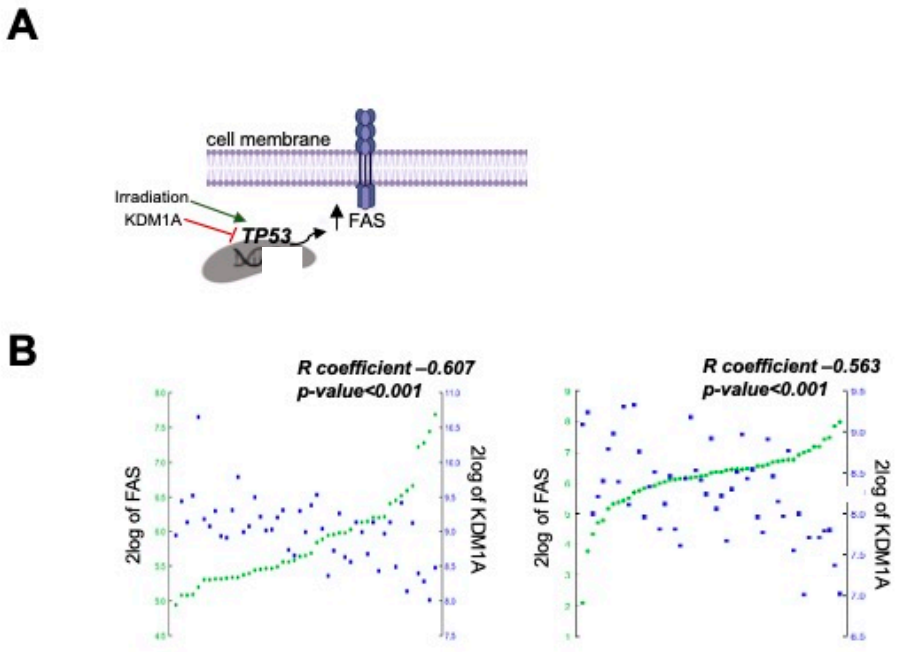


Figure 1. IFNG released by CAR T cells failed to induce FAS expression in neuroblastoma cell lines. (**A**) Target antigen expression of selected neuroblastoma cell lines measured by flow cytometry. (**B**) Target antigen density of selected neuroblastoma cells assessed by standardized QuantiBRITE calibration beads (mean ± SD, $n = 3$). (**C**) CAR T cell-induced tumor cell lysis after 24 h of coculture with neuroblastoma cell lines in different E:T ratios followed by luciferase-based quantification of viable cells (mean ± SD, $n = 3$). (**D**) IFNG release by CAR T cells after 24 h of coculture with neuroblastoma cell lines at an effector:target of 5:1 (mean ± SD, $n = 3$). (**E**) Illustration of IFNG-mediated FAS regulation in tumor cells. (**F**) Flow cytometrically determined FAS expression in neuroblastoma cell lines after IFNG treatment for 24 h (mean ± SD, $n = 3$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

2.2. Inhibiting KDM1A Upregulates FAS Expression on Neuroblastoma Cell Lines

FAS receptor expression is upregulated by functional TP53 signaling, which is activated after exposure to agents causing DNA double-strand breaks, such as irradiation. KDM1A is an epigenetic regulator capable of suppressing TP53-directed transcriptional activity by demethylating TP53 lysine 370, thus, preventing DNA binding and expression of TP53-responsive genes [18]. We hypothesized that treatment with a small molecule inhibitor of KMD1A should upregulate FAS expression, among other TP53-responsive genes (Figure 2A). Pearson correlation analysis in two independent neuroblastoma cohorts consisting of 47 [22] and 51 [23] tumor samples demonstrated that *KDM1A* and *FAS* expression were negatively correlated (Figure 2B). For subsequent experiments, we used the IMR-5/75 and SH-SY5Y neuroblastoma cell lines because our focus in this study was on tumors with low antigen expression. Since both cell lines harbored wildtype *TP53*, we included the SK-N-BE(2) neuroblastoma cell line, which harbored a missense *TP53* mutation producing a nonfunctional protein, as negative control. All three neuroblastoma cell lines expressed high KDM1A protein levels (Figure S2A). The IC50 of the KDM1A small molecule inhibitor, SP-2509, was assessed in all three cell lines with doses ranging between 0.5 and 500 μM to determine the optimal treatment concentration. As expected, neuroblastoma cell lines harboring functional TP53 were sensitive to lower doses of the inhibitor (1–3 μM), whereas the SK-N-BE(2) cell line with inactive TP53 required higher doses (8.9 μM) to induce cell death (Figure S2B). Treatment with 3 μM SP-2509 upregulated FAS expression in both cell lines with functional TP53 but not the cell line lacking TP53 activity. To investigate whether we could further enhance FAS levels on the tumor cells, we combined the KDM1A inhibitor treatment with low-dose irradiation (2 Gy), but the combination therapy did not induce higher FAS expression levels than KDM1A inhibitor treatment alone (Figure 2C). Low-dose irradiation was also used to induce TP53 activity and FAS surface levels in neuroblastoma cells as a positive control. Western blots for TP53 and FAS showed an increase in TP53 levels in both IMR-5/75 and SK-N-BE(2) cells, but not SH-SY5Y, after SP-2509 treatment. FAS levels were only enhanced by KDM1A inhibitor pretreatment in the cell lines with wildtype *TP53* (IMR-5/75 and SH-SY5Y) (Figure 2D, Figures S3–S5). This finding was consistent with our flow cytometry data and indicates that functional TP53 is required for FAS upregulation via KDM1A inhibition. We concluded that KDM1A inhibition upregulates FAS expression on neuroblastoma cells in a TP53-dependent manner.



