CSPG4-Specific CAR T Cells for High-Risk Childhood B Cell Precursor Leukemia

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Der Medizinischen Fakultät
der Friedrich-Alexander-Universität
Erlangen-Nürnberg
zur
Erlangung des Doktorgrades Dr. med.
vorgelegt von

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Als Dissertation genehmigt von der
Medizinischen Fakultät der Friedrich-Alexander-Universität
Erlangen-Nürnberg

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Tag der mündlichen Prüfung: 23. April 2020

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1. Summary in German

Titel:

CSPG4-spezifische CAR-T-Zellen als Therapieoption bei pädiatrischen Hochrisiko-B-Zellvorläuferleukämien

Hintergrund und Ziele: Mit der Entwicklung von CD19-spezifischen CAR-T-Zellen (*chimeric antigen receptor* T-Zellen) ist das Arsenal der Krebsimmuntherapie für die akute lymphatische Leukämie (ALL) und das diffus-großzellige B-Zell-Lymphom (DLBCL) um eine neue erfolgsversprechende Therapiemodalität erweitert worden. Die Besonderheit der chimären Antigenrezeptoren (CARs) besteht darin, dass sie nach dem Einbau in T-Zellen antigenspezifisch an Oberflächenmoleküle von Krebszellen binden und nachfolgend eine T-Zellaktivierung induzieren können, die zur Zerstörung der gebundenen Krebszellen führt. Synthetisiert werden diese CARs durch die Fusion des intrazellulären Teils des T-Zell-Rezeptors mit der Antigenbindungsdomäne von tumorspezifischen Antikörpern. Das Auftreten einer Reihe von Leukämierezidiven gekennzeichnet durch CD19-negative Krebszellen nach initial erfolgreicher Therapie mit CD19-spezifischen CAR-T-Zellen akzentuiert die Relevanz der Suche nach Ersatzantigenen für CD19. Ein mögliches Ersatzantigen ist das Transmembranprotein Chondroitinsulfat Proteoglykan 4 (CSPG4), das vornehmlich auf Leukämiezellen nachgewiesen wurde, die eine prognostisch ungünstige Alteration des MLL (*mixed-lineage-leukemia*)-Gens im Lokus 11q23 aufweisen. Das Ziel der vorliegenden Studie war es, den Einsatz von CSPG4-spezifischen CAR-T-Zellen gegen leukämische Blasten zu erforschen, die Veränderungen im MLL-Gen aufweisen. Als Modell diente die pädiatrischen Hochrisiko B-Zell Vorläufer Leukämie Zelllinie KOPN8 (MLL-MLLT1 Translokation).

Methoden: Die CSPG4-Expression auf den verwendeten Zelllinien T2.A1 (TxB Zell Hybridom), A375M (Melanomzelllinie) und KOPN8 (B-Zellvorläufer-Leukämiezelllinie mit MLL-MLLT1-Translokation) wurde durchflusszytometrisch mit einem CSPG4-spezifischen Antikörper (Klon: 9.2.27) analysiert. Für die Generierung von CSPG4-spezifischen CAR-T-Zellen wurden T-Zellen zuerst selektiv durch die Stimulation mit einem monoklonalen Antikörper (Klon: OKT3) gegen die CD3 Komponente des T-Zell-Rezeptors und IL-2 aus monozytendepletierten PBMZs (mononukleäre Zellen des peripheren Blutes) expandiert. Anschließend wurden diese T-Zellen mit mRNA, die für einen CSPG4-spezifischen CAR mit CD28-Kostimulation kodierte, elektroporiert. Als Negativkontrollen fungierten ohne RNA elektroporierte T-Zellen und T-Zellen, die mit einem analog aufgebauten CAR gegen das Kontrollantigen CEA (Carcinoembryonales Antigen) transfiziert wurden. Danach wurden die verschiedenen T-Zell-Konditionen mit KOPN8 Zellen ko-inkubiert, und verschiedene T-Zell-Effektorfunktionen analysiert. Die Expression von Aktivierungsmarkern, die Zytokinsekretion und die Degranulationsaktivität wurden durchflusszytometrisch erfasst. Die Zytotoxizität wurde

mit einem Chrom-Lyse-Test ermittelt. T2.A1 Zellen fungierten als Negativkontrollen und A375M Zellen als Positivkontrollen für antigenspezifische T-Zellaktivität.

Ergebnisse und Beobachtungen: Durch Färbung mit dem CSPG4-spezifischen Antikörper 9.2.27 sowie dem CEA-spezifischen Antikörper B1.1/CD66 konnte auf den KOPN8 Zellen eine uniforme CSPG4-Expression und ein geringfügige CEA-Expression im Vergleich zur Isotyp-Kontrolle demonstriert werden. Die T2.A1 Zellen waren sowohl CSPG4 als auch CEA negativ. Die A375M Zellen waren CSPG4 positiv und CEA negativ. Mittels selektiver T-Zellexpansion basierend auf OKT3 und IL-2 Stimulation konnte eine homogene CD3-postive T-Zellpopulation amplifiziert werden, die überwiegend aus CD8-positiven zytotoxischen T-Zellen bestand. Nach der CAR-Transfektion wurde sowohl der CSPG4-CAR als auch der CEA-CAR von mehr als 80% der eingesetzten T-Zellen exprimiert. Nach einer Ko-Kultur mit KOPN8 Zellen zeigten CSPG4-CAR-T-Zellen im Vergleich zu ohne RNA transfizierten T-Zellen und CEA-CAR-T-Zellen eine signifikante antigenspezifische Induktion der Aktivierungsmarker CD25 und CD69. Ferner sezernierten CSPG4-CAR-T-Zellen antigenspezifisch signifikant mehr TNF und IFNγ als ohne RNA transfizierte und CEA-CAR-T-Zellen nach Ko-Inkubation mit KOPN8 Zellen. Schließlich konnte demonstriert werden, dass CSPG4-CAR-T-Zellen spezifische zytotoxische Effekte gegen KOPN8 Zellen bewirken können, die sich in einer signifikanten antigenspezifischen Degranulation sowie in einer antigenspezifischen Lyse der Leukämiezellen manifestierten. CSPG4-negative T2.A1 Zellen wurden nicht lysiert.

Schlussfolgerung: CSPG4 ist ein etabliertes Zielantigen in der CAR-T-Zelltherapie des Melanoms. Die vorliegenden Ergebnisse zeigen, dass CSPG4-CAR-T-Zellen auch für die CAR-T-Zelltherapie pädiatrischer Hochrisiko-B-Zellvorläuferleukämien mit Alterationen im MLL-Gen Verwendung finden könnten.

2. Introduction

Adoptive T-cell therapy: from TILs to CARs

Fueled by impressive clinical results and driven by recent advances in synthetic biology, adoptive T-cell therapy (ATT) has transitioned from a merely experimental procedure to a promising therapeutic option in advanced malignancies. Capitalizing on the capacity of T cells to antigen-specifically recognize and eliminate target cells, three major forms of ATT have emerged: TIL (tumor-infiltrating-lymphocytes) – therapy, TCR (T-cell receptor) – T-cell therapy and CAR (chimeric antigen receptor) – T-cell therapy [1]. The first successful application of adoptively transferred tumor-specific T cells was marked by T-cell therapy with tumor-infiltrating lymphocytes, which requires the *a priori* infiltration of tumor-tissue with T cells cognizant of tumor antigens [1]. In order to generate TILs, autologous T cells are extracted from tumor biopsies and expanded *ex vivo* to sufficient numbers before re-infusion [1]. The clinical efficacy of this approach was demonstrated in spectacular fashion by investigators from the National Cancer Institute (NCI) reporting complete remission of melanoma metastases after adoptive transfer of expanded TILs [2]. Nevertheless, two major obstacles currently impede the success of TIL-therapy: First, a substantial portion of cancer patients does not show T-cell presence inside the tumor, compromising TIL extraction [1]. Second, T cells in the tumor microenvironment frequently display an exhaustive phenotype characterized by the up-regulation of inhibitory receptors and impaired effector functions [3]. Hence, only a fraction of expanded TILs can mediate anti-tumor activity after transfer [1]. Finally, the generation of TILs is a very strenuous and time-consuming process covering several weeks, which compounds the battle against highly progressive and refractory tumors [1]. To detach from the dependence on pre-existing tumor-reactive T cells in the tumor microenvironment, Steven Rosenberg`s group at the NCI retrovirally transduced peripheral blood mononuclear cells (PBMCs) with a MART-1/HLA-2-specific TCR isolated from TILs of a melanoma patient [4]. Of note, the MART-1 antigen is expressed in melanoma cells but also in normal melanocytes. Upon e*x vivo* expansion and transfer of these engineered T cells into 17 patients suffering from metastatic melanoma, two patients evinced an objective anti-tumor response [4]. In a follow-up trial using T cells engineered with a higher affinity TCR reactive to MART-1/HLA-2, objective anti-tumor response rates were increased to 30% (6/20 melanoma patients) [5]. Strikingly, in this trial severe, but reversible, side-effects ranging from skin rash and vitiligo to hearing problems and vision impairments occurred, which could be traced back to on-target/off-tumor toxicity emanating from engineered T cells leading to the destruction of pigmented cells in the skin, eyes, and ears [5]. The results of those pilot trials underscore the feasibility of reprogramming T cells to tumor antigens by introducing tumor-specific antigen receptors and provide proof-of-principle data for the clinical efficacy of TCR-T-cell therapy. Apart from melanoma, TCR-modified T cells have been successfully employed in synovial cell sarcoma [6], multiple

