Aus der Klinik für Pädiatrie mit Schwerpunkt Onkologie und Hämatologie
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DISSERTATION

Selektiver Einfluss von extrazellulären Neuroblastomvesikeln auf $CD4^+$ CD171-spezifische CAR-T-Zell Effizienz gegen NTRK exprimierende Neuroblastomzellen

Selective impact of neuroblastoma-derived extracellular vesicles on $CD4^+$ CD171-specific CAR T cell efficacy against NTRK expressing neuroblastoma cell lines

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1. Abstract

1.1. Abstract Deutsch

Chimerische Antigenrezeptor (CAR) T Zelltherapie basiert auf der genetischen Integration eines tumorspezifischen CAR-Konstruktes in Patienten eigene T-Zellen, um tumorspezifische zytotoxische Reaktionen zu verstärken. Bei hämatologischen Malignitäten hat dies große Erfolge erzielt, während bei soliden Tumoren ein ähnlicher Erfolg noch ausgebleibt. Die CAR-T-Zelltherapie bei soliden Tumoren wird durch Faktoren erschwert, die mit dem direkten, aber auch entfernten Tumormicromilieu zusammenhängen. Das Neuroblastom macht einen großen Teil der pädiatrisch-onkologischen Todesfälle aus, da es meistens in bereits fortgeschrittenen Stadien diagnostiziert wird, was die verfügbaren Möglichkeiten zur kurativen Behandlung stark einschränkt. Die Expression immunogener Tumoroberflächenantigene, wie der Neurotrophen Rezeptorkinasen 1 und 2 (NTRK1, NTRK2) spielen eine führende Rolle in der Neuroblastombiologie. Neuroblastome und solide Tumoren im Allgemeinen schützen sich vor dem Immunsystem durch die Produktion und den weitrechenden Einfluss von extrazellulären Vesikeln. CD171 ist ein reichlich exprimiertes Neuroblastom-spezifisches Oberflächenantigen und das Ziel von CD171-spezifischen CAR-T-Zellen. Wir untersuchten die Zytotoxizität, Aktivierung, Erschöpfung und Zytokinproduktion von CD171-CAR-T-Zellen aus $CD4^+$- und $CD8^+$-Fraktionen nach Kokulturen mit einem SH-SY5Y-Neuroblastom-Zelllinienmodell. Wir beobachteten kongruente Effekte von NTRK1- oder NTRK2-Expression in Neuroblastomzellen auf die Effizienz von CAR-T-Zellen. Die NTRK1-Expression verbesserte die Zytotoxizität in beiden CD171-CAR-T-Zellfraktionen. Wir untersuchten ebenfalls die spezifischen Effekte von Tumorvesikeln unserer Neuroblastomzellen durch vorausgehender Kokultur mit den CD171-CAR-T-Zellen und darauffolgender Kokultur mit den entsprechenden Tumorzellen. Vorausgehender Kontakt mit den Neuroblastomvesikeln hatte keinen Einfluss auf die Viabilität, Aktivierung oder Zytokinproduktion von CAR T-Zellen, jedoch beeinträchtigte vorausgehende Kokultur mit SH-SY5Y Vesikeln die Zytotoxizität von CD171-CAR-T-Zellen der $CD4^+$-Fraktion signifikant, unabhängig von NTRK1- oder NTRK2-Expression. Unsere Ergebnisse zeigen zum ersten Mal den Einfluss von extrazellulären Tumorvesikeln und nicht zellvermittelten tumorsuppressiven Effekten auf die Wirksamkeit von $CD4^+$ CD171-spezifischen CAR-T Zellen in einem präklinischen Setting. Wir schließen daraus, dass bei der Entwicklung von CAR T-Zelltherapien diese Variablen berücksichtigt werden sollten, um die Wirksamkeit der CAR T-Zellen gegen solide Tumoren zu verbessern.

1.2. Abstract English

Chimeric antigen receptor (CAR) T cell therapy decribes the genetic introduction of a tumor specific CAR construct into patient derived T cells in order to reinforce tumor specific cytotoxic responses. This has shown great success in hematological malignancies, whereas for solid tumors similar success has yet to be achieved. CAR T cell therapy in solid tumors is aggravated through multiple factors related to the imminent and distant tumor microenvironment. Neuroblastoma makes up a large part of pediatric cancer deaths, as it is most commonly diagnosed in advanced stages, which limits currently available curative treatment options. The expression of immunogenic tumor surface antigens such as neurotrophic receptor kinases 1 and 2 (NTRK1, NTRK2) have shown to play a leading role in neuroblastoma biology. Another way neuroblastoma and solid tumors in general exert their immunoescape mechanisms is through tumor-derived extracellular vesicles across distant cell sites. CD171 is an abundant neuroblastoma-specific surface molecule that is targeted by CD171-specific CAR T cells. CD171-CAR T cells of $CD4^+$ and $CD8^+$ subgroups were evaluated in co-culture experiments with an SH-SY5Y neuroblastoma cell line model to assess CAR T cell cytotoxicity, activation, inhibition and cytokine production. We observed concurrent effects with NTRK1 or NTRK2 expression in neuroblastoma on CAR T cell cytotoxicity. NTRK1 expression improved cytotoxicity in both CD171-specific CAR T cell subgroups. We evaluated the isolated role of neuroblastoma derived extracellular vesicles by co-culture with the CD171-CAR T cells prior to tumor cell co-culture. This had no influence on CAR T cell viability, activation or cytokine production, yet SH-SY5Y derived extracellular vesicles impaired CD171-CAR T cell cytotoxicity of the $CD4^+$ subgroup significantly, independent of NTRK1 or NTRK2 expression. For the first time, our findings demonstrate the influence of tumor-derived extracellular vesicles and non-cell-mediated tumor-suppressive effects on the efficacy of $CD4^+$ CAR T cells in a preclinical setting. We conclude that for the development of CAR T cell-based treatments, these variables should be considered to improve CAR T cell therapy effectiveness against solid tumors.

2. Manteltext

2.1. Introduction

2.1.1. Combating cancer strategies

Cancer is an umbrella term describing a broad group of diseases that collectively share characteristics of disordered, uncontrolled and infiltrative cell proliferation. Depending on its potential for dissemination and malignancy, its clinical manifestation can take many forms. Therefore, a multifaceted disease like cancer requires versatile therapeutic approaches**.** Oncological therapy concepts can range from physically minimizing tumor burden through surgery or radiation therapy, to systemic therapies like chemotherapy, which unselectively targets all fast-proliferating cells in the body, or targeted therapies that specifically aim at and eliminate tumor cells. Ultimately, the choice and combination of anti-cancer approaches are among other things determined by multiple factors like the cancer type, tumor stage, progression rate, sensitivity, proteogenomic factors and many more parameters. Additionally, factors determined by the patient, like their current and evolving physical state, their age, their response to previous therapies and last but not least their mental and physical capacity to go through cancer therapy have to be taken into consideration for the choice of therapy (1). The lack of specificity in oncological therapy may cause a wide array of adverse effects, some of which are permanent. In this regard, some patient groups are more sensitive towards collateral damage caused by oncological therapies. Children for example are particularly sensitive towards lasting side effects, which can have a significant impact on their quality of life after oncological treatment and patients who do survive often suffer permanent long-term health consequences such as hearing loss, growth retardation and secondary malignancies (2).

2.1.2. Current state of neuroblastoma therapy

Pediatric malignancies remain a significant cause of death in children around the world (3). Among the most common types of pediatric malignancies are solid tumors and tumors originating from the central nervous system. “Neuroblastoma is a childhood tumor derived from primordial neural crest cells and is the most common extracranial solid tumor of childhood” (4). Due to its biological characteristics neuroblastoma’s clinical manifestation can vary dramatically, which leads to diverse outcomes that can range from spontaneous regression to wide-spread metastasis.

Children diagnosed with neuroblastoma are included into the GPOH multicenter prospective randomized controlled trials for treatment of the disease (ClinicalTrials.gov identifier: NCT 03042429). Prognostic factors are assessed during risk-stratification, which is defined by the International Neuroblastoma Risk Group (INRG) and includes among other parameters imaging stage, age of the patient, tumor histology, chromosomal ploidy and presence of chromosomal abnormalities or oncogene amplification, such as *MYCN* amplification, which is found in 20% of all neuroblastoma patients (5). Current neuroblastoma treatments mainly consist of resection or radiation of the tumor, multi-agent chemotherapy, hematopoietic stem cell transplantation and immunotherapy. Despite these multimodal therapeutic regiments and ongoing adjustments after evaluation of applied therapies, overall survival remains low. While neuroblastoma makes up 10% of all pediatric malignancies and is far less common than leukemia and lymphoma in children, it is responsible for 15% of all pediatric cancer deaths (4). When detected in very early stages and in combination with other prognostically positive parameters, neuroblastoma has a high potential to be curable or even regress spontaneously. Low-risk neuroblastoma patients have an excellent 5-year survival of >90%, whereas high-risk cases have a long term survival of only around 40% (5). Unfortunately, at time of diagnosis around 35% of all neuroblastoma patients present with lymphatically spread disease (5). General screening efforts in order to reduce the incidence of progressed disease by earlier diagnosis did not lead to an advantage in neuroblastoma diagnosis and are not established (6). Therefore, more effective therapeutic approaches are direly needed.

2.1.3. NTRK expression as influential factor in neuroblastoma biology

Apart from *MYCN* amplification, the presence of surface receptors of the neurotrophic tyrosine receptor kinase (NTRK) family can make valuable predictions and are directly correlated to neuroblastoma malignancy (7). NTRK receptors exist in three different isoforms NTRK1, NTRK2 and NTRK3, which bind to nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3), respectively. When activated by their neurotrophins, NTRK receptors activate differentiation and survival cascades in neurons, a process that is especially crucial during embryogenesis (8). Expression of NTRK1 or NTRK2 has repeatedly shown to be a reliable predictor for favorable or unfavorable neuroblastoma biology, respectively. NTRK1 expression leads to positive clinical outcomes through multiple synergistic ways such as increased sensitivity to chemotherapy and less metastatic potential, whereas NTRK2 expression is directly correlated to chemotherapy resistance, metastatic phenotype, oncogene amplification,

yielding to an overall bad clinical outcome (7, 8). Therefore, determining NTRK status of a patient's tumor histology provides further information regarding the course of the disease based on the malignancy of the tumor.

Apart from neuroblastoma, NTRK plays a role in multiple other solid tumors as well. Small proteins targeting NTRK were mostly investigated in the context of NTRK-fusion genes. In a group of solid tumors, fusion of the *NTRK* gene with another unrelated gene leads to oncogenic drive, increasing NTRK activity leading to uncontrolled cellular activation. This is the case in infantile fibrosarcoma, but can also be observed in gliomas, thyroid malignancies and lung cancer (9). As of February 2021 the FDA has approved two NTRK-targeting therapies: ROZYLTREK® (entrectinib, Gentech Inc.) and VITRAKVI® (larotrectinib, Loxo Oncology Inc., Bayer) (10, 11). Both approved NTRK inhibitors are pan-NTRK inhibitors, meaning that they target NTRK1, NTRK2 and NTRK3 collectively. They are approved for patients with solid tumors expressing NTRK gene fusion protein and in the case of ROZYLTREK®, also for ROS1-positive non-small-cell lung carcinoma. Selective targeting of NTRK2 has appeared to be difficult but is desired in order to bypass negative side effects that occur with NTRK1 targeting, like peripheral edema, extremity pain, neurosensory symptoms, as well as cognitive changes, especially in children (12, 13).

2.1.4. Tumor-derived vesicles as extended part of the solid tumor microenvironment

Independent of neuroblastoma biology, what generally contributes to aggravated effectiveness of systemic cancer therapies is the fact that neuroblastoma is a solid tumor. Solid tumor therapy is complicated by the presence and influence of the tumor microenvironment (TME). Any anti-tumor efforts, either through the patient's own immune system or through administered therapy, are faced with the additional challenge of homing to the tumor site, which involves locating the tumor, passing through blood vessels and the lymphatic system, only to be met by a pro-tumorigenic environment enclosed in a dense extracellular matrix that is enriched in various immunogenic cells and factors. An unphysiological metabolic environment, cancer-associated fibroblasts, tumor-associated macrophages and immune cells of immunosuppressive phenotype like regulatory T cells and MDSCs are only a few key players in creating and maintaining this tumorigenic environment (14, 15).

The establishment and maintenance of the TME is made possible through reliable cell-to-cell communication. Extracellular vesicles (EVs) have emerged as an efficient way of

information transmission between tumor as well as healthy cells, especially across distant cell sites. Initially EVs were thought to be a way to get rid of cellular waste, however they soon proved to be powerful communication tools utilized by almost every cell type in the human body (16). EVs are categorized into three separate subgroups: exosomes, microvesicles and apoptotic bodies, depending on their size and biogenesis. Exosomes are the smallest type of EV with up to 150 nm in diameter and are generated by the endosomal fusion of multiple vesicular bodies with the help of characteristic proteins like ESCRT and nSMase2. Microvesicles are 1 µm in diameter and are formed and released through outward budding of their parent cell membrane. Apoptotic bodies are the biggest kind of EV and are secreted by cells during apoptosis. EVs carry biologically active proteins on their surface and transport DNA and RNA species as well as enzymes, cytokines and other proteins as cargo**,** through which they can directly illicit diverse signals to their recipient cells (17). Depending on the type of cell they derived from, EVs mirror their parent cell in the composition of proteins.