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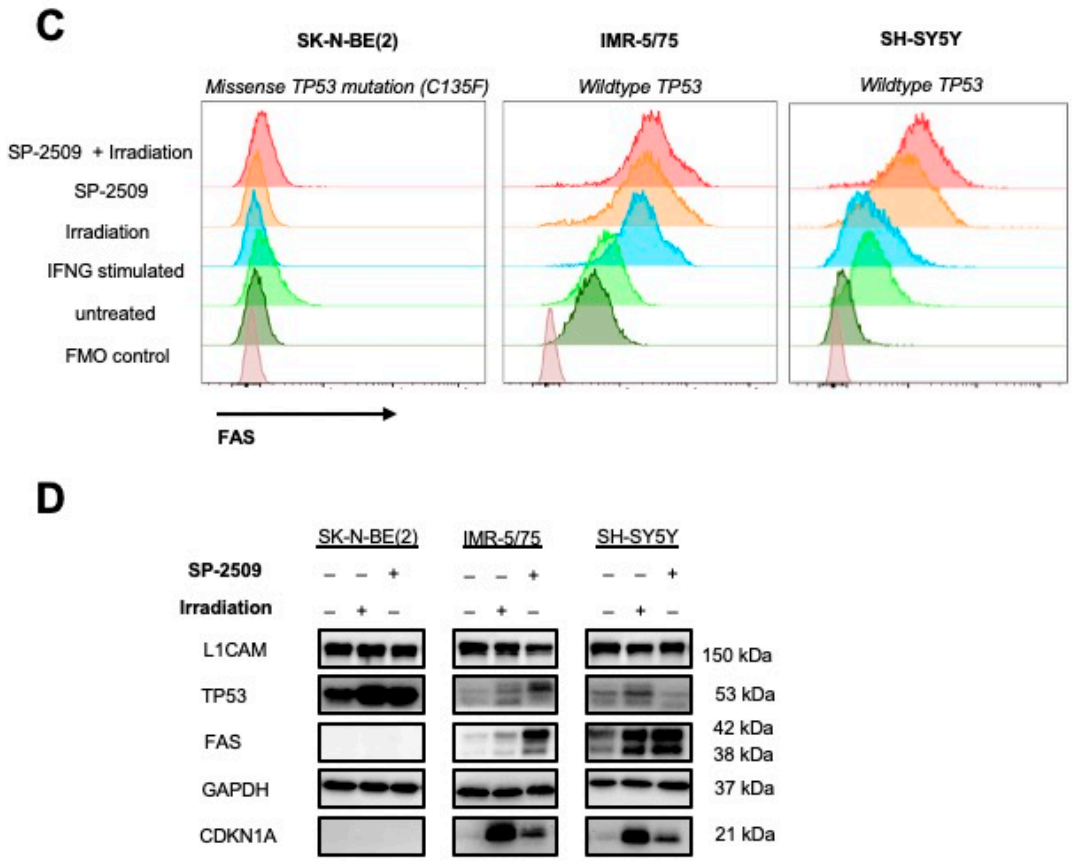
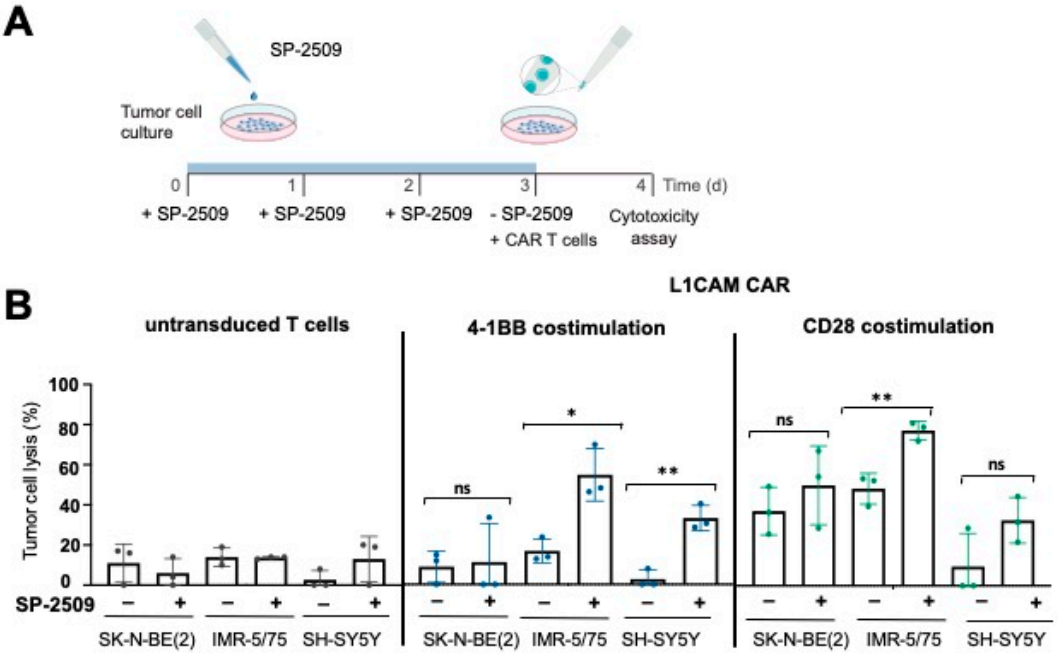


Figure 2. Inhibiting KDM1A strongly enhanced FAS on neuroblastoma cell surface. (**A**) Illustration of FAS regulation in tumor cells. (**B**) Pearson correlations between FAS and KDM1A expression on the left side across 47 samples [22] and on the right side across 51 samples from primary neuroblastomas [23] (source: R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl, accessed on 27 September 2021)). (**C**) Flow cytometrically determined FAS surface expression in neuroblastoma cell lines after 72 h of treatment with 0.5 µM KDM1A inhibitor (SP-2509), irradiation with 2 Gy, or a combination of both (mean ± SD, $n = 3$). (**D**) L1CAM, TP53 and FAS protein expression in neuroblastoma cells after 72 h of treatment with 0.5 µM KDM1A inhibitor (SP-2509), irradiation with 2 Gy, or a combination of both. GAPDH served as loading control.