myeloma [7], and esophageal cancer [8]. The Achilles´ heel of TCR-T-cell therapy, however, is posed by human leukocyte antigen (HLA)-restriction, which becomes apparent in the inability to target cancer cells with down-regulated or abrogated expression of MHC class I molecules to evade TCR-mediated immunity [1]. The quest for a T-cell-based targeting platform independent of HLA-restriction, has led to the development of chimeric antigen receptor (CAR)-T cells. The basic concept underlying the CAR-T cell approach is based on redirecting T cells to antigens expressed on the surface of malignant cells irrespective of HLA-expression [9]. Chimeric antigen receptors are synthetic molecules assembled in modular fashion by merging the single-chain variable fragment (scFv) derived from a monoclonal antibody to the intracellular signaling domain of a TCR [9]. A scFv is created by linking the antigen binding part of the heavy chain of an antibody in cis with the antigen binding part of the corresponding light chain [10]. The result is a compact linear molecule with the same specificity as the original antibody [10]. Interposed between the scFv and the intracellular part are an extracellular spacer (hinge) and a short transmembrane protein [11]. The hinge, usually IgG4 or CD8α, confers spatial flexibility on the extracellular part, which improves antigen-binding [11]. Upon binding of the scFv to the cognate target antigen, the intracellular part of the CAR subsequently mediates and modulates T-cell activation [9]. The first CAR-constructs presented by Zelig Eshhar`s group, approximately three decades ago, featured an extracellular antigen-binding domain and an intracellular CD3ζ chain [12]. Chimeric antigen receptors signaling exclusively through the CD3ζ chain were later termed first-generation CARs [1]. Initial trials evaluating the efficacy of T cells transduced with first-generation CARs in ovarian cancer and renal cancer failed to demonstrate any significant anti-tumor responses [13,14]. These negative outcomes were primarily rationalized by deficient proliferation and long-term persistence of transferred T cells [13,14]. Thus, efforts to improve the CAR design for long-term engraftment of CAR-T cells focused on the addition of a co-stimulatory domain linked in cis with the CD3ζ chain. CAR-T cells incorporating one or two additional co-stimulatory domains were denoted second- or third-generation CARs, respectively [1]. Currently, the best characterized co-stimulatory domains include CD28, which is a member of the immunoglobulin superfamily, and the tumor necrosis factor (TNF) – family member 4-1BB [9]. Both co-stimulatory domains improved the proliferative capacity of CAR-T cells, augmented the persistence and dramatically improved the performance in clinical trials as testified to by a multitude of complete tumor regressions achieved via a single injection of second-generation CAR-T cells [1,15]. Conspicuously, CD28-co-stimulation outperforms 4-1BB-co-stimulation with respect to magnitude of T-cell effector functions and initial proliferative activity [16]. In the long run, however, 4-1BB-co-stimulated CAR-T cells display prolonged persistence and a less exhausted phenotype [16]. This difference is partially explained by distinct metabolic signatures intrinsic to the different CAR variants [16]. Whereas CD28 expedites glycolytic pathways necessary for swift execution of

potent effector functions, 4-1BB imparts a metabolic profile relying on fatty acid consumption, which is essential for long-term T-cell persistence [16]. Of note, third-generation CARs encompassing CD28 and 4-1BB did evince pronounced cytolytic capacities and cytokine secretion, but they did not match the proliferative potential of second-generation CARs [17]. The latest addition to the CAR family are fourth-generation CARs or T cells redirected for universal cytokine signaling (TRUCKs), which deliver an additional payload, for instance IL-12, after CAR activation [18].

CAR-T cells against acute lymphoblastic leukemia: clinical experience

Acute lymphoblastic leukemia (ALL), which is more prevalent among pediatric patients than adults, requires intensive treatment regimens including extensive chemotherapy applications and, depending on the genetic and clinical risk stratifications, an allogeneic stem cell transplantation [19]. Over the past decades, durable complete remissions could be attained with these protocols in the majority of patients [19]. Nevertheless, a small portion of ALL patients is primarily refractory to induction chemotherapy and a sizable percentage of patients relapse after successful first-line treatment [19]. Both scenarios refractory- and relapsing disease are associated with a poor prognosis rendering the quest for novel therapeutic strategies for refractory and relapsing ALL (r/r ALL) an urgent medical need [19]. Encouraged by positive data obtained from murine ALL models, CAR-T cells specific for the antigen CD19 were prepared for clinical application [20,21,15]. Importantly, while uniformly expressed on lymphatic blasts, CD19 is also present on the surface of normal B cells, but not on other non-malignant tissues [15]. Hence, the only tangible on-target/off-tumor toxicity is B-cell aplasia and ensuing hypogammaglobulinemia, which can be managed with immunoglobulin substitution therapy [15]. The first patient with ALL treated with CD19-CAR-T cells, received a single dose of engineered T cells after induction of complete remission as a bridging therapy to consolidating allogeneic stem cell transplantation [22]. After CAR-T-cell transfer, no side-effects, except for the expected decrease of normal B cells was observed [22]. The first data on clinical efficacy of CD19-CAR-T cells in ALL were published in 2013 by investigators from the Memorial Sloan Kettering Cancer Center (MSKCC) [23]. Five patients with relapsed ALL underwent conditioning chemotherapy with cyclophosphamide before receiving a single dose of 1.5-3 × 10^6 /kg autologous CD28-co-stimulated CD19-CAR-T cells [23]. Upon infusion, CD19-CAR-T cells showed robust proliferation and demonstrated brisk tumor eradication reflected in MRD-negative remissions achieved in all five patients [23]. Besides demonstrating the potency of CD19-CAR-T cells in ALL, this pioneer study also emphasizes the benefit of using conditioning chemotherapy prior to CAR-T-cell infusion. Mechanistically, conditioning chemotherapy causes a lymphodepletion creating a niche for the engineered T cells, which is a vital prerequisite for persistence [22]. Moreover, evidence gathered in recent years suggests that conditioning chemotherapy promotes proliferation of CAR-T cells by elevating pro-

inflammatory cytokines, e.g. IL-12 [24]. In subsequent trials analyzing CD19-CAR-T cell activity in larger cohorts of pediatric and adult patients with ALL impressive complete remission rates beyond 90% could be observed [15]. All these trials relied on autologous CAR-T cells and employed various conditioning chemotherapy protocols [15]. Motivated by the excellent performance of CD19-CAR-T cells in early clinical studies, the multi-center phase 2 trial ELIANA was launched to assess the clinical application of tisagenlecleucel, autologous CD19-CAR-T cells co-stimulated by 4-1BB, in young patients with r/r ALL [25]. Of the 75 patients, who were all enrolled after failing several lines of therapy, 81% achieved MRD-negative disease control within 3 months after a single application of tisagenlecleucel [25]. Follow-up analyses conducted 12 months after CAR-T-cell infusion revealed an event free survival of 50% and an overall survival rate of 76% [25]. Against the backdrop of those impressive results tisagenlecleucel (Kymriah®, Novartis) was approved for the treatment of CD19-positive r/r ALL by the U.S. Food and Drug Administration (FDA) in 2017 and by the European Medicines Agency (EMA) in 2018 [26]. Having reached this historic milestone, the current focus has now shifted from achieving high response rates to improving progression free survival and overall survival by perpetuating the duration of complete remissions. A major barrier to durable responses with CAR-T cell therapy in general is created by relapse of antigen-negative tumor cells evading destruction by CAR-T cells by virtue of antigen-loss or antigen-downregulation [27]. As for CD19, mutational loss, as well as shutdown due to posttranscriptional modifications has been observed [27]. Moreover, recent findings implicate the process of trogocytosis, which refers to the transfer of membrane-bound antigens from one cell to another, in the generation of antigen-negative target cells [28]. In several *in vivo* and *in vitro* co-culture experiments of CD19-CAR-T cells and the CD19-positive ALL cell line NALM6, Hamieh *et al.* could demonstrate that CD19 is transferred from NALM6 cells to CD19-CAR-T cells [28]. As a result, leukemia cells eluded elimination and CD19-CAR-T cells became susceptible to premature exhaustion and fratricide killing [28]. To re-impose tumor control and counteract target antigen trogocytosis various combinations of CARs specific for CD19 or CD22 with either CD28 or 4-1BB co-stimulation were assayed [28]. In this preclinical study CD19-CAR-T cells with CD28 co-stimulation combined with CD22-CAR-T cells with 4-1BB co-stimulation emerged as the most promising approach to combat antigen loss by trogocytic exchange [28]. Clinical evidence supporting the efficacy of CD22-CAR-T cells as a salvage therapy for scenarios with relapse of CD19-negative ALL was provided by a phase 1 clinical trial investigating CD22-CAR-T cells in 22 patients with ALL [29]. Of note, 17 of those patients were enrolled after disease progression following CD19-directed treatment [29]. Ensuing CD22-CAR-T cells infusion, 83% of the patients treated at the higher dose-levels (≥ 1×10^6 CD22-CAR/kg) entered complete remission within 6 months [29]. Importantly, complete remission was also achieved in patients with dim or absent CD19 expression qualifying CD22-CAR-T cells as back-up therapy for ALL

refractory to CD19-CAR-T cells [29]. Nevertheless, most of the patients with complete remission relapsed within 12 months following CD22-CAR-T cell application, which could be largely rationalized by a diminished CD22 expression on the recurrent leukemia cells undermining recognition by CD22-CAR-T cells [29]. In aggregate, this study illustrates the dynamics of antigen-shutdown in ALL and highlights the necessity to build up a thorough arsenal of back-up antigens for CD19 to prepare for the outgrowth of antigen-negative cancer leukemia cells and to ensure lasting tumor control by CAR-T cells.