Through their ubiquitous presence, far reaches across distant cell sites and distinctive proteogenomic features EVs have the potential for versatile uses. For example, EVs can be a practical non-invasive tool for diagnosing cancer or monitoring cancer therapy, because burden of EVs derived from cancer patients has shown to be correlated with cancer burden. Furthermore, EVs can be repurposed for therapeutic intentions, especially in anti-cancer therapy (18). Immune cell-derived EVs have shown to exert anti-tumor activity (19). Additionally, EVs can be used to transport drugs into recipient tumor cells through encapsulation (20).

In the context of solid tumor formation and perseverance however, tumor-derived EVs (TEVs) have shown to play a crucial role in the establishment of a pre-metastatic niche, facilitating metastatic spreading (21). Apart from securing tumor spreading, TEVs have demonstrated to play a vital part in solid tumor maintenance and malignancy by promoting angiogenesis, modulating immune cells to a pro-tumorigenic phenotype, inhibiting T cell and NK cell activity directly by expressing inhibitory tumor antigens on their surface and increasing tumor cell chemotherapy resistance (21, 22).

2.1.5. Utilization of the immune system in anti-cancer therapy

Immunotherapy is an individualized approach to beat cancer. The body’s natural anti-cancer machinery, the immune system, can be harnessed through multiple ways to combat cancer cells: Through checkpoint inhibitors immune cell damping can be reversed. Monoclonal

antibodies can be used to attach to a tumor antigen and trigger an immune response. Cytokines can be administered to exert a wide range of immunomodulatory responses and mitigate immune cells. Cellular immunotherapy, for example in the form of adoptive T cell therapy, utilizes autologous T cells that were altered *ex vivo* in a way to express a heightened anti-tumor response, either by selectively expanding tumor-infiltrating lymphocytes or equipping these T cells with a tumor-selective T cell receptor (TCR) or chimeric antigen receptor (CAR) (1).

The main effector immune cells dictating anti-tumor responses, the T cells, proved to be ideal candidates for genetically editing to increase anti-tumor efficacy. In the physiological setting, T cells detect antigens through their TCR. Tumor antigens are presented to the TCR through the major histocompatibility complex (MHC) proteins of surrounding antigen-presenting cells. TCR activation results in downstream signaling that among other things leads to T cell activation, proliferation and effector functions to eliminate the detected antigen (23). This sequence of actions has acquitted itself well for the detection of foreign and harmful antigens, however, it is not perfect and does not always yield to the necessary immune response. Immune evasion happens when tumor cells manage to escape the body's immune system through different mechanisms. These include shifting the immune cells into a more tumor tolerating phenotype and directly preventing immunorecognition by downregulating MHC molecules resulting in an omitted anti-tumor immune response by T cells (24).

Immunotherapy in neuroblastoma was brought into focus when GD2 antibody therapy had shown to increase overall survival of high-risk patients by 10%. GD2 is a well-studied tumor-associated antigen expressed on neuroblastoma cells. This prompted the incorporation of GD2 antibody therapy into the standard protocol for the treatment of patients with high-risk neuroblastoma (25). The success of GD2 antibodies has been a landmark for neuroblastoma treatment. It has hence become a popular target for GD2-specific CAR T cell therapy as well.

2.1.6. CAR T cell therapy

To combat tumor-induced immune evasion, it is advantageous to involve patient-derived immune cells in the development of individualized potent anti-tumor therapies. Cellular immunotherapy went through a paradigm shift when immune cells could be genetically edited in a way to boost tumor-targeting mechanisms, as well as cytotoxic reactions after docking to the target antigen. In addition to that, by bypassing MHC I-conveyed antigen

presentation, detection and effector mechanisms of T cells in reaction to tumor cells could be made less evadable. To achieve that, T cells are genetically engineered to express an additional synthetic surface receptor that specifically targets tumor-associated or tumor-specific surface antigens. This additional synthetic receptor is referred to as chimeric antigen receptor (CAR). Through the expression of a CAR, T cells combine the specificity of an antibody, with the cytotoxicity of a T cell and can target antigens independent of MHC I presentation. The CAR is structurally made up of an extracellular antigen-recognizing region, analogous to a single chain variable fragment (scFv) region of an antibody. The scFv domain can be designed to bind to the desired tumor antigen and directs specificity and binding affinity (26). The scFv region is connected to the hinge domain. The hinge domain structurally resembles an antibody's Fc region and contributes to antigen binding by determining the CARs flexibility and distance to the antigen binding site (27). The hinge region is linked to the transmembrane domain, which anchors the CAR into the T cell membrane. The choice of transmembrane domain has also shown to determine a CAR's structure and persistence, as it is involved in signal transduction (28). The endodomain carries a CD3zeta molecule which conveys TCR signaling. CARs can be categorized into first, second and third generation CARs, depending on the number of co-stimulatory domains in addition to CD3zeta. First generation CARs carry only the CD3zeta stimulatory domain, whereas second and third generation CARs carry two or three costimulatory domains in addition to CD3zeta respectively. Co-stimulatory domains mainly include CD28 and 4-1BB, but ICOS, CD27 and OX40 have shown efficacy as well (29, 30). The choice of co-stimulatory domain has substantial effects on CAR T cell proliferation, persistence and efficacy (26). The intricate composition of each element making up the CAR determines its activity, persistence, efficacy and homing to the tumor. Apart from CAR composition, the choice of targeted tumor antigen is imperative. Ideally, tumor antigens should be homogenously and stably expressed on the surface of every tumor cell, and most importantly exclusively by the tumor to prevent on-target off-tumor reactions (31).

The only approved CAR T cell therapies are targeting hematological malignancies. As of February 2021, three CD19-specific CAR T cell therapies have been approved by the FDA and have found their way into the clinic: KYMRIAH™ (Tisagenlecleucel, Novartis Pharmaceuticals Corp.) was the first CAR T cell therapy approved by the FDA and is used in the treatment of children and adults of up to 25 years of age with B cell precursor acute lymphoblastic leukemia and for adults with relapsed or refractory diffuse large B-cell lymphoma (32). YESCARTA™ (Axicabtagene ciloleucel, Kite Pharma Inc.) is approved for

the treatment of adults with certain kinds of relapsed or refractory B cell lymphoma (33). Most recently, TECARTUS™ (brexucabtagene autoleucel, Kite Pharma Inc.) was approved as the first CAR T cell treatment of relapsed and refractory mantle cell lymphoma in adults (34). The choice of targeted tumorantigen plays a major part in the success of CD19 CAR T cell therapy. CD19, is abundantly and stably expressed by B cell lineages. Even though it is expressed on malignant as well as healthy B cells, the CD19 CAR T cell-induced B cell aplasia is a manageable side effect. Furthermore, with B cell lymphomas being part of hematological malignancies, CD19 CAR T cells do not have to overcome tumor microenvironment-related obstacles that would complicate homing to the target.

2.1.7. CD171-specific CAR T cell therapy targeting neuroblastoma

In the context of neuroblastoma, CD171 (L1CAM) has shown the potential to be a suitable tumor-associated antigen. CD171 is an adhesion molecule that is abundantly expressed in neurological tissues but overexpressed on neuroblastoma cells. In contrast to the CD171 that is expressed on healthy tissues, neuroblastoma-associated CD171 shows proteomic alterations in the form of glycosylation of the CE7 epitope (35). Despite CD171 being an abundant molecule on healthy tissues, tumor-specificity is provided by targeting the glycolyzed CE7 epitope on neuroblastoma CD171. The selectivity of CE7-specific CD171-CAR T cells has been demonstrated in safety and toxicity studies (35).

In order to further advance CD171-CAR T cell therapy against neuroblastoma, we were interested to assess the influence of two factors that are, by themselves and in combination, responsible for low efficacy of standard neuroblastoma therapy: malignant tumor phenotype through the expression of NTRK2 and the presence of neuroblastoma-derived TEVs, which enable the hampering effects of the TME through distant cell sites.

2.2. State of research

2.2.1. Neuroblastoma-targeting CAR T cell therapies

Immunotherapy in neuroblastoma was brought into focus when GD2 antibody therapy had shown to increase overall survival of high-risk patients by 10%. As February 2021, CliniclalTrials.gov has registered 11 active studies involving GD2-specific CAR T cell therapy for neuroblastoma (36). In a recent phase I study (NCT 02761915) GD2-CAR T cells have shown promising results by displaying antitumor activity in patients with relapsed and refractory neuroblastoma (37).

CD171-specific CAR T cell therapy against neuroblastoma is currently under evaluation in the ENCIT-01 phase I clinical trial at Seattle Children's Hospital (NCT 02311621). As of February 2021, this is the only CD171-specific CAR T cell therapy against neuroblastoma registered in ClinicalTrial.gov (38). The ENCIT-01 study assesses third generation CD171-specific CAR T cells harboring CD24 and 4-1BB as costimulatory domains, as well as two second generation, 4-1BB-harboring, CD171-CAR T cell constructs with either a short or a long spacer lengths. Generated CAR T cells are derived from autologous $CD4^+$ and $CD8^+$ T cell subsets and are reintroduced to the patient in a 1:1 ratio. The importance of the choice of T cell subset ratio for overall outcome has been shown previously in the context of Non-Hodgkin Lymphoma (39).

2.2.2. NTRK2-specific inhibition as therapeutic approach in solid tumors

Expression of NTRK2 is associated with aggressive disease and poor survival of patients with neuroblastoma. As of February 2021, only pan-NTRK-specific therapies (see 2.1.3), but no NTRK2-specific therapies have been clinically approved by the FDA. A potential NTRK2-specific molecule, GZD2202, is under preclinical evaluation and shows 10-fold less potency against NTRK1 than NTRK2 (40). In a xenograft mouse model, GZD2202 administration led to 36.1% growth inhibition of the NTRK2-expressing neuroblastoma model SH-SY5Y-NTRK2. However, no NTRK2-specific targeted therapy has entered clinical testing yet.

2.2.3. Tumor-derived vesicles as therapeutic target

Neuroblastoma-derived extracellular vesicles have shown to induce tumor-progressing factors like cytokines and chemokines in mesenchymal stromal cells (41). Therefore, blockage of TEV production or reduction of TEV burden in order to disrupt cancer crosstalk has been a recently explored approach. Inhibition of TEV production has been assessed by blockage of proteins involved in the biogenesis of TEVs. Through screening efforts of commonly available drugs, multiple inhibitors of TEV production in prostate cancer cells were identified: tipifarnib, neticonazole and ketoconazole inhibited TEV production by blocking RAB27A, which is involved in exosome production and signal transduction. Compounds like GW4869 and Manumycin A were shown to selectively block TEV production in prostate cancer cells through inhibition of neutral sphingomyelinase (42, 43). Apart from directly inhibiting the TEV production machinery, it is possible to reduce EV production in other ways. It has been reported that a low pH in the TME has shown to be

beneficial for tumor growth as well as TEV production. Common proton pump inhibitors like lansoporazole and omeprazole reached significant reduction of TEV production in melanoma cells by raising the pH through V-ATPase blockage (42, 44). Inhibition of proton exchanger carbonic anhydrase IX has shown promise in reducing TEV burden by raising the pH and is currently being explored in combination with Gemcitabine in patients with metastatic pancreatic ductal cancer in an open-label multicenter phase Ib trial (NCT 03450018). Another component displaying TEV inhibition properties is cannabidiol, which showed reduced TEV production in prostate cancer, breast adenocarcinoma and hepatocellular cancer cell lines (45). Despite showing promising preliminary data *in vitro*, TEV inhibitors have to be further explored as adjuvant for cancer therapy in a clinical setting.

2.3. Subject relevance

Neuroblastoma-derived EVs had not yet been explored in the context of CD171-specific CAR T cell therapy against NTRK-expressing neuroblastoma. CAR T cell development is becoming increasingly sophisticated, with more and more adjustments made on the T cells and the CAR construct itself. We aim to identify components that can be possible future targets for accessory adjustment in assistance of CD171-specific CAR T cell therapy in neuroblastoma by exploring to what degree environmental components like TEVs and NTRK expression have an influence on CAR T cell efficacy. This way we want to contribute to the advancement of individualized immunotherapy in the form of CD171-specific CAR T cell therapy for solid malignancies like neuroblastoma.

2.4. Methods

The following description of methods corresponds to the methods described in the publication “Tumor-Derived Extracellular Vesicles Impair CD171-Specific $CD4^+$ CAR T Cell Efficacy” (46).

2.4.1. Neuroblastoma cell lines and NTRK expression

All experiments were conducted *in vitro*, using established cell line models and methods of measurement.

The cell line SH-SY5Y is a subclone of the human neuroblastoma cell line SK-N-SH and is commonly used for neuroblastoma research. SH-SY5Y parental cells were maintained in RPMI Medium (Gibco) supplied with 10% fetal calf serum (FCS). Wildtype SH-SY5Y is endogenously NTRK negative, which is why we used SH-SY5Y generated to stably express

NTRK1 or NTRK2 via retroviral transduction of NTRK expressing cDNA in order to obtain comparable data concerning the role of NTRK receptors as previously described (7). NTRK expression was confirmed genomically via PCR and proteomically by western blotting for pan-Trk and activated NTRK expression phospho-Trk. SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 were cultivated in RPMI medium, supplied with 10% FCS and 500µg/ml G418 (Sigma). All cell lines underwent Short Tandem Repeat DNA genotyping for cell line identification as well as weekly testing for mycoplasma using the PlasmoTestTM Kit (Invitrogen). The general number of passages between thawing and use was <20 for all experiments performed.