2.3. Combining KDM1A Inhibition with CAR T Cell Therapy Kills Neuroblastoma Cells Expressing Low Antigen Levels via the FAS-FASL Axis

To test the functional significance of FAS induction on neuroblastoma cells for CAR T cell efficacy, we combined SP-2509 and L1CAM-specific CAR T cell treatments in vitro. To minimize inhibitor-related cytotoxicity in the experiment, KDM1A inhibitor doses were selected that were high enough to induce FAS expression but low enough to be tolerated by the neuroblastoma cells. FAS expression on IMR-5/75 cells was detected via flow cytometry after treatment with titrated SP-2509 concentrations to select this dose. KDM1A inhibitor doses as low as 0.5 µM could induce FAS expression on tumor cells (Figure S2C), and FAS upregulation by the KDM1A inhibitor remained stable for up to 48 h after inhibitor removal (Figure S2D), allowing subsequent T cell treatment to test combination therapy. To exclude that CAR T cells themselves were affected by KDM1A inhibition, we first assessed KDM1A expression with and without exposure to SP-2509. Untransduced control T cells and CAR T cells expressed much lower KDM1A protein levels than IMR-5/75 neuroblastoma cells (Figure S6A). Although T cells were also sensitive to lower doses of the inhibitor (1.9–2.5 µM) to induce cell death (Figure S6B), 0.5 µM SP-2509 did not impair cytokine release by CAR T cells and was sufficient to upregulate FAS at the tumor site (Figure S6C). For combination experiments, the three neuroblastoma cell lines were

exposed to fresh medium or pretreated for 72 h with 0.5 μM SP-2509 before exposure to L1CAM-specific CAR T cells in vitro (Figure 3A). After 24 h of coculture, we measured tumor cell lysis via a bioluminescence-based killing assay. To calculate the impact of FAS upregulation on CAR T cell efficacy, toxicity caused by the inhibitor alone was factored out by using the neuroblastoma cells pretreated for 72 h with SP-2509 alone as the reference baseline. Our results show that cytotoxicity induced by L1CAM-specific CAR T cells was significantly increased by SP-2509 pretreatment of neuroblastoma cells (Figure 3B). We observed a 3.2-fold increase in L1CAM-4-1BB CAR T cell-induced cytotoxicity and a 1.5-fold increase in the L1CAM-CD28 CAR T cell-induced cytotoxicity against neuroblastoma cells expressing intermediate levels of the target antigen (IMR-5/75) compared to CAR T cell treatment alone. The effect was heightened against neuroblastoma cells expressing low L1CAM levels (SH-SY5Y), with a 12.6-fold increase in the L1CAM-4-1BB CAR T cell-directed cytotoxicity and a 3.4-fold increase in L1CAM-CD28 CAR T cell-directed cytotoxicity. Costimulating 4-1BB signaling enhanced the increased cytotoxicity induced by L1CAM-targeting CAR T cells above that from CD28 costimulation. The cytotoxicity induced by either CAR T cell construct against SK-N-BE(2) neuroblastoma cells (lacking functional TP53) was unchanged by SP-2509 pretreatment, supporting that TP53-dependent FAS upregulation is the reason for enhanced CAR T cell efficacy. To exclude that enhanced killing efficacy observed against IMR-5/75 and SH-SY5Y was not due to alterations in tumor antigen expression, L1CAM expression was flow cytometrically assessed 72 h after SP-2509 treatment and shown to be unchanged (Figure S7A,B). To provide further evidence that FAS induction on the tumor cells was the mechanism responsible for enhancing the cytotoxic efficacy of L1CAM-specific CAR T cells, we conducted experiments to specifically block FAS activity. IMR-5/75 neuroblastoma cells, either untreated or pretreated with the KDM1A inhibitor, were incubated with an antibody that blocks FAS binding to its ligand without inducing FAS-directed cell death prior to exposure to CAR T cells. Blockade of FAS activity in the neuroblastoma cells eliminated the increase in tumor cell lysis observed in cells pretreated with the KDM1A inhibitor but did not impact CAR T cell-directed killing of untreated neuroblastoma cells (Figure 3C). Our findings suggested that KDM1A-mediated FAS upregulation at the tumor site is the mechanism behind enhanced CAR T cell efficacy (Figure 3D).



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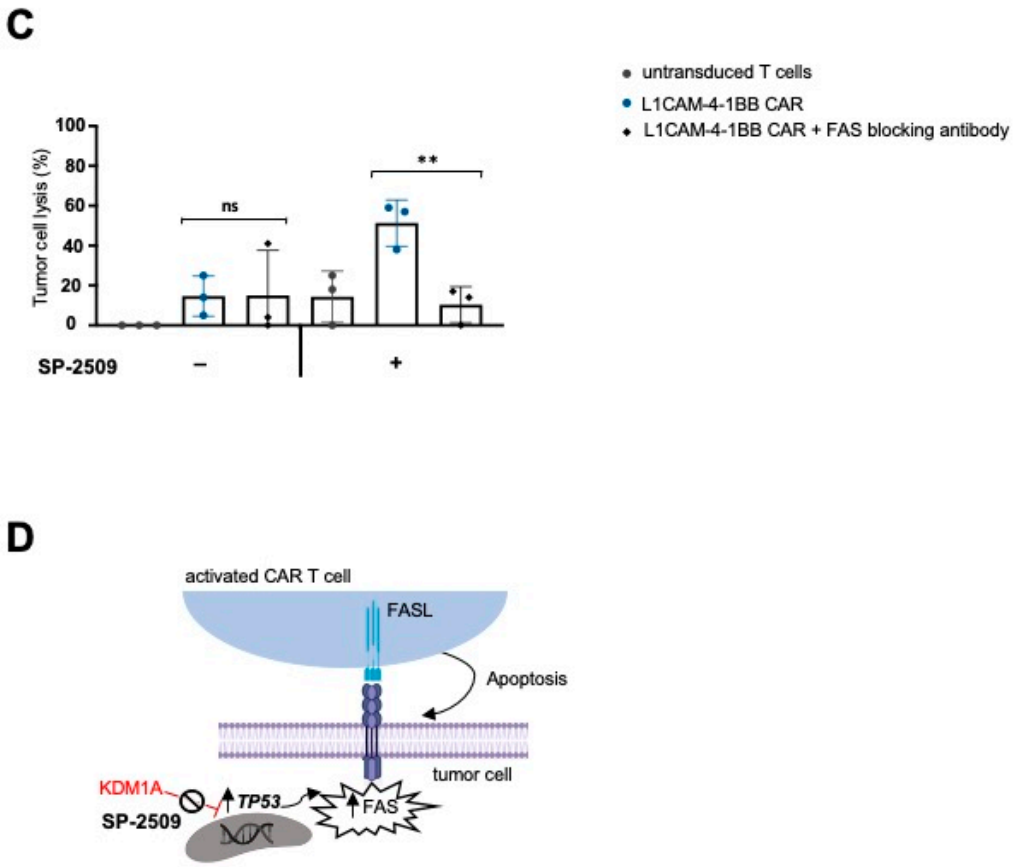