CAR-T cells against MLL-rearranged leukemias: a special challenge

Chromosomal alterations at the 11q23 locus define a certain subgroup of high-risk leukemias termed mixed lineage leukemia-rearranged leukemia (r-MLL) [30]. MLL-rearrangements can be detected in ALL, AML and in mixed phenotype acute leukemia (MPAL) [30]. The most frequent genetic alterations are the translocations t(4;11), t(9;11) and t(11;19) creating the fusion proteins MLL-AF4, MLL-AF9 and MLL-ENL, respectively [30]. Whereas t(4;11) is more common in B-cell ALL and t(9;11) is more frequent in AML, t(11;19) alterations are found both in ALL and AML [31,32]. Epidemiologically, MLL-rearranged leukemias account for 10% of all newly diagnosed leukemias, with a clear preponderance in pediatric patients making up >70% of all infant ALL cases, especially those manifesting in the first year after birth [30]. Furthermore, the occurrence of r-MLL in adult patients, mostly with an AML phenotype, is strongly associated with previous exposure to topoisomerase II inhibitors, such as VP16 and to a lesser degree doxorubicin [30]. In general, the presence of MLL alterations bodes prognostically dismal for the overall survival of leukemia patients [30]. This derives in part from the intrinsic resistance to important chemotherapeutic agents, such as prednisone and L-asparaginase [33]. Besides, clinical trials analyzing the use of allogeneic stem cell transplantation, which forms a major pillar of the therapy of acute leukemias, demonstrated sobering results in MLL-rearranged leukemia [34]. Finally, r-MLL evinces a more aggressive phenotype with hyperleukocytosis and early infiltration of the central nervous system [35]. In aggregate, the quest for new treatment strategies of r-MLL represents an urgent medical need. In order to enlarge the therapeutic arsenal for r-MLL, several novel drugs were subjected to clinical evaluation in recent years. Given a concomitant up-regulation of Fms-like receptor tyrosine kinase-3 (FLT-3) in r-MLL, FLT-3 inhibitors, such as quizartinib and lensartinib were examined yielding promising results *in vitro*, but equivocal results in small clinical trials [30]. Additionally, the proteasome inhibitor bortezomib revealed inhibitory effects on r-MLL by interfering with histone ubiquitination [30]. Moreover, hypomethylating agents, for instance decitabine and 5-azacytidine, and inhibitors of histone deacetylase (HDCA) are under investigation in r-MLL [30]. Given the paucity of efficacious curative options coupled with the mesmerizing activity of CD19-CAR-T cells against MLL-wild type ALL, the use of CAR-T cells against MLL-rearranged leukemia has gained increasing attraction. In 2016 investigators from

the Fred Hutchinson Cancer Research Center shared clinical courses of seven patients with MLL-rearranged B-ALL entering complete remission after a single application of autologous CD19-CAR-T cells administered after lymphodepleting chemotherapy [36]. Unfortunately, two of those patients, a 52-year-old woman and an 18-month-old girl, showed recurrent disease one month after CAR-T cell infusion [36]. Flow cytometrical analyses revealed the loss of CD19 on relapsing blasts in both patients as the reason for resistance to CD19-CAR-T cells [36]. Remarkably, the shutdown of CD19 occurred in the wake of a complete lineage switch from the lymphoid B-ALL phenotype to a myeloid AML phenotype [36]. Thus, the expression of conventional backup antigens for CD19-loss, such as the B-cell membrane protein CD22, was abrogated as well [36]. This is the first study to report on a lymphoid to myeloid lineage switch as a powerful mechanism of eliminating CD19 expression to confer resistance to CD19-CAR-T cells [36]. MLL-rearranged leukemias are probably particularly prone to undergo such lineage switches due to the pronounced intrinsic epigenetic deregulation originating in part from an altered activity of the methyltransferase MLL1 encoded by 11q23 leading to a wave of global demethylation and activation of gene expression [30]. Hence, the quest for target antigens, which are not affected by lineage conversion, has garnered special attention to improve CAR-T cell approaches for r-MLL.

Chondroitin sulfate proteoglycan 4 (CSPG4): a complex tumor antigen

Chondroitin sulfate proteoglycan 4 (CSPG4) is a single pass type 1 transmembrane protein, encoded by a gene located on chromosome 15 [37]. Former names include melanoma-associated chondroitin sulfate proteoglycan (MCSP) or high molecular weight melanoma-associated antigen (HMW-MAA) [37]. Due to neuronal-glial interactions in the rat brain the rodent homolog of CSPG4 was dubbed Neuron-Glia Protein 2 (NG2) [38]. Before expression on the cell surface, the protein backbone of CSPG4 is transformed into a glycoprotein by the addition of multiple sugar residues in the golgi apparatus [39,40]. Further modifications encompass the assembly of a 450 kDa proteoglycan by affixing glycosaminoglycans to the glycoprotein main structure [41]. The extracellular part of CSPG4, which represents the largest part of this proteoglycan, is split into three distinct morphological and functional domains [39]. The juxtamembrane domain D3 represents a binding site for lectins and integrins, for instance Galectin-3 and α1β3 [42]. The adjacent D2 domain forms the largest part of the extracellular domain and harbours a cluster of 15 CSPG-specific repeats, which act as ligands for integrins and collagen, as well as receptors for growth factors secreted into the extracellular matrix [39]. The distal D1 domain establishes contact with the extracellular matrix via two laminin G-type domains [39]. CSPG4 does not exhibit any overt immanent enzymatic activity, but the intracellular part is involved in several signaling networks by providing phosphorylation sites and docking places for adaptor molecules [39]. The key signaling pathways downstream of CSPG4 are the mitogen-activated-protein-kinase (MAPK) pathway providing growth and

survival signals [43], and the focal adhesion kinase (FAK) pathway, which functions as an interface to the extracellular matrix enabling refined responses to extracellular changes [43]. Additionally, CSPG4 is supposed to concentrate nearby growth factors by virtue of trapping those via its exterior D2 domain [39]. Subsequently, those growth factors, such as platelet-derived growth factor (PDGF) are transferred to corresponding receptors with kinase activity resulting in the initiation of growth promoting signaling [39]. Furthermore, CSPG4 is wired to the actin cytoskeleton via a plethora of adaptor molecules and integrins, which implicates CSPG4 in cell motility and tissue invasion [39]. Physiologically, CSPG4 plays a role in angiogenesis [42], neurogenesis [44], and in the homeostasis of keratinocytes and epidermal stem cells [45].

CSPG4-CAR-T cells against MLL-rearranged leukemia: benefits and barriers

Early studies relying on the CSPG4-specific monoclonal antibody 7.1 could detect CSPG4 expression in 18 of 166 AML specimen obtained from pediatric leukemia patients [46]. The presence of CSPG4 exhibited a close association with alterations at the 11q23 locus [46]. Moreover, CSPG4-expression was inversely correlated with progression-free survival and overall survival. Staining of ALL specimen with the 7.1 monoclonal antibody revealed CSPG4 upregulation in 9 out of 104 pediatric ALL patients [47]. Of note, all of those cases did bear a MLL-rearrangement (5 with MLL-AF4 and 4 with MLL-ENL) [47]. Similar to AML, the presence of CSPG4 was associated with a worse prognostic fate and a reduction in progression-free survival [47]. The results from those pilot investigations were subsequently corroborated by several other studies suggesting that over half of all newly diagnosed MLL-rearranged leukemias stain positive for CSPG4 [37]. Conversely, surface expression of CSPG4 can be used to screen for MLL-rearrangements in leukemic blasts, as approximately 90% of all CSPG4-positive leukemia cells possess an 11q23 alteration [37]. Thus, CSPG4 has garnered attention as a potential target for the CAR-T-cell therapy of r-MLL. The exact mechanisms underlying CSPG4 expression in r-MLL have not been properly elucidated so far. It is conceivable that CSPG4 upregulation is caused by the dysregulation of epigenetic control mechanisms leading to promotor demethylation and uncontrolled transcription of a variety of proteins. Contrary to CD19 and CD22, CSPG4 is not restricted to a certain hematopoietic lineage, which constitutes a major benefit given the intrinsic potential of r-MLL to simultaneously shutdown several antigens via lineage conversion [46]. Targeting leukemia cells with CAR-T cells harbours the potential to incur collateral damage on non-malignant blood cells ranging from selective B-cell depletion to eradication of hematopoietic stem cells with ensuing myeloablation [48,49]. The absence of B cells, which is a common side-effect following infusion of CD19-CAR-T cells, can be conveniently managed by immunoglobulin substitution therapy. In contrast, therapy-related myeloablation originating from targeting antigens that are co-expressed on hematopoietic stem cells (HSCs) creates an immediate emergency