Baseline proliferation and viability of SH-SY5Y, -NTRK1, and -NTRK2 was determined by incubation of the cell lines in their respective medium for eight days and counting total and living cells on days 2, 4, 6 and 8 using trypan blue.

CD171-high SH-SY5Y-NTRK2 cell line was generated by transfection of SH-SY5Y-NTRK2 with an epHIV7.2 viral vector carrying the CD171-expressing plasmid. Expression of our target antigen CD171 on the SH-SY5Y cell lines was measured via flow cytometry using a fluorophore-conjugated antibody for CD171 (cat#130-100-691, Miltenyi Biotec)**.**

2.4.2. PCR

DNA was isolated from SH-SY5Y parental cells and the stable expressing SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 cell models using the Nucleospin Tissue Kit (Macherey-Nagel). PCR-based detection of neurotrophin receptor expression was achieved by PCR-based amplification using primers for NTRK1 (forward: ACCATGCTGCCCATTCGCTG, reverse: GAGGGC AGGCCCCAGTATTC) or NTRK2 (forward: GCAATGATG ATGACTCTGCC, reverse: GGAACACTTTTCCAAAGGCT), and subsequent separation of PCR products in 1% agarose gels by electrophoresis.

2.4.3. Western Blotting

Tumor cells were detached by trypsin, washed twice with PBS and lysed in RIPA buffer including protease inhibitors and the Phosphatase Inhibitor Cocktail (Roche). Proteins were separated by SDS-PAGE before western blotting with pan-Trk (C-14; sc- 11, Santa Cruz Biotechnology) and phospho-Trk (#4621, Cell Signaling Technology) antibodies.

2.4.4. Flow Cytometry

Cell-surface expression of CD4 (BD Biosciences), CD8 (BioLegend), and CD171 (cat#130-100-691, Miltenyi Biotec) was detected by fluorophore-conjugated monoclonal antibodies. EGFRt expression was detected using biotinylated cetuximab (Bristol-Myers Squibb) and a phycoerythrin (PE)-conjugated streptavidin antibody (BioLegend). T cell activation and inhibitory markers were assessed by fluorophore-conjugated monoclonal antibodies detecting CD137 (BioLegend), CD25 (BioLegend), PD-1 (also known as PDCD1 or CD279, BioLegend), TIM3 (Biolegend), and LAG3 (BD Biosciences). Flow cytometry was performed on a Fortessa X-20 (BD Biosciences) and data were processed using FlowJo software (Tree Star Inc.). Dead cells were excluded from analyses using LIVE/DEADTM Fixable Green Dead Cell Stain Kit (Life Technologies).

2.4.5. Isolation and verification of TEVs

TEV isolation and verification was done by Vera Rebmann (University Duisburg-Essen). TEVs were derived from the respective SH-SY5Y cell line supernatant. To obtain extracellular vesicles released from SH-SY5Y, SH- SY5Y-NTRK1, and SH-SY5Y-NTRK2 cells, cells were cultured for 9 h in RPMI medium supplemented with 10% extracellular vesicle-depleted fetal bovine serum (FCS), 5% penicillin- streptomycin (Pen Strep, 10,000 U/mL, Life Technologies), and 1% L-glutamine (L-Glutamine, 200 mM, Life Technologies). Conditioned media was subjected to ultracentrifugation at 10,000 × g in a fixed angle Type 45 Ti rotor (Beckman Coulter) for 30 min in order to remove membrane patches, followed by a further ultracentrifugation step at 120,000 × g for 120 min at 4◦C using a swinging bucket SW 40 Ti rotor (Beckman Coulter). Pelleted TEVs were resuspended in 0.9% NaCl and stored at −20◦C until usage. The obtained TEV fractions were characterized by (i) SDS-PAGE and western blotting to verify typical extracellular vesicle marker expression (CD81, TSG101, syntenin) and the absence of intracellular proteins or endosomes (calnexin) according to consensus requirements defining extracellular vesicles **(28**) (47), (ii) nano-particle tracking analysis using ZetaView analyses (Particle Metrix, Diessen, Germany) to define size and particle concentration **(29)** and (iii) protein assay (Thermo Scientific, Darmstadt, Germany) to define protein concentration.

2.4.6. Generation and cultivation of CD171-specific CAR T cells

For generation of CD171 specific CE7-CAR T cells the CAR construct was cloned into a SIN epHIV7 lentiviral vector, and lentivirus was propagated in 293T cells. The scFv was codon-

optimized and subsequently linked to a 229-amino acid spacer domain from the human IgG4 hinge. The spacer domain was modified by two substitutions, L235D and N297Q, to reduce binding to the IgG Fc gamma receptor (48)**.** The spacer domain connects the antigen-binding domain to the CD28 transmembrane domain, which is followed by the signaling module containing the CD3zeta cytoplasmic domain and 4-1BB. The CAR construct also contained a T2A self-cleaving peptide and truncated epidermal growth factor receptor (EGFRt) allowing for CAR T cell detection and enrichment.

Apheresis products were obtained from healthy donors (Charité ethics committee approval EA2/216/18) and peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare). $CD4^+$ and $CD8^+$ T cells were obtained by positive selection using immunomagnetic microbeads (Miltenyi Biotec) and activated with anti-CD3/CD28 beads (Life Technologies). On day three, activated T cells were transduced with the CAR-containing lentivirus. $EGFRt^+$ CAR T cells were enriched by immunomagnetic selection with biotin-conjugated cetuximab (Bristol-Myers Squibb) and streptavidin microbeads (Miltenyi Biotec). Untransduced T cells were used as negative controls alongside CAR T cells in all experiments. CAR T cells and control T cells were cryopreserved until further use. Cryopreserved cells were thawed, stimulated with irradiated peripheral blood mononuclear cells, irradiated lymphoblastoid TMLCL cells, and OKT3 (30 ng/mL, Miltenyi Biotec), and expanded according to a rapid expansion protocol (35). $CD4^+$ T cells were supplied with IL2 (50 U/µl) and IL7 (10 ng/µl) and $CD8^+$ T cells were supplied with IL2 (50 U/µl) and IL15 (10 ng/µl) every other day following expansion. Functional *in vitro* assays were conducted between days 11 and 16 of culture.

2.4.7. TEV Co-culture and CAR T cell exposure

T cells (2×10^5) were co-cultured with 10 µg TEV protein per well in 96-well flat-bottom plates in triplicate for 24 hours in RPMI supplemented with 10% extracellular vesicle-depleted FCS. After 24 hours cells were pooled, washed twice with PBS and either used in FACS analyses or for viability assessment using trypan blue. T cells were then mixed with tumor cells at an effector:target ratio of 1:2. T cells primed with TEVs derived from parental SH-SY5Y cells were co-cultured with SH-SY5Y target cells. T cells primed with TEVs derived from SH-SY5Y-NTRK1 or SH- SY5Y-NTRK2 cells were co-cultured with SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 target cells, respectively. Viability, activation and inhibitory markers were assessed in CAR T cells after 24 hours using FACS-based detection of cell

surface markers. We determined the concentration of cytokines IL-1 and IFNG in the supernatant of TEV and CAR T cell co-cultures via OptEIA^{TM} ELISA.

2.4.8. Functional Assays

For cytokine release assays, 2 × 10^5 T cells were seeded together with stimulator cells at a 1:2 effector:target ratio. After 24h, conditioned media was collected and stored at −80◦C until analysis of IL2 and IFNG using the OptEIATM 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 the GFP-ffLuc_epHIV7 reporter plasmid served as tumor target cells. Target cells were co-cultured with negative control or CAR T cells. The maximal biophotonic luciferase signal was obtained by measuring luminescence of target cells in the absence of CAR T cells (RLUmax, maximal relative light unit). After 24 or 72 h, 0.14 mg D-luciferin (PerkinElmer Inc.)/ml medium was added to each well, and the biophotonic signal was detected. Lysis was determined as [1-(RLUsample/RLUmax)] × 100 in relation to untreated cells. For sequential treatment, the additive amount of tumor lysis was calculated related to viable tumor cells at day 3. If not indicated otherwise, all data points were obtained as technical triplicates.

2.4.9. Statistical Analysis

The differences between treatment groups were statistically analyzed using unpaired Student *t* tests in PRISM (GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ were considered statistically significant.

2.5. Results

2.5.1. TEV isolation and influence on CD171-CAR T cell viability and functionality

We confirmed NTRK1 and NTRK2 expression of the SH-SY5Y cell lines by PCR and proved constitutive activation by identifying presence and phosphorylation of said receptors via western blotting (46).

TEVs were successfully isolated by Vera Rebmann (University Duisburg-Essen) via established ultracentrifugation methods from supernatants of the cell lines SH-SY5Y, SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2. Their purity was validated by checking size using Nanoparticle tracking analysis and confirming exosomal size of <150nm. Expression of extracellular vesicle specific proteins TSG101, syntenin and CD81, and the absence of

intracellular proteins like calnexin was validated by SDS-PAGE and western blotting (46). Previous publications reported that TEVs had an impact on immune cell viability, e.g. TEVs derived from colorectal cancer patients or from the ascites of ovarian cancer patients directly induced apoptosis in T cells through the death receptor pathway (49, 50). After a 24 hour co-culture of TEVs derived from SH-SY5Y, SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 cell lines with CD171-specific $CD4^+$ or $CD8^+$ CAR T cells, no changes in CAR T cell viability were observed when CAR T cell viability was assessed via flow cytometry (46).

We further determined the expression of activation markers CD25, CD137 as well as inhibitory markers TIM3 and LAG3 on TEV primed and unprimed CAR T cells by flow cytometry. Expression of CD137 remained low in TEV primed and unprimed CAR T cells of both subpopulations (46). CD25 expression in CAR T cells and control T cells was comparibly high in both subgroups, however the co-culture without TEVs lead to high ectopic CD25 as well, most likely due to alloreactivity (46). Expression of TIM3 was more pronounced in CAR T cells compared to control T cells, but TEV priming did not lead to significant changes in TIM3 expression in either subgroup (46). TEV co-culture did not lead to an increase in PD-1 and PD-1 expression remained low in both CAR T cell subgroups and the control (46).

CAR T cell functionality can be determined through the measurement of produced cytokines IL2 and IFNG. We measured IL-2 and IFNG concentration in the supernatant of CAR T cells co-cultred with or without TEVs. We found that TEV co-culture did not induce cytokine production in CAR T and control T cells of either subgroup.

Priming CD171-CAR T cells of $CD4^+$ and $CD8^+$ subpopulations with TEVs derived from SH-SY5Y, SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 did not lead to changes in viability, expression of activation or inhibitory markers, and cytokine production (46).

2.5.2. Influence of TEV-priming on CD171-CAR T cell cytotoxicity

TEVs have repeatedly shown to be involved in the formation of a pre-metastatic niche by differentiating the surrounding environment into a tumor-friendly type and facilitating tumor establishment and metastasis. TEVs stimulate angiogenesis and induce fibroblast activation for the maintenance of the TME's extracellular structure (51, 52). When met with TEVs, antigen-presenting cells like monocytes differentiate into myeloid-derived suppressor cells (MDSCs), which exert tumor-tolerating effects. MDSCs in turn stimulate the proliferation of regulatory T cells, which promote pro-tumorigenic mechanisms (53). Other

immunodysregulatory effects of TEVs include the inhibition of activated T cells through downregulation of the CD3zeta chain on $CD8^+$ T cells (54).

Despite not having witnessed TEV induced apoptosis in our CAR T and control T cells, we were interested to see whether previous co-culture of our CAR T cells with TEVs from SH-SY5Y, SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 for 24 hours would have an impact on CAR T cell functionality. For that the CAR T cells, previously exposed to the different TEVs for 24 hours, were co-cultured with the respective SH-SY5Y cell lines for additional 24 hours in an effector: target ratio of 1:2. Resulting cytotoxicity was measured by the remaining biophotonic signal of living tumor cells carrying a GFP-ffLuc reporter. We found that prior CAR T cell exposure to TEVs lead to significantly impaired cytotoxicity of CD171-CAR T cells of the $CD4^+$ subpopulations when consecutively co-cultured with the respective SH-SY5Y cell lines (46). This finding was independent of NTRK expression. CAR and control T cells of the $CD8^+$ subpopulation were unaffected by prior TEV exposure and displayed similar cytotoxicity towards the tumor cells (**Figure 1**) (46).