Figure 3. Combining KDM1A inhibition with CAR T cell therapy killed neuroblastoma cells expressing low tumor antigen levels via the FAS-FASL axis. (**A**) Experimental timeline for the combination therapy. (**B**) Killing efficacy of L1CAM-specific CAR T cells after 24 h coculture of neuroblastoma cell lines treated with 0.5 µM SP-2509 (or untreated controls) at an effector:target ratio of 1:5 (mean ± SD, $n = 3$). (**C**) Untreated or KDM1A-pretreated SH-SY5Y cells were incubated for 1 h in the presence or absence of the human neutralizing anti-FAS antibody before measuring the tumor cell lysis by L1CAM-specific CAR T cells cocultured with the tumor cells at an effector:target ratio of 1:5 for 24 h (mean ± SD, $n = 3$). (**D**) Illustration of FAS regulation in tumor cells. ns not significant * $p \leq 0.05$, ** $p \leq 0.01$.

2.4. FAS Upregulation by KDM1A Inhibition Enables CAR T Cells to Eradicate Antigen-Negative Neuroblastoma cells via the FAS-FASL Axis

We next explored whether FAS upregulation by KDM1A inhibition was sufficient to kill even antigen-negative neuroblastoma cells via the FAS-FASL axis. To exclude any antigen-related killing, we generated an SH-SY5Y cell model with a complete L1CAM knockout (SH-SY5Y-L1CAM-ko; Figure S8) and exposed the cell model to untransduced T cells stimulated to induce FASL expression (Figure S9). We did not use the L1CAM-negative Raji cell line, derived from a Burkitt lymphoma, for this experiment because the primary focus for our study was to show an effect on a solid tumor such as neuroblastoma. SH-SY5Y-L1CAM-ko cells were pretreated for three days with 0.5 µM SP-2509 and labeled with one fluorescent dye Dil, or alternatively untreated and labeled with a different fluorescent dye DiO, (for flow cytometric identification) before 24 h coculture with either stimulated and unstimulated T cells (E:T = 1:1, Figure 4A). Different ratios (1:1, 5:1 and 10:1) were tested for this experiment because we hypothesized that the previously applied E:T of 1:5 might be unfavorable for the untransduced T cells, which, contrary to CAR T cells, cannot eradicate tumor cells via L1CAM antigen recognition. We showed the results for the experimental design using the lowest number of effector cells (1:1 ratio) that achieved a clear effect. KDM1A-inhibited but not untreated tumor cells were selectively killed by activated T cells, resulting in a ratio shift towards the untreated tumor cells in the resulting

population (Figure 4B,C). The ratio of both tumor cell populations remained nearly 1:1 in cultures with unstimulated T cells, validating that FASL-expressing T cells induced cytotoxicity. This finding demonstrated that inhibiting KDM1A was sufficient to assist either endogenous T cells or CAR T cells present in the tumor in killing a heterogeneous tumor cell population containing tumor cells completely lacking expression of the target antigen. Taken together, our results indicated that increasing FAS expression on tumor cells by pharmacologically inhibiting KDM1A activity could improve the efficacy of L1CAM-specific CAR T cell therapy against neuroblastomas with low or heterogeneous levels of target antigen expression by unleashing antigen-independent killing via the FAS-FASL axis.