necessitating rescue allogeneic bone marrow transplantation. In a preclinical study conducted at the university of Pennsylvania, CAR-T cells specific for CD123 were evaluated as salvage therapy for ALL after relapse to treatment with CD19-CAR-T cells [50]. High expression of CD123 could be detected on CD19-negative leukemia cells obtained from ALL patients [50]. Furthermore, CD123-CAR-T cells, unlike CD19-CAR-T cells could eliminate CD19-negative ALL blasts in a xenograft mouse model [50]. Unfortunately, CD123 is also abundantly expressed on HSCs, and myeloablation following CD123-CAR-T cell application has been observed in several studies [49,50]. Due to the absence of CSPG4 expression on HSCs and other blood cells [46], CSPG4-CAR-T cells are not associated with any serious hematotoxicity, such as myeloablation. An additional merit of CSPG4-CAR-T cells derives from the fact that CSPG4-expression in ALL coincides with augmented chemoresistance [51]. In a recent study it was established that the combination of standard chemotherapy agents, such as prednisone, vincristine and L-asparaginase with CSPG4-blockade enhanced the cytotoxicity towards ALL blasts [51]. At the mechanistic level, CSPG4-blockade resulted in pronounced translocation of leukemia cells from the bone marrow, the stroma of which creates a chemoprotective environment, into the blood stream leading to an increased exposure to chemotherapeutic agents and enhanced cytotoxicity [51]. Consequently, CSPG4-downregulation to evade CSPG4-CAR-T cell mediated elimination might re-sensitize r-MLL blasts to standard chemotherapeutic agents. Collectively, wide-spread expression of CSPG4 on r-MLL blasts coupled with no significant expression in HSCs, and augmented chemosensitivity in case of antigen-loss are vital benefits supporting further exploration of CSPG4-CAR-T cells for the therapy of r-MLL. Nevertheless, those beneficial traits are opposed by concerns on potential non-hematological on-target/off-tumor toxicities. At the RNA level CSPG4 could be detected in a multitude of normal tissues including central nervous system, eye, skin, adipose tissue, blood vessels, bladder, gastrointestinal tract, uterus, prostate, spleen, and thymus [52]. The ratio of CSPG4-expression in malignant tissue (melanoma) compared to healthy tissues averaged 6.6 to 1 [52]. Importantly, using immunohistochemical staining with the CSPG4-specific antibody TP41.2, tangible CSPG4 expression at the protein level could only be verified in some samples from the small bowel [53]. No CSPG4-protein expression could be detected in the brain, peripheral nerves, skin, mesothelium, breast, heart, kidney, adrenal glands, liver, lung, lymph node, muscle, ovary, pancreas, esophagus, prostate, spleen, stomach, thyroid, uterus [53]. These analyses mitigate the concerns on on-target/off-tumor toxicities emanating from CSPG4-CAR-T cells as CSPG4 presence at the protein level is required to induce CAR-T-cell activity. Functional corroboration for the non-reactivity of CSPG4-CAR-T cells to normal tissues with detectable CSPG4 RNA, but without CSPG4 protein expression, was provided by the group of Gianpietro Dotti [54]. They could show that CSPG4-CAR-T cells did not exert any significant cytotoxicity towards primary epithelial cell lines from prostate, lung, and kidney [54].

In aggregate, the expression profile of CSPG4 does not reveal any serious risks for on-target/off-tumor toxicities, which might preclude application of CSPG4-CAR-T cells in the first place but warrants a strict surveillance of patients during CSPG4-CAR-T cell therapy to detect toxicities at early stages. Another barrier to a successful CSPG4-CAR-T cell therapy of r-MLL is posed by CSPG4-negative r-MLL blasts, which account for approximately 40% of newly diagnosed r-MLL [37]. Moreover, CSPG4-negative escape variants are likely to evolve during CSPG4-CAR-T cell therapy by shutting down CSPG4 expression. As CSPG4 is not a crucial oncogenic driver in r-MLL, the oncogenic progression of which is governed mostly by the products of chromosomal alterations [30], it can be down-regulated without any immediate repercussions on the growth and survival of r-MLL blasts rendering CSPG4 a target at elevated risk for shutdown. Consequently, it would be beneficial to integrate CSPG4-CAR-T cells into a multimodal therapy for r-MLL combining different CAR-T-cell products, such as CD19- and CD22-CAR-T cells, with chemotherapeutic agents and new small molecule inhibitors tailored to r-MLL.

Practical experience with CSPG4-CAR-T cells: melanoma and glioblastoma

Given a uniform CSPG4-positivity of primary tumor cells and metastases, malignant melanoma emerged as the tumor model of choice for initial functional analyses of CSPG4-CAR-T cell activity [55]. The first proof-of-principle data showing that CSPG4-CAR-T cells are capable of killing CSPG4-positive tumor cells were established using melanoma cells as targets and T cells retrovirally transduced with a CSPG4-specific second-generation CAR as effectors [54]. *In vitro*, these CSPG4-CAR-T cells were capable of lysing melanoma cells in antigen-specific fashion [54]. Moreover, triggering of the CAR led to a pronounced secretion of IL-2 and IFNγ. *In vivo*, administering CSPG4-CAR-T cells to mice bearing melanoma cells significantly stunted tumor growth and improved the overall survival of the test subjects [54]. Contrary to leukemia, CSPG4 acts as an oncogenic driver in melanoma promoting growth and survival of malignant cells by signaling through various pathways [55]. Seeking to capitalize on the entanglement of CSPG4 with the MAPK signaling network, our group conducted a series of experiments to evaluate the feasibility of combining CSPG4-CAR-T cells with inhibitors of BRAF and MEK (BRAFi/MEKi), which represent central components of the MAPK pathway [56]. While all assayed BRAFi/MEKi impaired T-cell activation and diminished antigen-specific cytokine secretion in response to melanoma cells, the cytotoxicity of CSPG4-CAR-T cells was only affected by a single kinase inhibitor combination [56]. Hypothetically, simultaneously targeting components of the same signaling network might render downregulation of either of the components less likely due to the loss of compensation owing to the simultaneous blockade.

Besides melanoma, CSPG4-CAR-T cell activity was examined in a variety of glioblastoma models. Interestingly, CSPG4 was found on a certain subset of glioblastoma cells, which were

regarded as glioblastoma stem cells (GSCs) [53]. T cells equipped with a CSPG4-specific CAR exhibited antigen-specific cytotoxicity towards those GSCs providing the first report of CSPG4-CAR-T cell efficacy against glioblastoma [53]. Furthermore, intracranial application of CSPG4-CAR-T cells impeded glioblastoma growth in a xenograft mouse model [57]. No signs of antigen-loss or downregulation could be observed after CSPG4-CAR-T cell application [57]. Remarkably, the overall antigen-density of CSPG4 increased over the course of one week following CAR-T-cell infusion [57]. Efforts to dissect the mechanism underlying this peculiar phenomenon established a causal connection between TNF secreted by adjacent microglial cells resulting in NFκB signaling in glioblastoma cells and the augmented expression of CSPG4 on glioblastoma cells [57]. Whether TNF-mediated CSPG4 upregulation bears relevance in other malignancies remains to be elucidated.