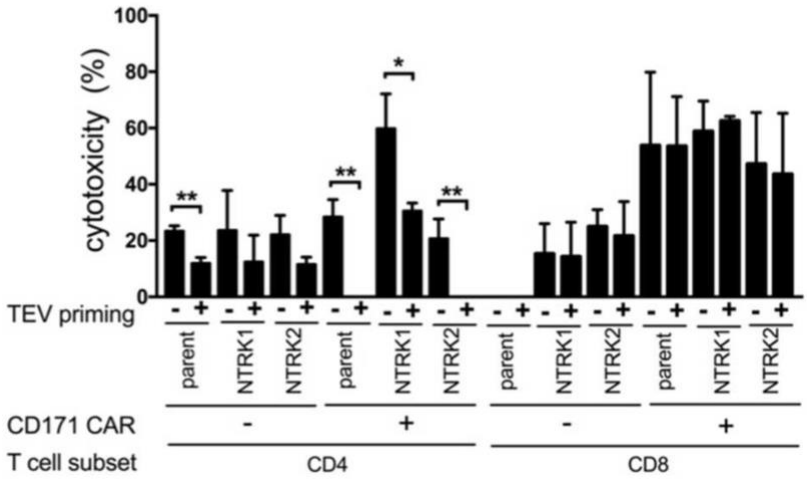

Figure 1 Cytotoxicity of TEV-primed and -unprimed CD171-CAR T cells of $CD4^+$ and $CD8^+$ subpopulations against NTK1 or NTR2 expressing SH-SY5Y. *(Ali S. et al. Tumor-Derived Extracellular Vesicles Impair CD171-Specific CD4(+) CAR T Cell Efficacy. Front Immunol. 2020;11:531.)*

2.5.3. CD171-CAR T cell funtionality after TEV exposure

To identify the cause of impaired cytotoxicity in the TEV-primed $CD4^+$ CAR T cells, we measured cytokine concentrations in the supernatant of the 24 hours CAR T cell and tumor cell co-cultures. We compared IL2 and IFNG production of TEV primed and unprimed CAR T cells after tumor co-culture and found that previous TEV exposure led to a consistently lower IL2 and IFNG release by $CD4^+$ and $CD8^+$ CAR T cell subpopulations. However, these

differences in concentration were discreet. Significantly lower IFNG production was only measured in TEV-primed $CD4^+$ CAR T cells when co-cultured with SH-SY5Y-NTRK2 compared to their unprimed counterparts (46). We concluded that while a discreetly lower cytokine production might have an effect on overall CAR T cell functionality, it did not explain significantly impaired CAR T cell cytotoxicity of the TEV primed $CD4^+$ CAR T cells against the tumor cell lines.

We next evaluated whether TEV priming lead to a difference in CAR T cell activation and inhibitory receptors. Expression of ectopic T cell activation markers (CD25 and CD137) and inhibitory markers (TIM3, PD-1 and LAG3) on TEV-exposed or -unexposed CAR T cells after tumor cell co-culture was measured via flow cytometry. Previous TEV exposure did not lead to changes in ectopic expression of T cell activation or inhibitory markers. To conclude, impaired cytotoxicity of $CD4^+$ CAR T cells was not attributed to impaired effector functions like cytokine production or CAR T cell activation (46).

Interestingly, TEVs isolated from NTRK1- or NTRK2-expressing cell lines impaired CAR T cell efficacy of the $CD4^+$ subpopulation to the same degree, meaning that TEV impairment was likely independent of NTRK expression. After demonstrating that previous 24-hour exposure of CD171-specific $CD4^+$ CAR T cells led to impaired cytotoxicity against SH-SY5Y cell lines regardless of NTRK expression we wanted to further investigate the role of ectopic NTRK expression.

2.5.4. Impact of NTRK1 or NTRK2 expression on CD171-CAR T cell cytotoxicity

To understand the involvement of NTRK1 or NTRK2 expression on CAR T cell cytotoxicity, we co-cultured $CD4^+$ and $CD8^+$ CD171-specific CAR T cells with SH-SY5Y, SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 for up to 72 hours in effector: target ratios of 1:2 and 2:1 without any previous TEV involvement and measured CAR T cell cytotoxicity after 24 and 72 hours. We observed that NTRK expression had a significant influence on CAR T cell cytotoxicity in both subpopulations. CAR T cells of both subpopulations displayed significantly higher cytotoxicity towards SH-SY5Y-NTRK1 compared to SH-SY5Y and SH-SY5Y-NTRK2 (46). This remained consistent in both timepoints and in both effector:target ratios.

2.5.5. Role of NTRK2 and target antigen expression on CAR T cell cytotoxicity

We investigated the influence of NTRK2 expression in SH-SY5Y by comparing baseline proliferation rates and viability of the three cell lines SH-SY5Y, SH-SY5Y-NTRK1 and SH-

SY5Y-NTRK2. We found that NTRK2 expressing SH-SY5Y showed comparably lower viability compared to the parental cell line. Viability of SH-SY5Y-NTRK2 ranged from 59.6-89.9%. SH-SY5Y-NTRK1 cells displayed highest viability ranging from 84.3-100%. Similarly, SH-SY5Y-NTRK2 displayed lowest proliferation rates compared to the parental cell line whereas SH-SY5Y-NTRK1 displayed higher proliferation rates than the parental control. These differences however remained discreet and not significant.

CAR T cell activation and cytotoxicity is among other things dependent on target antigen exposure. We measured expression of our target antigen CD171 on the tumor cell lines. Interestingly, NTRK expression influenced CD171 surface expression on the tumor cells. SH-SY5Y-NTRK1 cells expressed the highest levels of CD171, while SH-SY5Y-NTRK2 expressed lowest levels of CD171 in comparison (46). We investigated whether the higher tumor target antigen expression of ectopic CD171 in SH-SY5Y-NTRK1 was responsible for consistently higher CAR T cell cytotoxicity against SH-SY5Y-NTRK1. We virally transduced SH-SY5Y-NTRK2, which was naturally low in CD171 expression and towards which the CAR T cells showed least amount of cytotoxicity, to overexpress ectopic CD171 (46). Flow cytometric control showed successful overexpression of CD171 on transduced SH-SY5Y-NTRK2. Following that, we performed co-culture experiments analogous to the initial cytotoxicity assessment using naturally CD171-low SH-SY5Y-NTRK2 and our generated CD171-overexpressing SH-SY5Y-NTRK2. We could observe that CD171 overexpression on SH-SY5Y-NTRK2 did not result in increased CAR T cell killing, neither by $CD4^+$ nor by $CD8^+$ CAR T cells (46). CAR T cell cytotoxicity towards both CD171-low and -high NTRK2-expressing cell lines remained comparable over time and in different ratios (46). We measured the concentration of cytokines IL2 and IFNG in the supernatent of these co-cultures. Co-culture with CD171-high SH-SY5Y-NTRK2 did result in discreetly higher IL2 and IFNG production by both CAR T cell subpopulations, however the differences were not significant compared to cytokines produced after co-culture with SH-SY5Y-NTRK2. This demonstrates that the level of ectopic target antigen expression is not the main driver for impaired CAR T cell cytotoxicity in NTRK2-expressing SH-SY5Y.

2.5.6. Influence of NTRK1 or NTRK2 expression on CD171-CAR T cell functionality

To further investigate whether variable cytotoxicity of NTRK1- and NTRK2-expressing SH-SY5Y, was due to different CAR T cell effector functions, we analyzed CAR T cell cytokine

release via ELISA. CAR T cells produced significantly more IL2 and IFNG when co-cultured with the SH-SY5Y and NTRK model cell lines, compared to control T cells. CAR T cell co-culture with NTRK1 expressing SH-SY5Y tended to result in higher cytokine levels compared to the other cell lines. However, these differences were not significant (46).

After tumor co-culture we measured T cell activation markers (CD25 and CD137) as well as inhibitory markers (TIM3, PD-1 and LAG3). Co-culture with SH-SY5Y-NTRK1 led to significant higher CD25 expression in $CD4^+$ and $CD8^+$ CAR T cells compared to coculture with SH-SY5Y (46). CD137 was upregulated in both CAR T cell subpopulations after tumor co-culture, but more so in $CD8^+$ CAR T cells. NTRK1 and NTRK2 expression in SH-SY5Y cells lead to significantly higher CD137 expression on $CD8^+$ CAR T cells (46). Both CAR T cell subpopulations upregulated TIM3 after tumor co-culture. Again, CAR T cells tendentially expressed higher levels of TIM3 when co-cultured with NTRK1-expressing SH-SY5Y, however these differences were not statistically significant (46). None of the CAR T cells upregulated PD-1 on their surface and PD-1 expression remained low, comparable to control T cells (46). LAG3 expression was upregulated independent of NTRK1 or NTRK2 expression only in $CD8^+$ CAR T cells. Control T cells displayed very low expression levels of all activation and inhibitory markers (46).

We further analyzed the distribution of CAR and control T cells expressing either single, double or all of the three analyzed inhibitory receptors after tumor co-culture. We could detect a tendentially higher amount of total CAR T cell subpopulations expressing a single, double or all three inhibitory receptors after co-culture with SH-SY5Y-NTRK1 cells in $CD4^+$ and $CD8^+$ CAR T cells (46). The shift in populations expressing different degrees of inhibitory receptors was however not significant compared with the other neuroblastoma cell lines (46).

2.6. Applications

Our research aims to identify approaches to advance CD171-specific CAR T cell therapy in neuroblastoma. Because neuroblastoma is a solid tumor, new therapeutic concepts are not only faced with overcoming tumor cells directly, but also the machinery that is responsible for its establishment and maintenance made up of the TME. The formation of a solid tumor involves multiple players. That is why we believe that a multi-targeted approach to solid tumor therapy has to be considered, even when CAR T cell therapy has shown promising effects on its own.

Following the reports that TEVs play immunoregulatory roles in the interaction with immune cells, we decided to investigate TEV involvement in the context of CAR T cell therapy against neuroblastoma. We showed for the first time, that certain CAR T cell subsets are not spared from TEVs inhibitory effects. TEVs play a crucial impairing role in the functionality of CD171-specific $CD4^+$ CAR T cells. This is valuable information for the modification and advancement of CAR T cell therapy against neuroblastoma and potentially has implications in the choice of T cell subsets used for CAR T cell generation. $CD8^+$ CAR T cells were not affected by the impairing effects of prior TEV contact.

It has been reported that TEVs carry surface proteins that resemble their parent cells surface protein composition. However, we observed that TEVs from NTRK1- or NTRK2-expressing cell lines did not reflect their parent cells and exerted inhibitory effects on $CD4^+$ CD171-specific CAR T cells to a similar degree. Despite NTRK1 expression leading to increased CAR T cell functionality, it did not positively affect NTRK1-derived TEVs. TEVs impaired $CD4^+$ CAR T cells independent of NTRK expression. Hence, it can be hypothesized that NTRK might not be present in SH-SY5Y-NTRK-derived TEVs. This would imply that inhibition of TEVs would be useful, regardless of prognostically good or bad protein expression on the tumor cell lines.

We further reinforced the role of NTRK1 and NTRK2 expression in neuroblastoma. We observed that NTRK1 expression was continuously accompanied by increased CAR T cell functionality. NTRK2 expression in the neuroblastoma cell line led to significantly worse CAR T cell functionality compared to CAR T cells faced with NTRK1-expressing neuroblastoma, but not significantly worse functionality when compared with CAR T cells faced with the NTRK negative parental neuroblastoma cell line. This has to be further investigated and considered in the development of NTRK2-inhibiting targeted therapies, as advantageous neuroblastoma phenotype is observed with the presence of NTRK1, rather than the lack of NTRK2.

Our $CD4^+$ CD171-CAR T cells displayed impaired cytotoxicity when previously primed with TEVs but showed moderate to good tumor cell killing when they were directly confronted with the tumor cell lines. This might indicate that CAR T cells would benefit from localized application into the tumor site, so that prior systemic TEV priming is limited.

2.7. Limitations

The results of our experiments give valuable implications about further fields of interest concerning CD171-CAR T cell therapy against NTRK-expressing neuroblastoma. However,

all our experiments were conducted *in vitro* and with variations of one neuroblastoma cell line. More extensive *in vitro* research followed by experimental *in vivo* models is needed to understand the interaction of CAR T cells and TEVs more specifically. Furthermore, we used the two subsets of our CAR T cells separately in our experiments. In a physiological setting $CD4^+$ and $CD8^+$ CAR T cells would naturally both be present at the tumor site. Mixing CAR T cells of different subpopulations in various ratios would give valuable information about how the CAR T cells might work synergistically against the tumor. Ultimately, to understand the role of TEVs in CD171-CAR T cell therapy of NTRK expressing neuroblastoma *in vivo* experiments are indispensable.

2.8. Further questions

Following the results of our experiments, more questions need to be investigated and answered in order to sufficiently understand the role of TEVs in CD171-specific CAR T cell therapy against NTRK-expressing neuroblastoma. Mostly questions regarding the mechanisms through which TEVs exert their inhibitory effects on CAR T cells of the $CD4^+$ subpopulation have to be tackled. We could observe that TEVs do not exert their impairing influence by reducing viability, cytokine production or expression of CAR T cell activation markers. It has been reported that T cells do not internalize TEVs and rather exert their effects through the interaction of surface receptors and that TEVs induced their immunoregulatory effects by changing the transcriptome in T cells (55, 56). This has to be further evaluated as a potential mechanism through which TEVs might inhibit $CD4^+$ CAR T cell cytotoxicity.

It has to be evaluated whether the selective use of $CD8^+$ subpopulations of CAR T cells would be an effective way to circumvent impairing immunomodulatory effects of TEVs. Some of our results stand in contrast with previous reports indicating that TEV contact induces $CD8^+$ T cell apoptosis and formation of $CD4^+$ and $CD8^+$ T cell suppressors (54, 55, 57). However, these previous reports are based on TEVs derived from other malignancies than neuroblastoma. More specific research of neuroblastoma-derived EVs has to be conducted in order to make valuable statements concerning their involvement in immunotherapy.