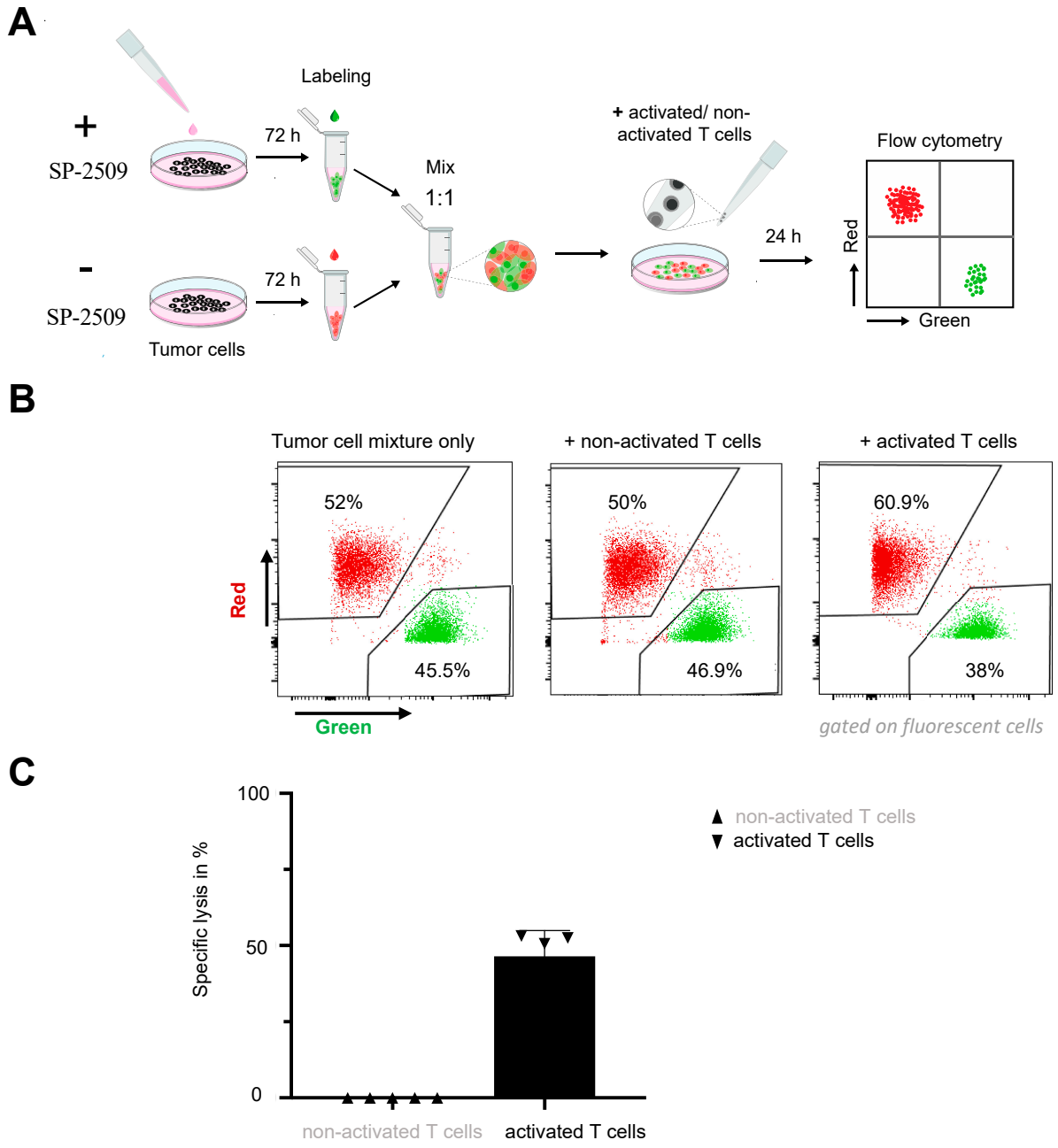

Figure 4. Combination therapy was beneficial for eradicating tumor cells lacking the tumor antigen. (**A**) Experimental timeline for the combination therapy. (**B**) Killing efficacy of activated or unactivated untransduced T cells after 24 h coculture with SH-SY5Y-L1CAM-ko cells treated with 0.5 µM SP-2509 (or untreated controls) at an effector:target ratio of 1:1. (**C**) Specific lysis of SP-2509 pretreated SH-SY5Y-L1CAM-ko cells by unactivated or activated T cells (mean ± SD, $n = 4$).

3. Discussion

To date, CAR T cells have shown unprecedented success in the treatment of hematological malignancies [24,25]. However, tumor evasion by downregulation or loss of the target antigen often ultimately leads to therapeutic failure at a later timepoint [26,27]. Therapy success against solid tumors has been very limited, due to antigen heterogeneity among tumor cells, an immunosuppressive tumor microenvironment and limited CAR T cell trafficking and infiltration at the tumor site [28]. Despite all these important factors, the strong heterogeneity in antigen expression within a solid tumor remains one of the greatest challenges limiting CAR T cell efficacy [29–32]. Developing strategies that extend CAR T cell efficacy against tumor cells with low or absent target antigen expression could provide a route to counteract tumor escape achieved by heterogeneous antigen expression per se or therapy-induced antigen downregulation. Here, we showed that pharmacological KDM1A blockade in neuroblastoma cells with the small molecule inhibitor, SP-2509, dramatically increased FAS cell-surface expression in a strictly TP53-dependent manner. FAS upregulation sensitized neuroblastoma cells to death via the FAS-FASL axis and enabled L1CAM-directed CAR T cells to eradicate antigen-negative tumor cells in vitro.

Several groups have reported failure of CAR T cell therapy against tumors which either exhibited low or heterogeneous antigen expression per se or lost antigen expression after a successful initial treatment. Xu et al. reported, that 10–20% of patients with B-cell acute lymphoblastic leukemia (B-ALL) relapsed after CD19-specific CAR T cell treatment due to emergence of CD19-negative leukemic cells [4]. Similarly, tumor escape via CD22 antigen loss has been observed in up to 30% of patients with B-ALL treated with CD22-specific CAR T cells [33]. Using ALL cell lines with variable CD22 expression, Ramakrishna et al. demonstrated that low CD22 expression (621 molecules/cell) impaired functionality as well as in vivo persistence of CD22-targeting CAR T cells [34]. We used established neuroblastoma cell lines with high, low, or heterogeneous antigen expression, and showed that the in vitro efficacy of L1CAM-directed CAR T cells was significantly reduced when the tumor antigen was less strongly or heterogeneously expressed on tumor cells. This is in line with recent literature reporting reduced CAR T cell activation, proliferation, persistence, and cytokine production as well as reduced cytotoxicity against tumors expressing low levels of target antigen [35]. We also observed differences in the efficacy of our neuroblastoma-specific CAR T cells, depending on the CAR construct employed. The CAR utilizing 4-1BB costimulation performed worse against neuroblastoma cells expressing low antigen levels, compared with the CAR imparting CD28 costimulation. These findings are in line with a report by Majzner et al. that the higher signal strength of CD28-harboring CARs was responsible for their higher efficacy against tumors with low tumor antigen density. They showed that T cells transduced with CD28-harboring CARs manifested more rapid and robust calcium influx than T cells with 4-1BB-harboring CARs [36]. These findings showed that low or heterogeneous tumor antigen expression remains one of the main reasons for therapeutic failure of CAR T cell therapy, especially for the 4-1BB-harboring CAR. To date, novel dual-antigen targeting CARs [37], tandem CARs [38] and CAR T cells secreting a bispecific T cell engager (BiTE) [39] are being investigated to tackle antigen heterogeneity within tumors. However, all these strategies remain dependent on sufficient tumor antigen expression. The advantages of the strategy we propose here, rely on epigenetically reviving an antigen-independent pathway to enhance the CAR T cell potency even in the absence of high-level tumor antigen expression. Since KDM1A is overexpressed on neuroblastoma cells [17], it remains a tumor-specific approach.