RNA-CAR-T cells: safety first

Despite the repetitive demonstration of pronounced efficacy against hematological malignancies the broad use of CAR-T cells is hampered by concerns arising from the occurrence of distinct side-effects related to CAR-T-cell therapy [58]. Across the majority of preclinical and clinical evaluation of CAR-T-cell products, three major side-effects assumed relevance: on-target/on-tumor toxicities, on-target/off-tumor toxicities, and neurotoxicity, the cause of which remains to be further elucidated [58]. The most notorious on-target/on-tumor toxicity is represented by cytokine release syndrome (CRS), which is prevalent in 77% of ALL patients treated with CD19-CAR-T cells [25]. The clinical manifestations of cytokine release syndrome include a broad spectrum of symptoms ranging from mild fever with headache and myalgia to high fever, hypotension, acute respiratory distress syndrome, disseminated intravascular coagulation, organ failure and death [59]. Diagnostic parameters encompass elevated values for C-reactive protein and IL-6 as well as signs of multi-organ failure, deranged coagulation parameters and cytopenias [59]. The results of a recent study seeking to decipher the pathophysiology of CRS prompted a paradigm shift in the perception of CRS. Contrary to previous assumptions that CRS is primarily driven by cytokines secreted from activated CAR-T cells, macrophages producing IL-1β and IL-6 were identified as the central mediators of CRS severity [60]. Apart from symptomatic interventions, e.g. providing blood pressure support and mechanical ventilation, the therapy of CRS is based on administering corticosteroids and the FDA-approved IL-6 receptor antagonist tocilizumab [59]. Upon infusion of tocilizumab, the symptoms of CRS usually resolve within a few hours [59]. Unlike tocilizumab, corticosteroids were shown to significantly impair the anti-tumor activity of CAR-T cells [59]. Thus, corticosteroid application is usually confined to second-line treatment after an insufficient response to tocilizumab [59]. Nevertheless, even combined application of tocilizumab and corticosteroids does sometimes not suffice to curb CRS and prevent lethal multi-organ failure [59]. On-target/off-tumor toxicity constitutes another dreaded side-effect of CAR-T-cell therapy,

which can be traced back to concomitant expression of the target antigen on normal tissues, and subsequent destruction of those non-malignant cells by CAR-T-cell activity. Plastic evidence for the severity of on-target/off-tumor toxicity is provided by early CAR-T-cell trials. T cells redirected to carboxy-anhydrase-IX (CAIX) via a first-generation CAR to treat metastatic renal carcinoma were found to induce serious biliary toxicity attributed to aberrant expression of CAIX on bile duct epithelial cells [14]. Another case highlighting the lethal potential associated with on-target/off-tumor toxicity was shared by investigators from the NCI. Shortly after infusing T cells expressing an ERBB2-specific CAR to a patient with metastatic colon cancer, clinical symptoms of acute respiratory distress syndrome were observed necessitating mechanical ventilation [61]. Unfortunately, the patient died 5 days later [61]. The cause of death was assumed to be on-target/off-tumor toxicity elicited by low levels of ERBB2 on epithelial cells in the lung. Remarkably, the CAR was derived from the FDA-approved monoclonal antibody trastuzumab, which has been widely used without the occurrence of severe pulmonary toxicities [62]. Collectively, limiting on-target/off-tumor toxicities remains a general challenge in CAR-T-cell therapy. Apart from CRS and on-target/off-tumor toxicities, CAR-induced neurotoxicity constitutes another primary side-effect observed after CAR-T-cell application [58]. Neurotoxicity can manifest itself in a plethora of symptoms comprising transient cognitive impairments, hallucinations and delirium [58]. More severe manifestations include encephalopathy and seizures [58]. Rarely, rapid onset cerebral edema refractory to corticosteroids and tocilizumab is observed, with often times fatal consequences [63]. To date, the pathogenesis of CAR-induced neurotoxicity is not clear and effective therapies are still lacking. In sum, alleviating the trinity of toxicity comprising CRS, on-target/off-tumor toxicity, and CAR-induced neurotoxicity represents an urgent challenge in CAR-T-cell therapy. Hence, mechanisms to obviate those toxicities associated with CAR-T-cell therapy, such as transient receptor transfer using RNA-based transfection, have garnered attraction. RNA-CAR-T cells display a temporally limited CAR expression, which is beneficial as CAR-associated toxicities are thought to cease after the CAR-expression has declined. The electroporation of receptor-encoding mRNA has emerged as the primary method to transiently equip T cells with CARs. This procedure is based on complex physicochemical mechanisms leading to plasma membrane perforation upon application of electric fields allowing for subsequent entry of mRNA into the cytosol [64]. Pilot studies conducted more than a decade ago by our group using mRNA-electroporation to transfer an additional T-cell receptor into T cells demonstrated robust transfection efficiencies and solid antigen-specific T-cell functionality after receptor transfer [65]. Equally, the transfer of CAR-encoding mRNA into T cells can be accomplished with high efficiency resulting in a homogenous CAR expression, which can be detected within one hour after electroporation [66]. Moreover, the introduced CAR capacitated T cells to antigen-specifically eliminate tumor cells [66]. The expression kinetics of CARs transferred via

electroporation are dependent on the CAR-backbone [66]. While first-generation CARs displayed a very short expression with a maximum 4 to 8 hours after electroporation and an overall expression of less than 24 hours, second-generation CARs co-stimulated with CD28 evinced a maximum expression 8 to12 hours after electroporation and an overall expression of approximately 120 hours [66]. Correspondingly, RNA-CAR-T cells transfected with a second-generation construct exhibited the strongest antigen-specific effector response at 24h after electroporation [66]. In more advanced settings, the feasibility of a concomitant transfection of T cells with a CAR and a TCR via co-electroporation of receptor encoding mRNA has also been proven [67]. This highlights the potential of mRNA-electroporation as an efficient tool to rapidly multi-modify T cells with several receptors. While the lion's share of clinical trials is based on permanent DNA-based transduction of T cells, clinical data on RNA-CAR-T cells deployed against several different entities is steadily mounting. Regarding hematological malignancies, transient responses in patients suffering from Hodgkin lymphoma were achieved via a single infusion of T cells transfected with mRNA coding for a CAR specific for CD19 [68]. The presence of these CD19-CAR-T cells was detected for at least 48 hours to a maximum of 7 days [68]. In solid tumors RNA-CAR-T cells redirected to the tumor antigen cMET were injected into lesions of metastatic breast cancer [69]. After local application cMET-CAR-T cells induced necrosis within the tumor. Importantly, some of the injected cMET-CAR-T cells entered the blood stream and could be monitored in the circulation for a short time [69]. In sum, RNA-CAR-T cells can be prepared for clinical application. Moreover, these T cells are capable of inducing transient tumor regression. Nevertheless, the major shortcoming of RNA-CAR-T cells is the short window of anti-tumor activity, which closes as CAR-expression declines. Thus, repetitive injections of RNA-CAR-T cells are required. Another promising approach is constituted by the RNA-CAR / DNA-CAR sequence starting with repetitive injections of RNA-CAR-T cells followed, provided that no toxicities occurred, by a single (or more) injection of DNA-CAR-T cells. This strategy is especially attractive in phase 0/1 clinical trials exploring new tumor antigens for CAR-T-cell therapy with an unknown clinical safety profile.

CSPG4-CAR-T cells against mixed lineage leukemia: road to clinical application

To translate the concept of CSPG4-CAR-T cells against r-MLL from a simple idea to a clinically applicable drug, an elaborate roadmap is warranted. First, albeit several analyses confirm the presence of CSPG4 on r-MLL, a more comprehensive screening with different antibodies is required. Due to the various glycoforms of CSPG4, we recommend the use of the anti-CSPG4 9.2.27 antibody, which stains CSPG4 independently form the glycosylation status. Second, owing to the exaggerated potential for on-target/off-tumor toxicity (see section 5) a RNA-CAR-T cell / DNA-CAR-T cell sequence seems reasonable. Third, a good manufacturing practice (GMP) compliant production process for CSPG4-CAR-T cells has to be established to

surmount regulatory hurdles for clinical application. Fourth, proof-of-principle *in-vitro* studies are needed to evaluate the capacity of CSPG4-CAR-T cells to mount effector functions, especially cytotoxicity, against r-MLL cells. At this step it is important to explore possible combination partners for CSPG4-CAR-T cells, such as CAR-T cells with other specificities or kinase inhibitors. Fifth, confirmation of those *in-vitro* results using clinically relevant animal models for r-MLL is indicated. Finally, the initiation of phase 0/1 clinical trials to gauge the safety, feasibility, and efficacy of CSPG4-CAR-T cell therapy for r-MLL will be necessary. In the present work we focused primarily on demonstrating for the first time ever that CSPG4-CAR-T cells can recognize and kill r-MLL cells, represented by the high-risk childhood B cell precursor ALL cell line KOPN8. Furthermore, we generated CSPG4-CAR-T cells via mRNA-electroporation based on a protocol, which is adaptable to GMP-compliant standards.

3. Literature

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International Journal of
Molecular Sciences

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CSPG4-Specific CAR T Cells for High-Risk Childhood B Cell Precursor Leukemia

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† The present work was performed in fulfillment of the requirements for obtaining the degree Dr. med. at the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

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Received: 16 April 2019; Accepted: 27 May 2019; Published: 5 June 2019

Abstract: The advent of CD19-specific chimeric antigen receptor (CAR) T cells has proven to be a powerful asset in the arsenal of cancer immunotherapy of acute lymphoblastic leukemia and certain B cell lymphomas. However, a sizable portion of patients treated with CD19-CAR T cells relapse with CD19-negative cancer cells, necessitating the quest for back-up antigens. Chondroitin sulfate proteoglycan 4 (CSPG4) expression has been reported on leukemic blasts bearing the ill-fated *MLL* 11q23 rearrangement. We aimed at exploring the use of CSPG4-specific CAR T cells against mixed-lineage leukemia (MLL)-rearranged leukemic blasts, using the precursor B cell leukemia cell line KOPN8 (MLL–MLLT1 translocation) as a model. First, we confirmed CSPG4 expression on KOPN8 cells. Bulk T cells electroporated with mRNA encoding a CSPG4-specific CAR upregulated activation markers and secreted the Th1 cytokines TNF and IFNγ in an antigen-specific manner upon co-culture with KOPN8 cells. More importantly, CSPG4-specific CAR T cells evinced specific degranulation towards KOPN8 cells and specifically lysed KOPN8 target cells in chromium lysis experiments. CSPG4 is a well-established CAR target in cutaneous melanoma. Here, we provide proof-of-principle data for the use of CSPG4-specific CAR T cells against MLL-translocated leukemias.