Furthermore, the role of NTRK1 and NTRK2 in the context of TEVs on a genomic and proteomic level has to be investigated more thoroughly as these are receptors playing influential roles in neuroblastoma biology. We observed that low cytotoxicity of NTRK2-

expressing neuroblastoma could not be overcome through the overexpression of our CAR T cells' target antigen CD171 in the NTRK2 cell line. Circumventing the increased neuroblastoma malignancy determined by NTRK2 expression through other ways is crucial to tackle the bad clinical prognosis of patients that present with NTRK2 positive neuroblastoma.

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3. Eidesstattliche Versicherung und Anteilserklärung

„Ich, Solin Ali, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: *Selektiver Einfluss von extrazellulären Neuroblastomvesikeln auf CD4⁺ CD171-spezifische CAR-T-Zell Effizienz gegen NTRK exprimierende Neuroblastomzellen / Selective impact of neuroblastoma-derived extracellular vesicles on CD4⁺ CD171-specific CAR T cell efficacy against NTRK expressing neuroblastoma cell lines*, 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.“

Datum 01.02.2021 Unterschrift

Anteilserklärung an den erfolgten Publikationen

Solin Ali hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1
Autoren:
Solin Ali, Karin Toews, Silke Schwiebert, Anika Klaus, Annika Winkler, Laura Grunewald, Lena Oevermann, Hedwig E. Deubzer, Alicia Tüns, Michael C. Jensen, Anton G. Henssen, Angelika Eggert, Johannes H. Schulte, Esther Schwich, Vera Rebmann, Alexander Schramm and Annette Künkele

Titel:
Tumor-Derived Extracellular Vesicles Impair CD171-Specific CD4⁺ CAR T Cell Efficacy

Zeitschrift:
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Erscheinungsjahr:
2020

Beitrag im Einzelnen:
Konzeption der Experimente, Zellkultur aller beteiligten Zelllinien, die in der Publikation in dem Methodenteil unter „Cell culture“ zusammengefasst werden, Zellkultur und Expansion der CD171-spezifischen CAR-T-Zellen, Gestaltung und Durchführung der Experimente für die Figures 1C, 2A-D, 3A-E, 4A-D sowie der Supplemental Figures 1-4, Datenanalyse und -interpretation, Gestaltung der jeweiligen Graphen, statistische Testung, sowie Auswertung der Ergebnisse für die Figures 1C, 2A-D, 3A-E, 4A-D sowie der Supplemental Figures 1-4, schriftliche Verfassung der Publikation bis auf „Isolation of Extracellular Vesicles“ und „CAR Constructs“ im Methodenteil der Publikation

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Unterschrift des Doktoranden/der Doktorandin

4. Auszug aus der Journal Summary List 2018

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"Immunology"** Selected Category Scheme: WoS
Gesamtanzahl: 158 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS IMMUNOLOGY	41,499	44.019	0.080220
2	NATURE IMMUNOLOGY	44,298	23.530	0.094250
3	IMMUNITY	51,051	21.522	0.126090
4	Annual Review of Immunology	17,013	21.429	0.022210
5	Lancet HIV	2,417	14.753	0.014270
6	JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY	51,978	14.110	0.076250
7	TRENDS IN IMMUNOLOGY	12,153	13.000	0.025470
8	IMMUNOLOGICAL REVIEWS	15,517	11.292	0.027050
9	JOURNAL OF EXPERIMENTAL MEDICINE	63,983	10.892	0.071790
10	Science Immunology	1,292	10.551	0.007580
11	CLINICAL INFECTIOUS DISEASES	64,031	9.055	0.119010
12	Journal for ImmunoTherapy of Cancer	2,716	8.676	0.011350
13	Cancer Immunology Research	5,420	8.619	0.025380
14	Cellular & Molecular Immunology	4,058	8.213	0.009160
15	AUTOIMMUNITY REVIEWS	9,127	7.716	0.018220
16	CURRENT OPINION IN IMMUNOLOGY	9,164	7.667	0.017440
17	Journal of Allergy and Clinical Immunology-In Practice	4,196	7.550	0.013090
18	JOURNAL OF AUTOIMMUNITY	6,900	7.543	0.015050
19	SEMINARS IN IMMUNOLOGY	5,016	7.358	0.010290
20	Mucosal Immunology	6,990	7.352	0.020730
21	CLINICAL REVIEWS IN ALLERGY & IMMUNOLOGY	3,149	7.328	0.006390

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
22	Clinical & Translational Immunology	998	7.271	0.003770
23	EMERGING INFECTIOUS DISEASES	30,311	7.185	0.059420
24	Seminars in Immunopathology	3,337	6.804	0.007890
25	ALLERGY	17,873	6.771	0.024250
26	EXERCISE IMMUNOLOGY REVIEW	754	6.455	0.000890
27	Emerging Microbes & Infections	1,941	6.212	0.006720
28	BRAIN BEHAVIOR AND IMMUNITY	14,533	6.170	0.025700
29	Advances in Immunology	2,544	5.771	0.004220
30	Journal of Neuroinflammation	11,767	5.700	0.023240
31	OncoImmunology	7,790	5.333	0.025470
32	Journal of the International AIDS Society	4,530	5.192	0.018770
33	JOURNAL OF INFECTIOUS DISEASES	45,452	5.045	0.076010
34	Allergy Asthma & Immunology Research	1,599	5.026	0.003240
35	npj Vaccines	282	5.020	0.001120
36	BIODRUGS	1,685	4.903	0.003370
37	CANCER IMMUNOLOGY IMMUNOTHERAPY	7,779	4.900	0.012870
38	Virulence	3,557	4.775	0.009120
39	Vaccines	1,077	4.760	0.003910
40	INFECTIOUS DISEASE CLINICS OF NORTH AMERICA	2,765	4.757	0.005160
41	CLINICAL AND EXPERIMENTAL ALLERGY	11,312	4.741	0.012900
42	JOURNAL OF IMMUNOLOGY	127,940	4.718	0.124400
43	Frontiers in Immunology	27,827	4.716	0.085880
44	EUROPEAN JOURNAL OF IMMUNOLOGY	22,037	4.695	0.024730

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5. Publikation

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ORIGINAL RESEARCH
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Tumor-Derived Extracellular Vesicles Impair CD171-Specific CD4$^+$ CAR T Cell Efficacy

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Chimeric antigen receptor (CAR) T cell efficacy against solid tumors is currently limited by several immune escape mechanisms, which may include tumor-derived extracellular vesicles. Advanced neuroblastoma is an aggressive childhood tumor without curative treatment options for most relapsed patients today. We here evaluated the role of tumor-derived extracellular vesicles on the efficacy of CAR T cells targeting the neuroblastoma-specific antigen, CD171. For this purpose, CAR T cell activation, cytokine production, exhaustion, and tumor cell-directed cytotoxicity upon co-culture was evaluated. Tumor-derived extracellular vesicles isolated from SH-SY5Y neuroblastoma cells neither affected CAR T cell activation nor expression of inhibitory markers. Importantly, exposure of CD4$^+$ CD171-specific CAR T cells to tumor-derived extracellular vesicles significantly impaired tumor cytotoxicity of CAR T cells. This effect was independent of neurotrophic receptor tyrosine kinases 1 or 2 (NTRK1, NTRK2) expression, which is known to impact immune responses against neuroblastoma. Our results demonstrate for the first time the impact of tumor-derived extracellular vesicles and non-cell-mediated tumor-suppressive effects on CD4$^+$ CAR T cell efficacy in a preclinical setting. We conclude that these factors should be considered for any CAR T cell-based therapy to make CAR T cell therapy successful against solid tumors.

Keywords: immunotherapy, pediatric oncology, neuroblastoma, solid tumors, neurotrophic receptor tyrosine kinase

INTRODUCTION

Solid tumor therapy is faced with multiple obstacles that complicate efficient reduction, and ultimately, elimination of cancer cells. Immune surveillance and reinforcement of immune cell activity as a therapeutic option has become increasingly successful. The introduction of checkpoint inhibitors into clinical practice and the advent of cellular immunotherapy, such as CAR T cell-based therapy, holds great promise. Redirection of autologous T cells to a specific tumor-associated antigen via a transfected chimeric antigen receptor (CAR) creates augmented target specificity and improved T cell cytotoxicity. To date, the success of CAR T cell therapy has mostly been limited to the treatment of hematopoietic cancers (1–5). CAR T cell therapy for solid tumors can be hampered by an anti-inflammatory tumor microenvironment, which promotes immune escape mechanisms (6, 7). Here, intercellular communication plays a crucial role in establishing a pro-tumorigenic micromilieu that facilitates cancer spread and immune cell exclusion or dormancy (8). It is increasingly clear that communication between cells can be mediated over distant sites by secretion and uptake of extracellular vesicles, which carry multiple biologically active proteins on their surfaces and generally reflect the molecular composition of their parent cells. Tumor-derived extracellular vesicles (TEVs) are enriched in immunoregulatory proteins including FasL, PD-L1, inhibitory cytokines, classical, and non-classical MHC molecules and the corresponding tumor-associated antigens (9–12). PD-L1 on tumor exosomes is especially relevant in settings where patients are treated with antibodies against this checkpoint inhibitor. Importantly, exosomal PD-L1 levels correlated with response to therapy and suppression of exosomal PD-L1-induced systemic anti-tumor immunity (13, 14). These findings suggest that exosomal composition can massively impact immune cell-directed therapies.

Neuroblastoma, the most-common pediatric extracranial solid tumor, derives from neural precursor cells and displays a high biological as well as clinical heterogeneity (15, 16). Neuroblastoma ranges from disease that can spontaneously regress to high-risk cases for which patient survival is below 40% regardless of current standard multimodal therapy consisting of surgery, polychemotherapy, and radiation (15). Influential factors that can be of prognostic value are e.g., oncogene amplification, ploidy, allelic loss, and tumor stage. Furthermore, mutually exclusive expression of the neurotrophic receptor tyrosine kinases 1 or 2 (NTRK1, NTRK2) has prognostic significance, and both kinases contribute to distinct neuroblastoma biologies, in particular to differences in tumor immunogenicity (17). NTRK1 expression in neuroblastoma has been reported to be correlated with low tumor stage, enhanced DNA repair, a retention of immunogenicity associated with higher degree of leukocyte infiltration, a higher degree of differentiation, higher chemotherapeutic sensitivity, and an inhibitory effect on angiogenesis. All of these features conceivably contribute to excellent patient survival when tumors express NTRK1 (18–22). In contrast, NTRK2 expression in neuroblastoma has been reported to be associated with *MYCN* amplification, enhanced migratory properties leading to early metastases and a resistance to first-line chemotherapeutics, all of which contribute to poor patient survival (20, 22–24).

Previously, we preclinically evaluated CAR T cells targeting the CE7 epitope of the CD171 tumor-associated antigen in neuroblastoma models for their therapeutic efficacy as well as toxicity and safety (25, 26). CD171-directed CAR T cells are currently being tested in a phase I trial for patients with recurrent or refractory neuroblastoma (ClinicalTrials.gov Identifier: NCT02311621). Here we investigate the potential influence of TEVs on the efficacy of CD171-specific CAR T cells from $CD4^+$ and $CD8^+$ T cell subsets in preclinical neuroblastoma models and assess a potential differential involvement of neurotrophin receptors in this process.

MATERIALS AND METHODS

Cell Culture

SH-SY5Y parental cells were maintained in RPMI Medium (Gibco) supplied with 10% fetal calf serum (FCS). Stable expression of NTRK1 or NTRK2 in SH-SY5Y human neuroblastoma cells was achieved as described before (27). SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 were cultivated in RPMI medium, supplied with 10% FCS and 500 µg/ml G418 (Sigma). All cell lines underwent Short Tandem Repeat DNA genotyping for cell line identification as well as weekly testing for mycoplasma using the PlasmoTest™ Kit (Invitrogen). The general number of passages between thawing and use was <20 for all experiments performed.

Isolation of Extracellular Vesicles

To obtain extracellular vesicles released from SH-SY5Y, SH-SY5Y-NTRK1, and SH-SY5Y-NTRK2 cells, cells were cultured for 9 h in RPMI medium supplemented with 10% extracellular vesicle-depleted fetal bovine serum (FCS), 5% penicillin-streptomycin (Pen Strep, 10,000 U/mL, Life Technologies), and 1% L-glutamine (L-Glutamine, 200 mM, Life Technologies). Conditioned media was subjected to ultracentrifugation at 10,000 × g in a fixed angle Type 45 Ti rotor (Beckman Coulter) for 30 min in order to remove membrane patches, followed by a further ultracentrifugation step at 120,000 × g for 120 min at 4°C using a swinging bucket SW 40 Ti rotor (Beckman Coulter). Pelleted TEVs were resuspended in 0.9% NaCl and stored at −20°C until usage. The obtained TEV fractions were characterized by (i) SDS-PAGE and western blotting to verify typical extracellular vesicle marker expression (CD81, TSG101, syntenin) and the absence of intracellular proteins or endosomes (calnexin) according to consensus requirements defining extracellular vesicles (28), (ii) nano-particle tracking analysis using ZetaView analyses (Particle Metrix, Diessen, Germany) to define size and particle concentration (29) and (iii) protein assay (Thermo Scientific, Darmstadt, Germany) to define protein concentration.