The main killing mechanism of CAR T cells is mediated via the perforin and granzyme pathway, which relies on the presence of the target antigen on the tumor cell surface [1]. Blockade of this pathway by the Ca^{2+} chelator, egtazic acid (EGTA), blocks cytotoxic granule exocytosis and almost completely diminishes CAR T cell-mediated tumor cell lysis [11]. The FAS-FASL axis is an alternative antigen-independent killing mechanism, and CAR T cells capable of killing via the FAS-FASL axis have been demonstrated to more efficiently lyse cells from tumors with low or heterogeneous antigen expression [1,40]. This path-

way might remain inactive, however, since FAS expression is downregulated on cells in a number of tumors, including gastric [41], colon [13], thyroid [42], and small cell lung [43] carcinomas, consistent with our observations in neuroblastomas. Known mechanisms responsible for FAS absence on the cell surface at the tumor site are transcriptional repression, promoter hypermethylation [44], histone acetylation [45] m and generation of secreted soluble FAS proteins that lack the transmembrane domain via alternative mRNA splicing [46]. Nevertheless, it has been demonstrated that FAS cell surface expression can still be induced in the presence of proinflammatory cytokines. For example, upon antigen recognition, CAR T cells produced proinflammatory cytokines, such as IFNG, which sensitized tumor cells to death receptor-mediated cell death by upregulating FAS expression [47]. Indeed, we observed FAS upregulation in neuroblastoma cells, but only in the presence of very high IFNG concentrations, which would be unrealistic in an in vivo setting. We did not observe FAS upregulation on neuroblastoma cells after exposure to IFNG concentrations equivalent to those produced by T cells upon in vitro tumor cell encounter. An exception was the NB-1 cell line, which expressed high levels of FAS protein on the cell surface even in the absence of IFNG. NB-1 was not included in further experiments due to its high L1CAM surface expression, but we hypothesize that the FAS-FASL axis plays a secondary role in cell lines with high antigen expression. Therefore, we aimed to epigenetically increase FAS levels on neuroblastoma cells to unleash this antigen-independent killing pathway and improve CAR T cell efficacy against neuroblastomas with heterogeneous or low target antigen expression. The epigenetic regulator, KDM1A, is known to suppress TP53-mediated transcriptional activation by maintaining TP53 in an inactive state and preventing its binding to DNA [18]. Müller et al. reported a TP53-responsive element in the *FAS* gene [14]. These reports are in line with our observation, that FAS upregulation via KDM1A inhibition was TP53-dependent. Over 98% of primary neuroblastomas harbor wildtype *TP53* at diagnosis [48]. However, the incidence of dysfunctional TP53 increases at disease relapse. Carr-Wilkinson et al. identified a TP53 dysfunction in almost half the cases (49%) of relapsed neuroblastoma in 41 patients, with the dysfunction stemming from a TP53 mutation in 15% of the cases and an upstream defect (affecting p14ARF or MDM2) in 35% of the cases [49]. TP53 activation via pharmacological MDM2 inhibition was recently shown to sensitize tumors to T cell-mediated killing [50]. Other agents suitable to revive TP53 functionality might, therefore, present alternative strategies to improve CAR T cell therapeutic efficacy by promoting antigen-independent killing via the FAS-FASL axis.

Irradiation is also known to upregulate FAS expression by inducing TP53 protein activity [51]. One study in nasopharyngeal carcinoma shows that irradiation over time increases epigenetic silencing of *TP53* via the DNMT3B DNA methyltransferase [52]. Radiation therapy not only induces a number of early side effects due to damage of surrounding healthy tissue but can also cause late adverse effects such as second malignancies. We showed that KDM1A inhibition in IMR-5/75 neuroblastoma cells induced FAS cell surface expression to higher levels than irradiation in a direct comparison. Maecker et al. demonstrated that epigenetic silencing of TP53 gene targets, such as *FAS*, can occur via TP53-independent mechanisms in cells that retain functional TP53 [53]. Huang et al. reported decreased histone acetylation and increased H3K9Me3 at the *FAS* promoter in patients with idiopathic pulmonary fibrosis, where treatment with histone deacetylase (HDAC) inhibitors restored FAS expression [45]. These findings from us and others demonstrate the critical role histone modification plays in epigenetically modulating *FAS* expression.

A possible limitation of the strategy we propose here is the biological role that KDM1A has in normal hematopoietic and neuronal stem cells [14]. Inhibiting KDM1A in these normal tissues could potentially cause treatment toxicity. However, the KDM1A inhibitor, tranylcypromine (TCP), is in clinical use as a monoamine oxidase (MAO) inhibitor to treat therapy-resistant depression. TCP was later identified as an irreversible and weak KDM1A inhibitor [54]. In recent clinical trials, treatment with 40 mg of TCP was well tolerated with an acceptable safety profile in patients with relapsed/refractory AML and myelodysplastic syndromes [55]. We selected the SP-2509 inhibitor, which allosterically

inhibits KDM1A by targeting its H3 pocket, for this study because of its high specificity for KDM1A. Furthermore, tumor-bearing mice treated with SP-2509 (15–25 mg/kg b.i.w. via IP injection) for three weeks exhibited no signs of toxicity [56], suggesting an acceptable safety profile.

Combining the inhibitor, SP-2509, with neuroblastoma-specific CAR T cells improved their efficacy, especially against neuroblastoma cells with low levels of tumor antigen, where insufficient upregulation of FAS by T cell-produced proinflammatory cytokines was observed. Ectopic FAS expression at the tumor site was recently shown to improve CAR T cell efficacy against embryonal carcinomas by enabling antigen-independent FASL-dependent tumor cell lysis [40]. Evidence for FAS-dependent bystander killing of antigen-negative tumors by T cells was also delivered by Upadhyay et al., who postulated that combining CAR T cells with small molecules targeting the FAS pathway may potentiate this mechanism [57]. Monoclonal antibodies that act as a FAS agonist have also been developed as an alternative strategy to activate tumor cell killing via the FAS-FASL axis. However, systemic toxicities caused by FAS expression in normal tissues and a 100-fold lower antibody efficacy compared to FASL expressed on T cells limited the further development of this approach [58]. In contrast, the FASL-mediated killing mechanism exploited by CAR T cells is strictly regulated, because activation-dependent upregulation of FASL on CAR T cells can only occur at the tumor site upon antigen encounter [1]. Overall, we propose that a combination of small molecule KDM1A inhibitors and CAR T cell therapy might present a promising strategy to efficiently attack neuroblastoma and other solid tumor entities with low or heterogeneous antigen expression by enabling eradication of an antigen-negative tumor fraction. Investigation of the toxicity and effectiveness of this strategy in vivo will be necessary before translating these findings to clinical therapy.