Keywords: CAR T cells; CSPG4; MLL-translocated leukemias; back-up target antigen; high-risk childhood B cell precursor leukemia

1. Introduction

CD19-specific chimeric antigen receptor (CAR) T cells have mediated substantial tumor regressions in patients with relapsed or refractory B-cell malignancies, resulting in their recent FDA approval for acute lymphoblastic leukemia (ALL) and certain non-Hodgkin lymphomas [1]. Impressive complete remission rates above 80% in B-ALL and around 40% in diffuse large B cell lymphoma were achieved by a single infusion of autologous CD19-CAR T cells [2–4].

CARs are composed of an extracellular single chain Fv detecting membrane-bound antigens and an intracellular CD3ζ activation motif linked in *cis* with a co-stimulatory domain, such as CD28 or 4-1BB [5]. To maximize durable response rates, it is warranted to select target antigens that exhibit consistent expression on malignant cells without inducing serious on-target/off-tumor toxicities. Whereas depletion of conventional CD19-positive B cells can be easily compensated for by immunoglobulin substitution therapy, CD19 shut down represents a major immune escape mechanism compromising ongoing responses to CD19-CAR T cell therapy [6]. Thus, albeit initial CD19 positivity and stringent complete responses after CD19-CAR T cell infusion, a sizable portion of patients suffering from B-ALL

relapse with CD19-negative blasts [2,7,8]. CD19 negativity originating from either mutational loss or posttranscriptional editing can be counteracted by using alternative B cell target antigens such as CD22 [9,10]. Recently, CD19 loss predicated on a myeloid lineage switch conferring negativity for all B cell specific antigens has been reported [11,12]. In those cases, B cell mixed-lineage leukemia blasts bearing CD19 and CD22 morphed into CD19/CD22 double-negative myeloid blasts. Hence, creating a diversified portfolio of back-up antigens for CD19 beyond B cell antigens is an urgent need.

Chondroitin sulfate proteoglycan 4 (CSPG4), formerly denoted as melanoma-associated chondroitin-sulfate proteoglycan (MCSP) or high-molecular-weight melanoma-associated antigen (HMW-MAA) is a heavily glycosylated transmembrane protein [13], which is overexpressed in a variety of prognostically unfavorable entities, such as melanoma [14], glioma [15], and triple-negative breast cancer [16]. Moreover, CSPG4 has been found on the surface of MLL-rearranged leukemia cells [17–21], a form that accounts for around 10% of all leukemias [22]. The MLL protein, encoded by the *KMT2A* gene, is a regulator of gene expression owing to its intrinsic methyltransferase activity [23]. In MLL leukemias, the *KMT2A* gene is disrupted ensuing chromosomal translocation [22]. This results in abrogated MLL protein expression and subsequent global demethylation, which provides a possible rationale for the correlation of CSPG4 upregulation and MLL rearrangement. In light of high relapse frequencies and a significantly reduced overall survival associated with MLL leukemia [22], novel treatment strategies are highly required. Hence, antigen-specific targeting of CSPG4 has garnered increasing interest, especially given an elevated resistance of MLL cells to standard chemotherapy [24].

Initial efforts to specifically attack CSPG4 on cancer cells have encompassed monoclonal antibodies and immunotoxins [25]. A variety of antibodies, such as monoclonal antibody 9.2.27 against melanoma [26], monoclonal antibody 225.28 against breast cancer [16], TP41.2 against mesothelioma [27], as well as a single-chain Fv construct, scFv–Fc21 [28], have been successfully employed to stunt tumor progression in animal models, which is largely ascribed to blockade of CSPG4-mediated pro survival signals [16,25,26,29]. Besides, antigen-specific direct cytolysis of CSPG4-expressing targets could be induced using immunotoxins, merging a CSPG4 scFv with a cytotoxic protein, e.g., Exotoxin A [30,31] or microtubule-associated protein tau [32].

Other strategies to specifically eliminate CSPG4-positive targets include fusion proteins linking a CSPG4 binding domain to soluble TRAIL (TNF-related apoptosis-inducing ligand) agonists to initiate cell death upon CSPG4 binding through the extrinsic apoptosis pathway [33]. Regarding MLL leukemia, data targeting CSPG4 are scant. A single study evaluating a CSPG4-specific monoclonal antibody did not show any significant impact on MLL cells in a NOD/SCID model [34]. To date, no further clinical or preclinical data assessing CSPG4 as a target antigen in B-ALL, especially MLL B-ALL, have been reported.

Consistently, all studies involving CSPG4-CAR T cells have been focused on solid tumors, and no data concerning leukemia have been published. T cells, retrovirally transduced with a CSPG4-specific CAR, exerted potent cytotoxicity in various CSPG4-expressing tumors, such as melanoma, breast cancer, mesothelioma, glioblastoma, and osteosarcoma [35,36], in animal models. Additionally, intracranial application of CSPG4-CAR T cells in a murine model of glioblastoma imposed efficient tumor control [37]. A potential caveat of CSPG4-CAR T cell therapy is posed by the fact that CSPG4 expression is not exclusively restricted to malignant cells. Among others, CSPG4 has been detected on activated pericytes [38,39] and, to a far lower extent, on smooth muscle cells [40]. In order to obviate concerns about potential on-target/off-tumor toxicities, we have previously demonstrated that transient transfection of T cells with CSPG4-CARs using mRNA electroporation might be an effective and safe tool in cancer immunotherapy [41,42].

The aim of the current study was to extrapolate our experience in targeting melanoma cells with CSPG4-CAR T cells to CSPG4-postiive MLL leukemias using MLL1-MLLT1-translocated KOPN8 B-ALL cells as target cells. We wish to raise initial awareness for CSPG4 as a possible back-up antigen in B-ALL, with special emphasis on MLL-rearranged leukemias. To our knowledge, this is the first study targeting leukemia cells.

2. Results

2.1. KOPN8 Leukemia Cells Express CSPG4

Mixed-lineage leukemias (MLL) are characterized by specific translocations involving the *MLL* gene on chromosome 11. The target cell line employed in this study, KOPN8, was derived from a patient with precursor B-ALL and harbors the MLL–MLLT1 translocation connecting chromosomes 11 and 19 (t(11;19)) [43]. It has been reported that CSPG4 is expressed on the surface of MLL-rearranged leukemias [17–21]. In order to examine the targetability of KOPN8 cells with CSPG4-CAR T cells, we first analyzed CSPG4 expression using the 9.2.27 anti-CSPG4 antibody, which can bind to diverse isoforms of CSPG4 originating from a variety of different glycosylation patterns. Importantly, the single-chain Fv of the CSPG4-CAR utilized in this study was derived from this antibody, suggesting that this antibody is particularly appropriate for probing the susceptibility to CSPG4-CAR T cells. Uniform expression of CSPG4 was detected on the surface of KOPN8 cells (Figure 1). CSPG4-negative T2.A1 cells did not display a difference between isotype staining and anti-CSPG4 staining. Human melanoma cells A375M, which evince a strong CSPG4 expression, served as positive control for CSPG4-CAR T cell activity throughout this study.

Collectively, uniform CSPG4 expression on KOPN8 leukemia cells suggested possible targetability by CSPG4-CAR T cells.

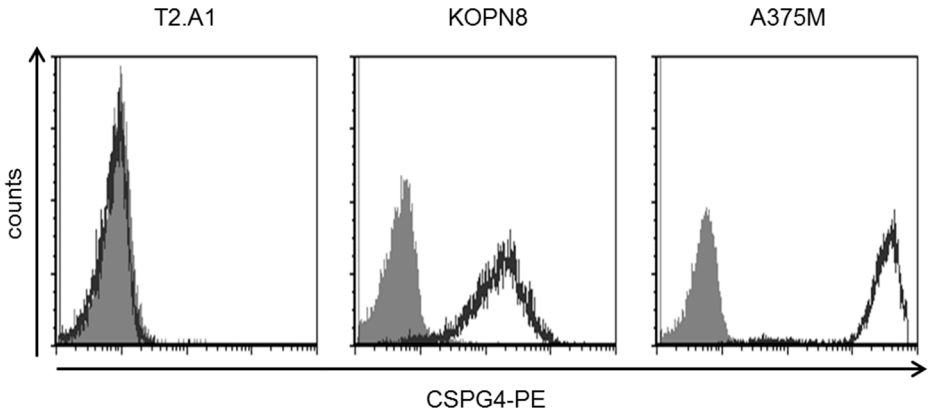

Figure 1. KOPN8 leukemia cells express CSPG4. Surface expression of CSPG4 on KOPN8 cells in comparison to CSG4-negative T2.A1 cells and CSPG4-positive A375M melanoma cells. One representative staining out of three independent experiments is shown.