CAR Constructs

The CD171-specific CE7-CAR was cloned into the SIN epHIV7 lentiviral vector, and lentivirus was propagated in 293T cells

(30, 31). The scFv was codon-optimized and subsequently linked to a 229-amino acid spacer domain from the human IgG4 hinge. The spacer domain was modified by two substitutions, L235D and N297Q, to reduce binding to the IgG Fc gamma receptor (32). The spacer domain connects the antigen-binding domain to the CD28 transmembrane domain, which is followed by the signaling module containing the CD3zeta cytoplasmic domain and 4-1BB. The CAR construct also contained a T2A self-cleaving peptide and truncated epidermal growth factor receptor (EGFRt) allowing for CAR T cell detection and enrichment.

Generation and Cultivation of CD171-Specific CAR T Cells

Apheresis products were obtained from healthy donors (Charité ethics committee approval EA2/216/18) and peripheral blood mononuclear cells were isolated using Ficoll-Paque (GE Healthcare). $CD4^+$ and $CD8^+$ T cells were obtained by positive selection using immunomagnetic microbeads (Miltenyi Biotec), and activated with anti-CD3/CD28 beads (Life Technologies). On day three, activated T cells were transduced with the CAR-containing lentivirus. $EGFRt^+$ CAR T cells were enriched by immunomagnetic selection with biotin-conjugated cetuximab (Bristol-Myers Squibb) and streptavidin microbeads (Miltenyi Biotec). Untransduced T cells were used as negative controls alongside CAR T cells in all experiments. CAR T cells and control T cells were cryopreserved until further use. Cryopreserved cells were thawed, stimulated with irradiated peripheral blood mononuclear cells, irradiated lymphoblastoid TMLCL cells, and OKT3 (30 ng/mL, Miltenyi Biotec), and expanded according to a rapid expansion protocol (26). $CD4^+$ T cells were supplied with IL2 (50 U/μl) and IL7 (10 ng/μl) and $CD8^+$ T cells were supplied with IL2 (50 U/μl) and IL15 (10 ng/μl) every other day following expansion. Functional *in vitro* assays were conducted between days 11 and 16 of culture.

PCR

DNA was isolated from SH-SY5Y parental cells and the stable expressing SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2 cell models using the Nucleospin Tissue Kit (Macherey-Nagel). PCR-based detection of neurotrophin receptor expression was achieved by PCR-based amplification using primers for NTRK1 (forward: ACCATGCTGCCCATTCGCTG, reverse: GAGGGC AGGCCCCAGTATTC) or NTRK2 (forward: GCAATGATG ATGACTCTGCC, reverse: GGAACACTTTTCCAAAGGCT), and subsequent separation of PCR products in 1% agarose gels by electrophoresis.

Western Blotting

Tumor cells were detached by trypsin, washed twice with PBS and lysed in RIPA buffer including protease inhibitors and the Phosphatase Inhibitor Cocktail (Roche). Proteins were separated by SDS-PAGE before western blotting with pan-Trk (C-14; sc-11, Santa Cruz Biotechnology) and phospho-Trk (#4621, Cell Signaling Technology) antibodies.

Flow Cytometry

Cell-surface expression of CD4 (BD Biosciences), CD8 (BioLegend), and CD171 (cat#130-100-691, Miltenyi Biotec) was detected by fluorophore-conjugated monoclonal antibodies. EGFRt expression was detected using biotinylated cetuximab (Bristol-Myers Squibb) and a phycoerythrin (PE)-conjugated streptavidin antibody (BioLegend). T cell activation and exhaustion were assessed by fluorophore-conjugated monoclonal antibodies detecting CD137 (BioLegend), CD25 (BioLegend), PD-1 (also known as PDCD1 or CD279, BioLegend), TIM3 (Biolegend), and LAG3 (BD Biosciences). Flow cytometry was performed on a Fortessa X-20 (BD Biosciences) and data were processed using FlowJo software (Tree Star Inc.). Dead cells were excluded from analyses using LIVE/DEAD^{TM} Fixable Green Dead Cell Stain Kit (Life Technologies).

TEV Co-culture and Exposure

T cells (2×10^5) were co-cultured with 10 μg TEV protein per well in 96-well flat-bottom plates in triplicate for 24 h in RPMI supplemented with 10% extracellular vesicle-depleted FCS. After 24 h cells were pooled, washed twice with PBS and either used in FACS analyses or for viability assessment using trypan blue. T cells were then mixed with tumor cells at an effector:target ratio of 1:2. T cells primed with TEVs derived from parental SH-SY5Y cells were co-cultured with SH-SY5Y target cells. T cells primed with TEVs derived from SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 cells were co-cultured with SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 target cells, respectively. Viability, activation and exhaustion were assessed in CAR T cells after 24 h using FACS-based detection of cell surface markers.

Functional Assays

For cytokine release assays, 2×10^5 T cells were seeded together with stimulator cells at a 1:2 effector:target ratio. After 24 h, conditioned media was collected and stored at −80°C until analysis of IL2 and IFNG using the OptEIA^{TM} 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 the GFP-ffLuc_epHIV7 reporter plasmid served as tumor target cells. Target cells were co-cultured with negative control or CAR T cells. The maximal biophotonic luciferase signal was obtained by measuring luminescence of target cells in the absence of CAR T cells ($RLU_{max,}$ maximal relative light unit). After 24 or 72 h, 0.14 mg D-luciferin (PerkinElmer Inc.)/ml medium was added to each well, and the biophotonic signal was detected. Lysis was determined as $[1\text{-}(RLU_{sample}/RLU_{max})] \times 100$ in relation to untreated cells. For sequential treatment, the additive amount of tumor lysis was calculated related to viable tumor cells at day 3. If not indicated otherwise, all data points were obtained as technical triplicates.

Statistical Analysis

The differences between treatment groups were statistically analyzed using unpaired Student *t* tests in PRISM (GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ were considered statistically significant.

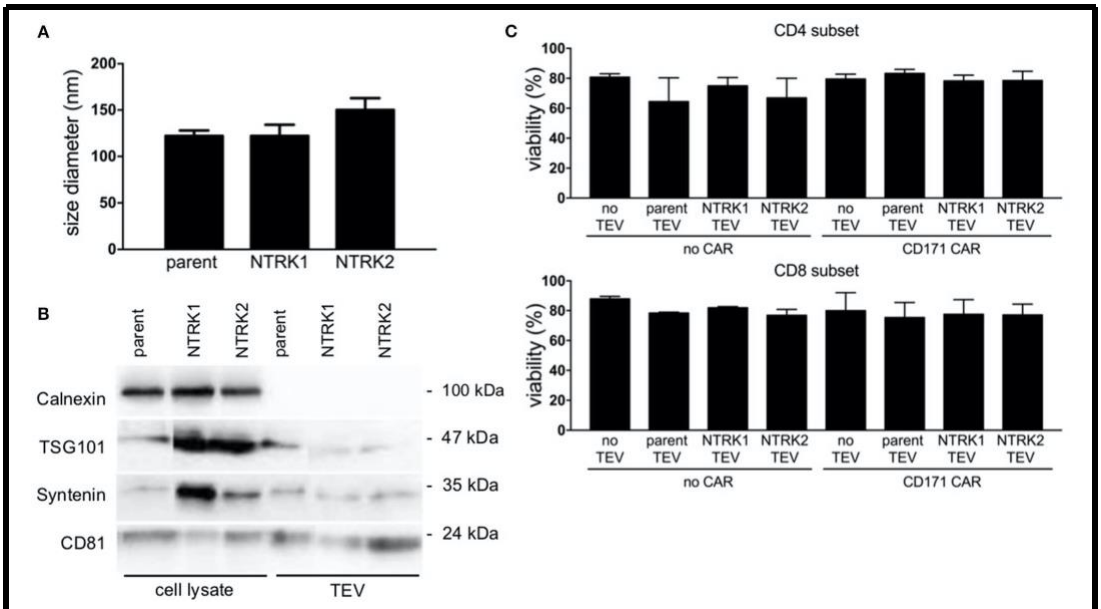

FIGURE 1 | Characterization of extracellular vesicles preparations derived from SH-SY5Y and SH-SY5Y-NTRK cells by Nanotracking analysis and immunoblotting. **(A)** The mean TEV size did not vary among the different TEV preparations ($n = 9$). **(B)** Immunoblotting of TEV preparation and cell lysates for typical TEV markers TSG101, Syntenin, and CD81. Calnexin as protein of endoplasmic reticulum served as negative control. **(C)** $CD4^+$ and $CD8^+$ T cell viability after 24 h co-culture with 10 μg extracellular vesicles, accessed via flow cytometry after live-dead staining. Depicted is mean of three independent experiments with each using technical triplicates with error bars representing standard deviation (SD).

RESULTS

Neuroblastoma TEVs Do Not Impair CAR T Cell Viability

Since TEVs have been reported to modify immune effector functions (33, 34), we investigated the impact of extracellular vesicles derived from the human SH-SY5Y neuroblastoma cell line, which is lacking neurotrophin receptor expression, or neurotrophin-expressing models on CAR T cell efficacy. Models stably expressing each neurotrophin receptor were used to explore the impact of neurotrophin receptor expression on TEVs and CD171-targeting CAR T cell efficacy *in vitro*. Ectopic NTRK1 and NTRK2 expression in SH-SY5Y cell models (referred to hereafter as SH-SY5Y-NTRK1 and SH-SY5Y-NTRK2, respectively) was validated using RT-PCR and western blotting. Constitutive activation was verified for both receptors as previously reported (**Supplementary Figure 1**) (35). TEVs were isolated from medium conditioned by SH-SY5Y, SH-SY5Y-NTRK1, or SH-SY5Y-NTRK2 cells. Nanoparticle tracking-based characterization of the TEV preparation revealed that vesicle diameter ranged between 120 and 150 nm, which is typical for extracellular vesicles (for further details see **Supplementary Table 1**). The CD81 tetraspanin protein and the syntenin and TSG101 cytosolic proteins, which are either ESCRT complex-associated proteins or linked to vesicle release, were detected in all extracellular vesicles preparations, but the intracellular protein, calnexin, was not (**Figures 1A,B**). This marker profile confirmed TEV preparation purity, and excluded contamination with cellular debris. To investigate the impact of extracellular vesicles derived from SH-SY5Y parental or NTRK-expressing cells on CAR T cell viability, we co-incubated CAR T cells with 10 μg TEV, then subsequently assessed viability. Viability of $CD4^+$ or $CD8^+$ CAR T or control T cells was not significantly altered by 24 h of co-culture with extracellular vesicles derived from SH-SY5Y parental or NTRK-expressing cells (**Figure 1C**).

TEV Priming Impairs $CD4^+$ CAR T Cell Cytotoxicity, While $CD8^+$ CAR T Cell Activity Is Unaffected

Next, we explored whether TEVs could influence CAR T cell efficacy. CAR T cells were exposed to TEVs for 24 h before co-incubation with tumor cells and assessment of cytotoxicity (**Figure 2A**). For this purpose, SH-SY5Y, SH-SY5Y-NTRK1, and SH-SY5Y-NTRK2 cells were transduced with a GFP-firefly luciferase reporter plasmid to facilitate viable tumor cell quantification. Cytotoxicity was assessed following 24 h of co-culture measuring residual luminescence signals of viable tumor cells. TEV priming of $CD4^+$ CAR or control T cells