4. Materials and Methods

4.1. Generation of L1CAM-Specific CAR T Cells

CAR lentiviruses were propagated in 293T cells as described before [59] using the previously generated L1CAM-specific CE7-CAR cloned into the SIN epHIV7 lentiviral vector plasmid [20]. The single-chain variable fragment was codon optimized and subsequently linked to a 12-amino acid (short) spacer domain from the human IgG4 hinge. The spacer domain connected the antigen-binding domain to CD28 transmembrane domain followed by the signaling module containing either the 4-1BB or CD28 endodomain and the CD3zeta cytoplasmic domain. CAR constructs were linked downstream to a T2A self-cleaving peptide and truncated epidermal growth factor receptor (EGFRt) to allow CAR T cell detection and enrichment. CAR T cells were generated using T cells from healthy donors (Charité ethics committee approval EA2/262/20), then cryopreserved and expanded as previously described [60]. Untransduced T cells were used as negative controls alongside CAR T cells in experiments. Functional in vitro assays were conducted between 11 and 16 days after initiating T cell expansion.

4.2. Neuroblastoma Cell Lines

The human NB-1, IMR-5/75 and SH-SY5Y neuroblastoma cell lines were purchased from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany) and cultivated in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (MilliporeSigma, Burlington, MA, USA) and 500 µg/mL geneticin (G418, MilliporeSigma, Burlington, MA, USA). The human SK-N-BE(2) neuroblastoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and cultivated in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (MilliporeSigma, Burlington, MA, USA) and 500 µg/mL G418 (MilliporeSigma, Burlington, MA, USA). Cell line identity was confirmed using short tandem repeat DNA genotyping (Eurofins Scientific SE, Luxemburg, Luxemburg), and cultures were routinely tested for mycoplasma contamination using the PlasmoTest Kit

(Thermo Fischer Scientific, Waltham, MA USA). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

4.3. CRISPR-Mediated Generation of L1CAM Knockout in SH-SY5Y Neuroblastoma Cells

Three high-ranking guide RNAs targeting L1CAM exon 1 were designed using Dr. Feng Zhang's online CRISPR design tool [18] and synthesized with BbsI restriction overhangs. Guide RNA sequences excluding the BbsI-compatible overhangs are: RNA1, GACTGTTCCGTGATGACAGG and RNA2, CACCGCCTCGGGGATCTGGATAAGC. Respective oligonucleotides were annealed and ligated into the BbsI-digested pSpCas9(BB)-2A-Puro (PX459) V2.0. Generated vectors were validated by sequencing before transfection into SH-SY5Y neuroblastoma cells using the Effectene transfection reagent kit (cat #301425, Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, 5×10^6 SH-SY5Y neuroblastoma cells were seeded per well into a 6-well plate 24 h before transfection using 100 µL Effectene buffer, 1 µg plasmid DNA, 2 µL Enhancer, and 7.5 µL Effectene per well. Puromycin selection (0.5 µg puromycin/mL complete medium) was conducted 3 to 6 days after transfection. Single-cell clones were established from FACS-sorted L1CAM-negative cells. One single-cell clone with a bi-allelic L1CAM knockout confirmed by sequencing was selected and propagated for experiments.

4.4. IFNG Stimulation

Tumor cells were seeded into 6-well plates for 24 h treatment in triplicate with 15 ng/mL IFNG (#300-02-250UG, PEPROTECH, Hamburg, Germany).

4.5. KDM1A Inhibition

Tumor cells were seeded into 10 or 15 cm dishes and treated with 0.5–3.0 µM KDM1A inhibitor SP-2509 (M60160-2s Xcessbio, Chicago, IL, USA) for 3 days, during which treatment medium was replenished for cells every 24 h. Irradiated cells were used as a positive control for the induction of FAS. The cells were exposed to a total dose of 2 Gy at a rate of 0.848 Gy/min using a gamma irradiator (GSR D1; GSM GmbH) operating with a maximum surface dose rate of <5 µSv/h at a maximum activity of 200TBq Cs-137.

4.6. FAS Blockade

For antibody-based FAS-FASL blockade, 5×10^5 untreated or KDM1A-inhibited tumor cells were seeded into 24-well plates in triplicate and incubated with the anti-FAS antibody (CAT# 05-338, clone ZB4, MERCK, Darmstadt, Germany) at a concentration of 500 ng/mL for 1 h prior to coculture with CAR T cells.

4.7. FASL Induction in Untransduced T Cells

Untransduced T cells (3×10^7) were stimulated for 1 h with 20 ng/mL phorbol-12-myristate-13-acetate (#P8139 MilliporeSigma, Burlington, MA, USA) and 1 µg/mL ionomycin (#I0634 MilliporeSigma, Burlington, MA, USA), then for further 3 h with 10 µg/mL brefeldin A (#347688, BioLegend, San Diego, CA, USA) and 5 µg/mL monensin (#M5273 MilliporeSigma, Burlington, MA, USA) in culture medium at 37 °C with 5% CO_2.

4.8. Western Blotting

Tumor cells were detached by trypsin, washed twice with PBS, and lysed in RIPA buffer containing 15 mM HEPES, 150 mM NaCl, 10 mM EGTA and 2% Triton-X100 and protease inhibitors (#11697498001, MilliporeSigma, Burlington, MA, USA). Proteins (20 µg total protein mixed with SDS sample buffer) were resolved on SDS–PAGE and transferred onto nitrocellulose membranes. Blots were blocked with 5% non-fat dry milk diluted in Tris-buffered saline (pH 7.4) with 0.05% Tween 20 for 1 h at room temperature before incubating overnight at 4 °C with primary antibodies against L1CAM (diluted 1:1000, mouse UJ127.11, Thermo Fischer Scientific, Waltham, MA USA), KDM1A (diluted 1:1000, rabbit #2139, Cell Signaling Technology, Danvers, MA, USA), FAS (diluted 1:500, rabbit ab82419,

abcam, Cambridge, UK), TP53 (diluted 1:1000, mouse sc-126, Santa Cruz Biotechnology, Dallas, TX, USA) and GAPDH (diluted 1:5000, mouse sc-32233, Santa Cruz Biotechnology, Dallas, TX, USA). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (diluted 1:5000, anti-mouse: #730020, Thermo Fischer Scientific, Waltham, MA USA, anti-rabbit: #W10809, Thermo Fischer Scientific, Waltham, MA USA) for 2 h at room temperature, then developed using the ECL kit (RPN2232 GE Healthcare, Chicago, IL, USA). Signal intensities were measured using the Fusion FX software (Vilber Lourmat, Eberhardzell, Germany).