2.2. CSPG4-CAR T Cells are Activated by KOPN8 Leukemia Cells in An Antigen-Specific Fashion

Having confirmed the presence of our target antigen, we next sought to evaluate whether CSPG4-CAR T cells could antigen-specifically target KOPN8 leukemia cells. To this end, bulk T cells from monocyte-depleted, healthy donor-derived PBMCs were selectively expanded with OKT-3 and IL-2 for 10 days. This resulted in a homogenous $CD3^+$ T-cell population, with a dominant $CD8^+$ T-cell fraction (Suppl. Figure S1). Next, these T cells were electroporated with mRNA encoding a CSPG4-specific, CD28-co-stimulated, second-generation CAR (Figure 2a). Mock (no RNA)-electroporated T cells and T cells transfected with a carcinoembryonic antigen (CEA)-specific control CAR served as controls for CSPG4-CAR specificity. Surface staining for CEA on target cell lines revealed a very weak expression of CEA on KOPN8 leukemia cells and no detectable expression on T2.A1 and A375M cells in comparison to CEA-positive KATO III gastric carcinoma cells (Suppl. Figure S2). Capitalizing on the pronounced difference in antigen expression levels (CSPG4 high and CEA very low) on KOPN8 cells, CEA-CAR was used to control for unspecific tonic CAR signaling, in order to confirm antigen-specific reactivity of CSPG4-CAR against KOPN8 cells. Upon electroporation, similar expression of CSPG4 and CEA CARs was observed, with transfection rates around 86% (Figure 2b). To determine whether CSPG4-CAR T cells can react to KOPN8 leukemia

cells in an antigen-specific manner, mock T cells, control CEA-CAR T cells, and CSPG4-CAR T cells were co-incubated with T2.A1, KOPN8, and A375M cells at a 1:1 ratio, 24 h after electroporation. After a co-culture period of 4 h, upregulation of the activation marker CD69 was analyzed (Figure 2c). Additionally, CD25 expression was assayed following 20 h of co-culture (Figure 2d).

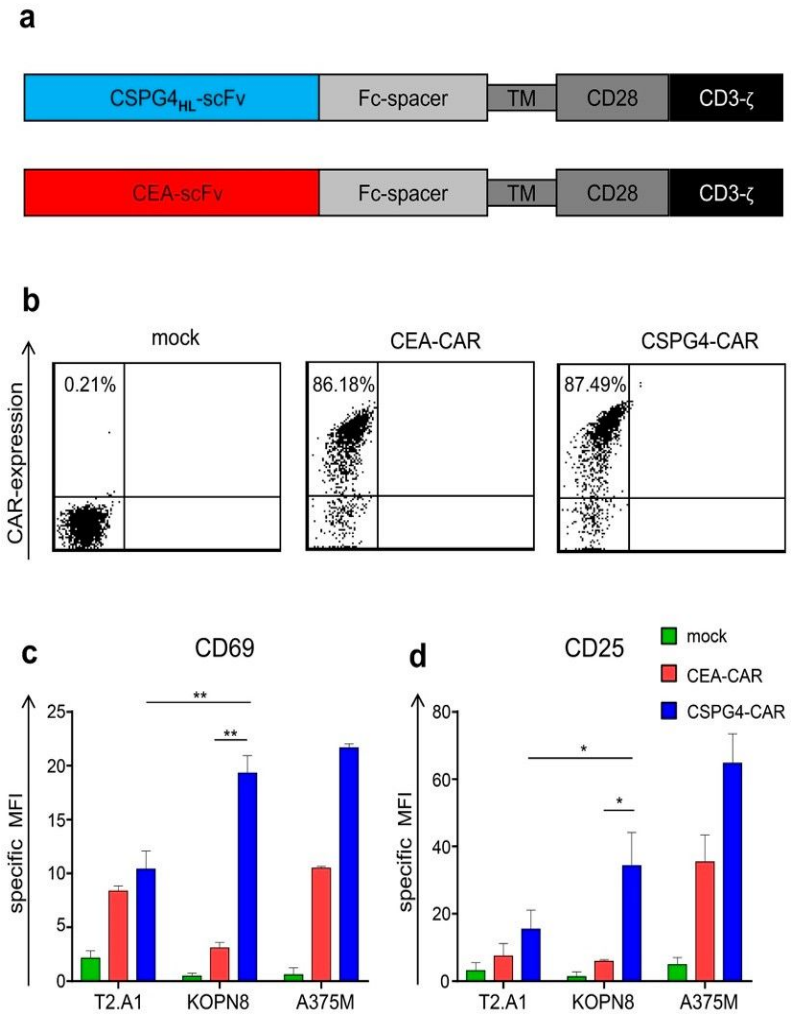

Figure 2. Chondroitin sulfate proteoglycan 4 (CSPG4)-chimeric antigen receptor (CAR) T cells are antigen-specifically activated upon stimulation with KOPN8 leukemia cells. Bulk T cells from healthy donors were selectively expanded using OKT3 and IL-2, as detailed in the Materials and Methods section. After 10 days, these cells were either mock (no RNA)-electroporated or transfected with CAR-encoding mRNA. (**a**) Schematic representation depicting the modular structure of the CSPG4-specific and the carcinoembryonic antigen (CEA)-specific CAR. (**b**) Equal expression of CSPG4 and CEA CARs was confirmed via flow cytometry 24 h after electroporation using a Phycoerythrin (PE)-labeled goat anti-human IgG antibody, binding the fc-part of the CARs. One representative donor out of three independent experiments is shown. (**c**,**d**) Twenty-four hours after electroporation, T cells were co-incubated at a 1:1 ratio with T2.A1 cells, KOPN8 cells, and A375M cells. Mock and CEA-CAR T cells served as negative controls. Upregulation of CD69 (**c**) was analyzed after 4 h of co-culture using anti-human CD69 staining, and upregulation of CD25 (**d**) was detected after 20 h of co-culture using an anti-human CD25 antibody. The specific mean fluorescence intensity (MFI) was calculated by subtraction of the background MFI obtained with isotype antibodies. Data represent geometric means ±SEM from three independent experiments; p values were calculated by paired t test, * indicates $p \leq 0.05$, and ** indicates $p \leq 0.01$.

Mock T cells did not upregulate CD69 or CD25 after co-incubation with KOPN8 leukemia cells (Figure 2c,d). CSPG4-CAR T cells evinced a significantly higher CD69 and CD25 upregulation compared to control CEA-CAR T cells following co-culture with KOPN8 leukemia cells, confirming antigen-specific activation mediated via CSPG4 CAR. Moreover, CSPG4-CAR T cells exhibited a significantly higher CD69 and CD25 expression upon co-culture with KOPN8 leukemia cells as compared to co-incubation with CSPG4-negative T2.A1 cells, further corroborating antigen-specific activity of CSPG4-CAR T cells in response to KOPN8 leukemia cells. Human melanoma cells (A375M) served as a positive control for the stimulation of CSPG4-CAR T cells and correspondingly induced upregulation of CD25 and CD69 expression on CSPG4-CAR T cells. Control CEA-specific CAR T cells slightly upregulated CD25 beyond background in response to melanoma cells but not to leukemia cells, which was unexpected considering the absence of CEA on A375M cells. A possible explanation for this conundrum might derive from a potential cross-reactivity of the CEA-CAR with surface molecules on melanoma cells that share antigenic sites of CEA [44].

In sum, these data indicated that KOPN8 leukemia cells can be antigen-specifically targeted by CSPG4-CAR T cells.

2.3. CSPG4-CAR T Cells Secrete Th1 Cytokines Following Co-Culture with KOPN8 Leukemia Cells

Next, we examined the cytokine secretion profile of CSPG4-CAR T cells in response to KOPN8 leukemia cells. Receptor-transfected T cells were stimulated with T2.A1 cells, KOPN8 cells, and A375M melanoma cells, and the secreted cytokines were quantified after overnight culture. CSPG4-CAR T cells produced significantly more IFNγ than control CEA-CAR T cells and mock T cells upon co-culture with KOPN8 leukemia cells (Figure 3). Additionally, CSPG4-CAR T cells did not secrete IFNγ upon co-incubation with T2.A1 cells, confirming antigen-specific IFNγ secretion towards CSPG4-positive KOPN8 leukemia cells (Figure 3). Moreover, CSPG4-CAR T cells exhibited IFNγ production upon co-culture with human melanoma cells A375M, serving as positive controls (Figure 3). Control CEA-CAR T cell IFNγ production was absent in response to T2.A1 cells but could be found upon co-culture with A375M melanoma cells. Regarding TNF secretion, a similar pattern was observed. In comparison to mock and CEA control CAR T cells, CSPG4-CAR T cells secreted significantly more TNF upon co-culture with KOPN8 leukemia cells (Figure 3). No tonic TNF production by CAR T cells was detected following co-culture with T2.A1 cells (Figure 3). As expected, CSPG4-expressing A375M melanoma cells evoked TNF production by CSPG4-CAR T cells (Figure 3). Whereas IFNγ and TNF are canonical pro-inflammatory cytokines, IL-2 has attained a more dichotomous role in cancer immunotherapy, with T cell stimulatory capacities on one hand and immunosuppressive characteristics on the other hand. Immunosuppression is largely mediated by IL-2 uptake by T regulatory cells, which are subsequently activated and promote immune evasion. Strikingly, while antigen-specific IL-2 production was detected following co-culture with A375M cells, hardly any IL-2 secretion by CSPG4-CAR T cells was observed upon stimulation with KOPN8 leukemia cells (Figure 3). Finally, we screened for secretion of the Th2 cytokine IL-4, which is considered to have immunosuppressive properties. No relevant IL-4 production by CSPG4-CAR T cells was measured after stimulation with KOPN8 leukemia cells and A375M target cells (Suppl. Figure S3). In aggregate, these results proved that CSPG4-CAR T cells evince an antigen-specific secretion pattern of Th1 cytokines in response to KOPN8 leukemia cells, without IL-2 and IL-4 production.