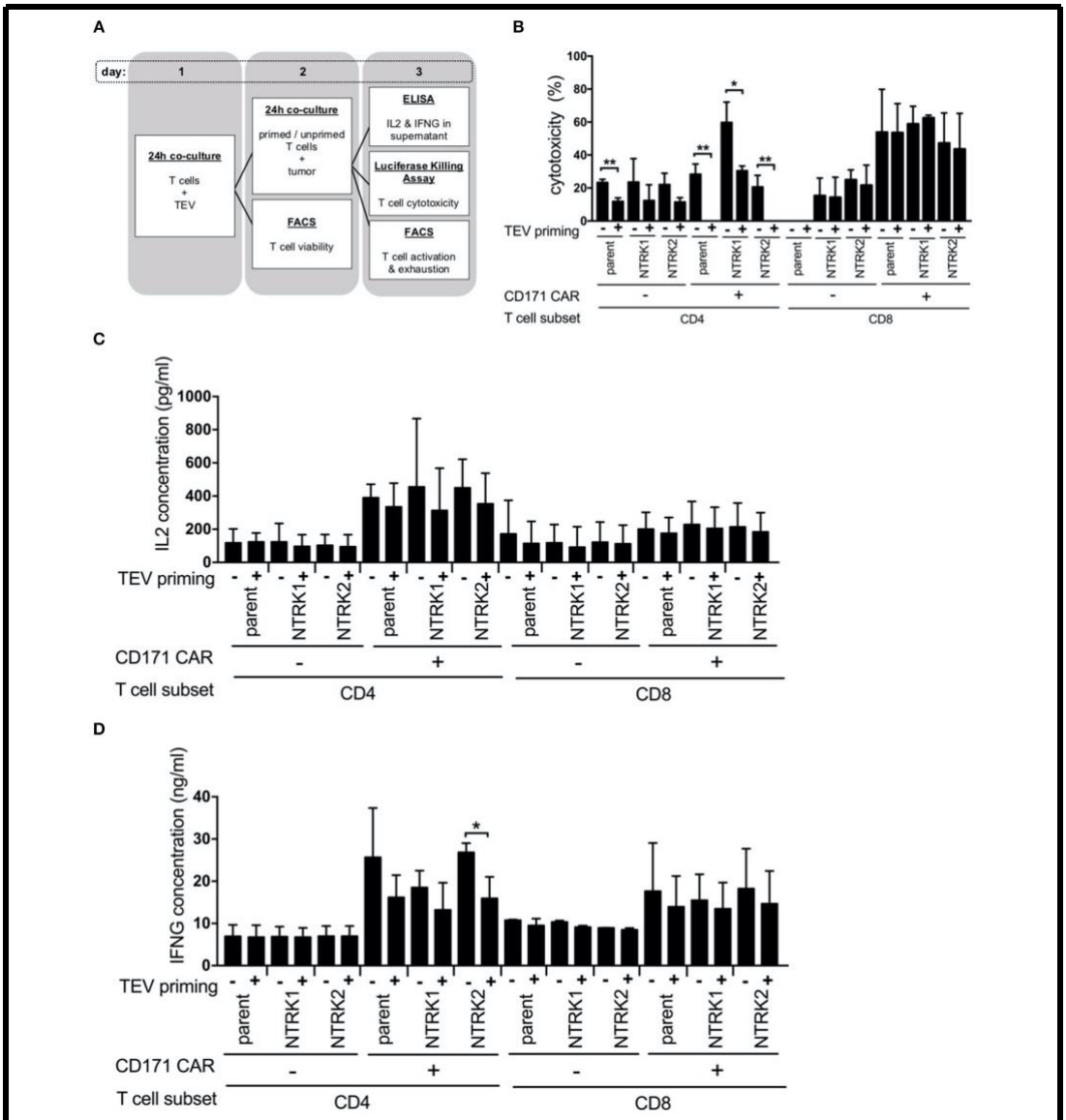

FIGURE 2 | TEV-priming results in impaired CD4$^+$ CAR T cell cytotoxicity. **(A)** Illustration of co-culture experiments with prior TEV-priming of CAR T cells. Co-culture experiments were conducted in three independent experiments with technical triplicates each. **(B)** Cytotoxicity assay of TEV-primed and unprimed CAR T cells measured biophotonically after co-incubation with GFP-ffluc expressing NTRK1 or NTRK2 expressing SH-SY5Y cell lines for 24 h at an effector:target ratio of 1:2. Co-culture experiments were conducted in three independent experiments with technical triplicates each. **(C)** Concentration of IL2 and **(D)** IFNG from supernatant of co-culture of TEV-primed and unprimed CAR T cells with tumor cells at an effector:target ratio of 1:2 quantified by ELISA. Cytokine measurements were conducted in three independent experiments with technical triplicates each. All experiments are depicted with error bars representing SD. *$p < 0.05$; **$p < 0.01$.

significantly impaired the killing activity of these cells, regardless of neurotrophin receptor expression in the SH-SY5Y target cells, whereas the TEV priming of CD8$^+$ CAR or control T cells did not affect neuroblastoma cell cytotoxicity (**Figure 2B**). Control T cells demonstrated a comparably low degree of cytotoxicity, most likely due to alloreactivity. Co-culturing TEV-primed

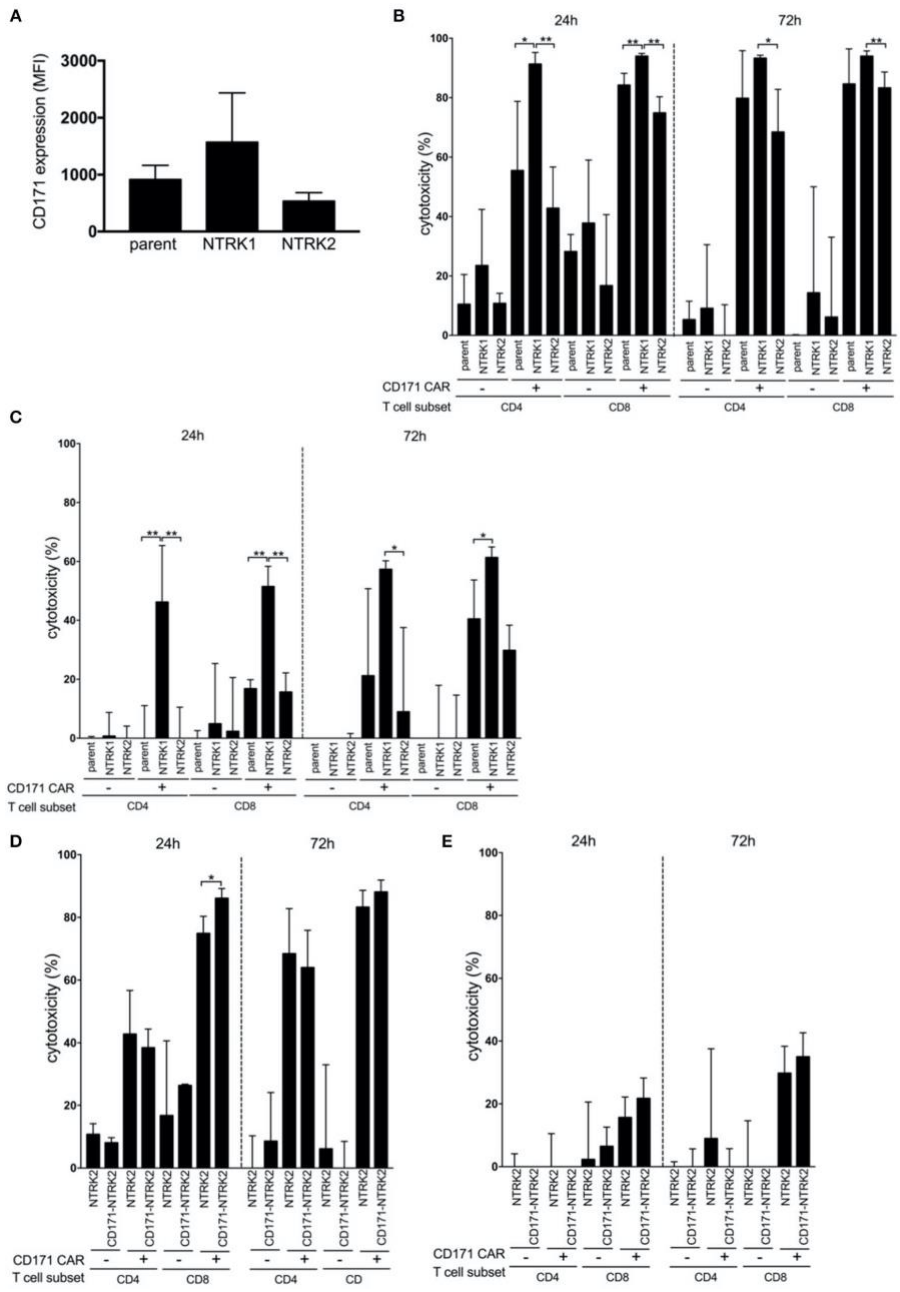

FIGURE 3 | SH-SY5Y-NTRK1 increases CD171 expression in tumor cell lines and leads to improved CAR T cell cytotoxicity that is not significantly dictated through CD171 expression on the target tumor cell line. **(A)** Mean CD171 surface expression intensity (MFI) on SH-SY5Y-NTRK1, SH-SY5Y-NTRK2, and parental control by

(Continued)

FIGURE 3 | flow-cytometric analysis. Assessment of CD171 surface expression was done in three independent experiments. Expressed in mean with error bars representing SD. **(B)** Luciferase-based cytotoxicity assay of CD171-specific CAR T cells from $CD4^+$ or $CD8^+$ subsets against GFP-ffluc expressing SH-SY5Y-NTRK1, -NTRK2 and parental control measured after 24 and 72 h in an effector:target ratio of 2:1 and **(C)** 1:2. **(D)** Luciferase-based cytotoxicity assay of CD171-specific CAR T cells from $CD4^+$ or $CD8^+$ subsets against CD171-high SH-SY5Y-NTRK2 measured after 24 and 72 h in an effector:target ratio of 2:1 and **(E)** 1:2. Cytotoxicity assays were conducted in four independent experiments with technical triplicates each and expressed in mean with error bars representing SD. $^*p < 0.05$; $^{**}p < 0.01$.

CD171-specific $CD4^+$ or $CD8^+$ CAR T cells with SH-SY5Y or SH-SY5Y-NTRK cells consistently reduced the release of IL2 or IFNG by either CAR T cell type compared to unprimed T cells, although comparisons only reached significance for the $CD4^+$ CAR T cells co-incubated with SH-SY5Y-NTRK2 cells (**Figures 2C,D**). FACS analyses revealed no differences in expression of activation markers or inhibitory receptors on primed vs. unprimed CD171-specific $CD4^+$ and $CD8^+$ CAR T cells after tumor cell co-culture (**Supplementary Figures 2A,B**). Our data demonstrate that TEV priming reduces CD171-specific $CD4^+$ CAR T cell efficacy, but does not affect $CD8^+$ CAR T cell-mediated cytotoxicity.

NTRK1 Expression Augments CAR T Cell Cytotoxicity

Since neurotrophin receptor expression on neuroblastoma cells is known to have an effect on immune cells, we investigated whether NTRK1 or NTRK2 expression influences CAR T cell efficacy. We first determined CD171 expression in SH-SY5Y and SH-SY5Y-NTRK cells using flow cytometry. Interestingly, CD171 expression was modulated by NTRK receptor expression (**Figure 3A**), and was highest in NTRK1-expressing SH-SY5Y cells. We next examined whether the level of target-antigen expression on tumor cells altered CAR T cell function. CD171-specific CAR T cells were co-cultured with neuroblastoma reporter cells at an effector:target ratio of 2:1 or 1:2, respectively. Cytotoxicity was assessed following 24 and 72 h of co-culture measuring residual luminescence signals of viable tumor cells. At an effector:target ratio of 2:1, neuroblastoma cell killing was significantly more effective in the presence of $CD4^+$ and $CD8^+$ CD171-specific CAR T compared to control T cells (**Figure 3B**). After 24 h, $CD4^+$ CAR T cell-directed SH-SY5Y-NTRK1 cytotoxicity was significantly higher than cytotoxicity to SH-SY5Y control cells ($p = 0.023$) or SH-SY5Y-NTRK2 cells ($p = 0.0005$). $CD8^+$ CAR T cells mediated cytotoxicity even more strongly compared to $CD4^+$ CAR T cells, and killing efficacy was significantly higher toward SH-SY5Y-NTRK1 compared to parental control cells ($p = 0.005$) or SH-SY5Y-NTRK2 cells ($p = 0.0005$, mean 94% vs. 84.2% vs. 74.9% after 24 h). These differences in killing efficacy were more prominent at effector:target ratios of 1:2. In contrast, control T cells displayed moderate or no cytotoxicity at effector:target ratios of 2:1 and 1:2, respectively (**Figures 3B,C**). In order to assess whether cytotoxicity induced by CD171-specific CAR T cells depended on antigen expression levels, we engineered SH-SY5Y-NTRK2 cells, which express only low CD171 levels, to over-express CD171 (**Supplementary Figure 3**). Cytotoxicity of $CD4^+$ CAR T cells (effector:target ratio of 2:1) was not significantly affected by CD171 expression on SH-SY5Y-NTRK2 cells after 24 h (mean 42.8% vs. 38.4%) or 72 h (mean 68.4% vs. 64%, **Figure 3D**). Control T cells displayed little or no cytotoxicity against target cells, regardless of CD171 expression level. $CD8^+$ CAR T cells displayed higher cytotoxicity against both cell lines when compared to $CD4^+$ CAR T cells, reaching a maximum killing efficiency at an effector:target ratio of 2:1 when CD171 expression was high. At lower effector:target ratios (1:2), tumor cell killing was decreased regardless of CD171 expression levels in SH-SY5Y-NTRK2 cells (**Figure 3E**). Again, we observed comparably low and unspecific alloreactive cytotoxicity of the control T cells. These experiments demonstrate that differential NTRK expression on neuroblastoma cells alters CD171-directed CAR T cell cytotoxicity, and that this effect is independent of endogenous CD171 expression levels on target cells.