4.9. Cell Viability Assay

For determining the half-maximal inhibitory concentration (IC50) of SP-2509, a total of 10,000–25,000 tumor cells were seeded into flat-bottomed 96-well plates in triplicate. Cells were allowed to settle for 3 h before inhibitor (1:10 and 1:100 dilutions, range from 1 to 300 µM) was added for 72 h treatment. Untreated cells were used as a negative control. The CellTiter-Glo® Luminescent Cell Viability Assay (G9242 Promega, Madison, WI, USA) was performed according to manufacturer's instructions. Viability was calculated relative to untreated tumor cells using GraphPad prism (Version 6.00, https://www.graphpad.com/scientific-software/prism/, accessed on 27 September 2021).

4.10. Flow Cytometry

Cell-surface expression of FAS and FASL was detected by the respective fluorophore-conjugated monoclonal antibody (#305611, DX2 and #306407, NOK-2; BioLegend, San Diego, CA, USA). L1CAM cell-surface expression was detected by the fluorophore-conjugated monoclonal antibody (#130-100-691, REA163, Miltenyi Biotec, Bergisch Gladbach, Germany) and standardized QuantiBRITE calibration beads (#655050, BioLegend, San Diego, CA, USA). Activation was assessed by fluorophore-conjugated monoclonal antibodies detecting CD8 (#344741, SK1, BioLegend, San Diego, CA, USA) and CD137 (#309819, 4B4-1, BioLegend). Flow cytometry was performed on an LSRFortessa X-20 (BioLegend, San Diego, CA, USA) and data were processed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Samples were acquired with at least 10,000 alive events. Dead cells were excluded from analyses using the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (cat#L23101, Thermo Fischer Scientific, Waltham, MA, USA).

4.11. Flow Cytometry-Based Cytotoxicity Assay

Following KDM1A inhibitor pretreatment (or control condition without treatment), SH-SY5Y tumor cells were labeled with Vybrant™ Dil and DiO Cell-Labeling Solution (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions, mixed in a 50:50 ratio with each other, and seeded at 5×10^5 tumor cells/well of a 12-well plate. Stimulated untransduced T cells were added (E:T ratio of 1:1) after tumor cells had settled 3 h. The ratio of living KDM1A-treated to untreated tumor cells remaining in each well was flow cytometrically determined after 24 h. Specific lysis was calculated using the formula, [1 − (Ratio untreated:treated tumor cells cocultured with unstimulated T cells/Ratio untreated:treated tumor cells cocultured with stimulated T cells)] × 100.

4.12. Functional Assays

For cytokine release assays, 2×10^5 T cells were seeded together with tumor cells at a 1:5 E:T ratio. Media conditioned by cocultures were collected after 24 h and stored at −80 °C until analysis of IL2 and IFNG using the OptEIA™ ELISA (BioLegend, San Diego, CA, USA) according to manufacturer's instructions. CAR T cell-induced cytotoxicity was quantified in a biophotonic luciferase assay in which the neuroblastoma cells stably transduced with a GFP-ffLuc_epHIV7 reporter, served as tumor target cells. Target cells were pretreated 72 h with 0.5–3 µM KDM1A inhibitor (technical triplicates), then cocultured with untransduced T cells (controls) or CAR T cells. After 24 h, 0.14 mg D-luciferin

(PerkinElmer Inc., Waltham, MA, USA) was added, and tumor cell lysis was determined as described before [60].

4.13. Statistical Analyses

Differences in cytotoxic activity, cell surface marker expression, and cytokine release between treatment and control groups were analyzed using paired or unpaired Student's T test in GraphPad prism 8 software (GraphPad Software, La Jolla, CA, USA). p values < 0.05 were considered statistically significant. All experiments were independently repeated ($n = 3$).

5. Conclusions

Epigenetic manipulation via KDM1A blockade upregulates FAS receptor expression in neuroblastoma cells with functional TP53 signaling. FAS upregulation sensitized neuroblastoma cells to FAS-FASL-dependent death, and enabled L1CAM-directed CAR T cells to eradicate antigen-negative tumor cells in vitro. This strategy demonstrates the potential to increase CAR T cell efficacy against high-risk neuroblastoma and potentially other solid tumors known to overexpress KDM1A.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13215489/s1, Figure S1: CAR constructs and transduction efficacy, Figure S2: Drug-mediated KDM1A inhibition induces FAS expression in neuroblastoma cell lines, Figure S3: Drug-mediated KDM1A inhibition induces the FAS protein expression in neuroblastoma cell lines. Figure S4: Drug-mediated KDM1A inhibition induces TP53 and CDKN1A protein expression in neuroblastoma cell lines. Figure S5: L1CAM protein expression remains the same after drug-mediated KDM1A inhibition in neuroblastoma cell lines. Figure S6: KDM1A inhibitor does not impair CAR T cell effector function, Figure S7: KDM1A inhibition does not affect target antigen expression on neuroblastoma cells, Figure S8: Validation of L1CAM knockout in SH-SY5Y cell model, Figure S9: FASL expression is upregulated on activated primary T cells.

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Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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11 Vollständige Publikationsliste

Sulejmani O, Grunewald L, Andersch L, Schwiebert S, Klaus A, Winkler A, Astrahantseff K, Eggert A, Henssen AG, Schulte JH, Anders K, Künkele A. Inhibiting Lysine Demethylase 1A Improves L1CAM-Specific CAR T Cell Therapy by Unleashing Antigen-Independent Killing via the FAS-FASL Axis. Cancers (Basel). 2021 Oct 31;13(21):5489. doi: 10.3390/cancers13215489. PMID: 34771652

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