NTRK1 Induces Elevated Expression of Activation Markers in CAR T Cells

NTRK-induced modulation of CAR T cell cytotoxicity was further analyzed using *in vitro* assays. Cytokine release (IL2 and IFNG) from CAR T cells following co-culture with SH-SY5Y, SH-SY5Y-NTRK1, and SH-SY5Y-NTRK2 cells was quantified by ELISA. Co-incubation with either the parental SH-SY5Y cell line or cell models expressing a neurotrophin receptor induced IL2 and IFNG release from CD171-specific CAR-T cells, but not control T cells (**Figure 4A**). Activation markers (CD25 and CD137) on $CD4^+$ and $CD8^+$ T cells harboring the CD171-specific CAR were induced when co-cultivated with SH-SY5Y cells, and this activation was independent of NTRK receptor expression (**Figure 4B**). CD25 upregulation on $CD4^+$ and $CD8^+$ CAR T cells was significantly more pronounced in the presence of SH-SY5Y-NTRK1 compared to parental control cells. Co-cultivation with either SH-SY5Y-NTRK1 or SH-SY5Y-NTRK2 (compared to parental control cells) significantly increased CD137 expression on $CD8^+$ CAR T cells. In contrast, co-incubation with different neuroblastoma cells yielded mixed results on the expression of PD-1, TIM3 and LAG3 inhibitory receptors on CAR T cells. While TIM3 expression was significantly induced by exposure to either SH-SY5Y cells or the NTRK-expressing models, PD-1 expression on CD171-directed CAR T cells remained low in all co-culturing conditions analyzed (**Figure 4C**). Induction of LAG3 expression was restricted to $CD8^+$ CD171-directed CAR T cells, independent of NTRK expression on target cells. Potential shifts in the distribution of the inhibitory receptor repertoire on CAR T cell subpopulations after exposure to SH-SY5Y cells and the NTRK-expressing derivates were investigated using pie charts visualizing the degree of inhibitory receptor expression in CAR T (**Figure 4D**) and control T cells (**Supplementary Figure 4**). While relative changes in $CD4^+$ and $CD8^+$ CAR T cell subpopulations expressing single,

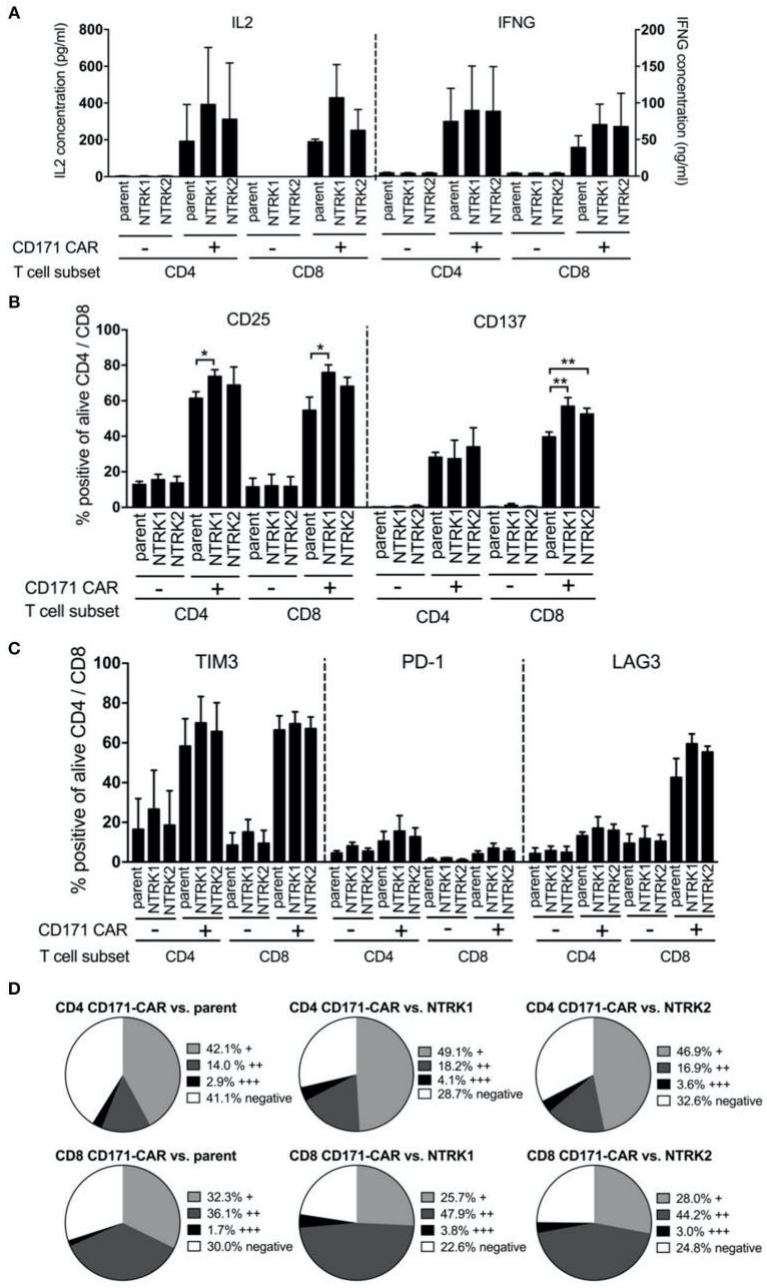

FIGURE 4 | SH-SY5Y-NTRK1 expression results in increased CAR T cell activity, whereas NTRK has no significant influence on cytokine production and expression of exhaustion markers in CAR T cells. **(A)** Concentration of cytokines IL2 and IFNG in supernatant of CAR T cell and tumor in an effector:target ratio of 1:2 after 24 h

(Continued)

FIGURE 4 | co-culture determined by sandwich ELISA. Flow cytometric analysis of T cell surface activation markers CD25 and CD137 on CAR and control T cells after 24 h tumor co-culture. Depicted are double positive CAR and control T cells for $CD4^+$ or $CD8^+$ and CD25 or CD137. Cells were gated from living single cells. **(B)** Flow cytometric analysis of surface activation markers: Proportion of CD25 and CD137 on CAR T cells after tumor co-culture for 24 h in an effector:target ratio of 1:1 are shown. Depicted are double-positive cells for $CD4^+$ or $CD8^+$ and CD25 or CD137. Cells were gated from living single cells. Flow cytometric analysis was conducted in three independent experiments. **(C)** Flow cytometric analysis of surface exhaustion markers: Proportion of TIM3, PD-1, and LAG3 on CAR T cells after tumor co-culture for 24 h in an effector:target ratio of 1:1. Depicted are double-positive cells for $CD4^+$ or $CD8^+$ and TIM3, PD-1, or LAG3. Cells were gated from living single cells. Flow cytometric analysis was conducted in three independent experiments. **(D)** Distribution of no, single (+), double (++), or triple (+ + +) expression of exhaustion markers on $CD4^+$ and $CD8^+$ CAR T cells after 24 h tumor co-culture in an effector:target ratio of 1:1. Single positivity (+) was defined as the sum of $TIM3^+/PD\text{-}1^-/LAG3^-$, $TIM3^-/PD\text{-}1^+/LAG3^-$ populations and $TIM3^-/PD\text{-}1^-/LAG3^+$ populations. Double positivity (++) was defined as the sum of $TIM3^+/PD\text{-}1^-/LAG3^+$ populations, $TIM3^+/PD\text{-}1^+/LAG3^-$ populations and $TIM3^-/PD\text{-}1^+/LAG3^+$ populations. Triple positivity (+ + +) was defined as $TIM3^+/PD\text{-}1^+/LAG3^+$ populations. Negative expression was defined as $TIM3^-/PD\text{-}1^-/LAG3^-$ populations. Bars showed mean values with error bars representing SD. $^*p < 0.05$; $^{**}p < 0.01$.

double or all three inhibitory receptors were highest after co-culture with SH-SY5Y-NTRK1 and lowest after co-culture with the parental cell control, these effects did not reach statistical significance. Our results demonstrate that co-cultivation with SH-SY5Y cells induced cytokine release, up-regulated different activation markers as well as the inhibitory LAG3 and TIM3 markers, but not PD-1, on CD171-specific CAR T cells.

DISCUSSION

Cell-based immunotherapies such as CAR T cells are now entering clinical testing for solid tumor treatment. Although the mechanism of action has been thoroughly investigated in childhood tumors including neuroblastoma (26, 36), little is known about host factors that could potentially modify therapeutic efficacy. TEVs have repeatedly shown to be key players in solid tumor formation and maintenance as well as immunosurveillance (13, 14). Here we analyzed the interplay between extracellular vesicles from preclinical neuroblastoma models with differential expression of the master regulators, NTRK1 and NTRK2, and CAR T cells, in order to identify molecular factors that can be investigated and maybe even manipulated to improve response to CAR T cell therapy. While CAR T cell viability, expression of activation and exhaustion markers as well as their cytokine production was largely unaffected, $CD4^+$ CAR T cell cytotoxicity was significantly reduced in the presence of neuroblastoma-derived extracellular vesicles independent of NTRK1/2 expression. These results are in line with previous reports highlighting $CD4^+$ T cell sensitivity toward exosomes and their suppressive potential (37, 38). However, we did not observe decreased T cell viability in the presence of TEVs that was described for TEVs derived from other tumor types (37, 39). Taken together these results suggest that suppressive effects of TEVs on T cell viability could vary depending on the tumor entity from which they are derived and on different CAR T cell subsets.

Even though NTRK1 and NTRK2 share extensive sequence similarity and similar proximal signaling targets, their expression is correlated with divergent effects on neuroblastoma biology and malignancy (27, 35). NTRK1 and NTRK2 expression are indicative of excellent and poor clinical outcome in neuroblastoma, respectively, yet there is no data on the role of NTRKs in cell-cell communication mediated by extracellular vesicles. We show that expression of the NTRK1 neurotrophin receptor in neuroblastoma cells augmented CAR T cell cytotoxicity and activation compared to cells not expressing NTRK1. In contrast, NTRK2 expression did not affect CAR T cell cytotoxicity. This effect could not be attributed to altered CAR T cell cytokine production, which was similarly induced upon co-incubation with tumor cells, irrespective of NTRK1 expression. These results are in line with previously published reports that NTRK1 expression on neuroblastoma cells enhanced proliferation, activation and IFNG production in healthy donor T cells (21, 27). These and other mechanisms, including MHC class I re-expression in neuroblastoma cells stably transfected to express NTRK1 and upregulation of immune modulating genes by NTRK1, corroborate the idea that excellent prognosis in patients with NTRK1-expressing neuroblastomas could be attributed at least in part through pro-immunogenic features mediated by NTRK1 (21).

It has been reported that TEVs reprogram $CD8^+$ T cells to become suppressor cells inhibiting proliferation of other T cells (40). TEVs also inhibit T cell proliferation and cytolytic activity in effector T cells and other effector immune cells such as natural killer cells, and promote regulatory T cell proliferation and formation of myeloid-derived suppressor cells through their immunosuppressive cargo (9, 40–42). Of note, cytotoxic activity of CD171-specific CAR T cells was independent of NTRK2 expression, which is a marker of aggressive neuroblastoma. It remains to be elucidated whether NTRK2 inhibition by small molecules, which is highly effective against preclinical neuroblastoma models (23, 43), would further improve CAR T cell therapy against NTRK2-expressing neuroblastomas. Single agent-targeting of NTRK2, such as larotrectinib for NTRK2-rearranged tumors in adults and children (44), has now been approved for clinical application. Since TEVs, regardless of their derivation from NTRK1- or NTRK2-expressing cells, display obstructing effects on $CD4^+$ CAR T cells *in vitro*, it should be tested whether $CD8^+$ CAR T cells are a better suited for cell-based neuroblastoma immunotherapy. Vice versa, Tang et al. showed that CAR T cell-derived extracellular vesicles had potent anti-tumor properties and preserved their tumor-targeting specificity (45). Thus, it is likely that the interplay of tumor-derived and CAR T cell-derived extracellular vesicles reprograms the tumor microenvironment and needs to be considered when optimizing CAR T cell therapy for solid tumors.

A limitation of this study is the use of only one cell line. Nevertheless, the neuroblastoma cell line SH-SY5Y ± NTRK1/2

expression is a well-accepted and commonly used model for neuroblastoma allowing in-depth analysis.

Here we demonstrate that CD171-specific CAR T cells are functional and cytotoxic in preclinical models of neuroblastoma with different neurotrophin receptor expression, thus, covering a wide range of neuroblastoma biology. NTRK1-expressing neuroblastoma cells were most susceptible to both $CD4^+$ and $CD8^+$ CAR T cell-mediated killing, while NTRK2-expressing cells were significantly more resistant. TEVs abrogated the effect of $CD4^+$, but not $CD8^+$, CAR T cells independent of whether the tumor cells from which they were derived expressed NTRK receptors. Our results demonstrate that non-cell-mediated tumor-suppressive effects must be taken into account for any CAR T cell-based therapy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Charité ethics committee approval EA2/216/18. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SA performed the experiments. SA, AS, VR, and AKü were major contributors to study concepts, study design, and manuscript writing. MJ provided the CD171-specific CE7-CAR construct. SA, AKü, KT, AKl, SS, AW, LG, LO, HD, and AH contributed to conduction of experiments, analysis of data, quality control, and interpretation of data. AT and AS provided the NRTK1 and NTRK2 expressing cell lines. VR and ES provided the tumor-derived extracellular vesicles. AE and JS were involved in revising the article for important intellectual content. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00531/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Lebenslauf

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

7. Publikationsliste

Publikation 1

Titel: Tumor-Derived Extracellular Vesicles Impair CD171-Specific CD4+ CAR T Cell Efficacy

Autoren: Solin Ali, Karin Toews, Silke Schwiebert, Anika Klaus, Annika Winkler, Laura Grunewald, Lena Oevermann, Hedwig E. Deubzer, Alicia Tüns, Michael C. Jensen, Anton G. Henssen, Angelika Eggert, Johannes H. Schulte, Esther Schwich, Vera Rebmann, Alexander Schramm and Annette Künkele

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