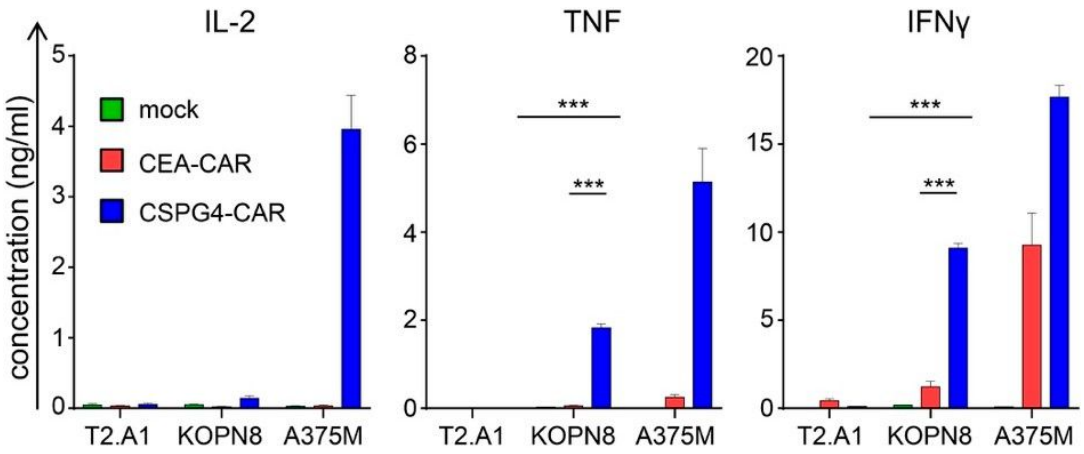

Figure 3. CSPG4-CAR T cells antigen-specifically produce Th1 cytokines in response to stimulation with KOPN8 leukemia cells. The different T cell conditions were generated as mentioned above (Figure 2). Mock (no RNA)-electroporated T cells and CEA-CAR T cells served as negative controls. Twenty-four hours after electroporation, T cells were co-incubated over-night at a 1:1 ratio with T2.A1 cells, KOPN8 cells, and A375M cells. Induced cytokine secretion was quantified in the supernatant with a cytometric bead array (CBA). Concentrations of IL-2, TNF, and IFNγ are depicted [ng/mL]; please note the different scales. Data represent means ± SEM from three independent experiments, p values were calculated by paired t test, *** indicates $p \leq 0.001$.

2.4. CSPG4-CAR T Cells Specifically Lyse Leukemia Cells

To test the cytolytic activity of CSPG4-CAR T cells, CEA control T cell, and mock T cells toward KOPN8 leukemia cells, we first performed a 4 h degranulation assay analyzing CD107a upregulation on $CD8^+$ T cells after co-incubation with T2.A1, KOPN8, and A375M cells. While neither mock-transfected nor CEA control CAR-expressing $CD8^+$ T cells displayed any relevant degranulation towards KOPN8 leukemia cells, $CD8^+$ T cells equipped with the CSPG4-CAR exhibited antigen-specific degranulation in response to KOPN8 cells (Figure 4a). Control CEA-CAR T cells showcased a slightly elevated degranulation against melanoma cells in comparison to background degranulation against T2.A1 cells. Thus, CSPG4-CAR T cells displayed antigen-specific cytotoxicity towards KOPN8 leukemia cells. Nevertheless, the most important feature of CAR-T-cell therapy is antigen-specific tumor destruction. Hence, we sought to corroborate the cytotoxic potential of CSPG4-CAR T cells toward KOPN8 leukemia cells by a 4–6 h chromium lysis assay. KOPN8 and T2.A1 cells were used as targets. Mock T cells, CEA-CAR T cells, and CSPG4-CAR T cells demonstrated equal background lysis on T2.A1 cells (Figure 4b). Mock T cells did not induce any lysis in KOPN8 cells beyond background (Figure 4c). CSPG4-CAR T cells antigen-specifically lysed KOPN8 leukemia cells even at low effector-to-target ratios (Figure 4c). Statistical significance for antigen-specific lysis in comparison to the CEA-specific control CAR was achieved at the 20:1 effector-to-target ratio and the 2:1 effector-to-target ratio. CEA-CAR T cells displayed an incrementing cytotoxicity from low to high effector-to-target ratios, presumably reflecting both the concomitant increase in overall tonic CAR signaling with higher effector-to-target ratios and an increasing on-target response against KOPN8 cells, which display a very low expression of CEA (Suppl. Figure S2). In sum, CSPG4-CAR T cells revealed a high cytotoxic potential as reflected in antigen-specific degranulation and antigen-specific lysis against KOPN8 leukemia cells. Concerning toxicity, no excessive activity against CSPG4-negative targets was observed.

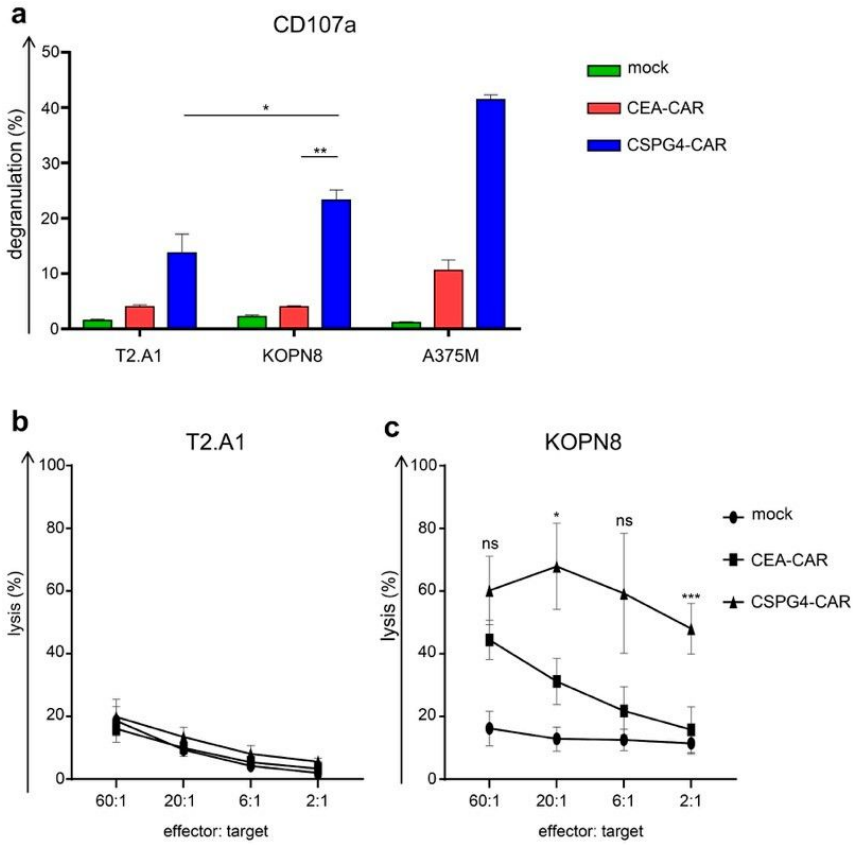

Figure 4. CSPG4-CAR T cells antigen-specifically kill KOPN8 leukemia cells. The different T cell conditions were generated as mentioned above (Figure 2). Mock (no RNA)-electroporated T cells and CEA-CAR T cells served as negative controls. Cytotoxicity towards T2.A1 cells, KOPN-8 cells, and A375M cells was determined 24 h after electroporation. (**a**) Upon 4 h of co-culture with the target cells, degranulation was assayed using CD107a and CD8 staining. Percent degranulation of $CD8^+$ T cells was calculated by dividing the portion of CD107a-positive/CD8-positive T cells by the portion of CD8-positive T cells. Data represent means ± SEM from three independent experiments; p values were calculated by paired t test, * indicates $p \leq 0.05$, and ** indicates $p \leq 0.01$. (**b**,**c**). Lytic capacity towards T2.A1 (**b**) and KOPN8 (**c**) cells was analyzed at the indicated effector-to-target ratios in a standard 4 h chromium lysis assay. Data represent means ± SEM from three independent experiments; p values were calculated by paired t test, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

3. Discussion

In the present study, we examined the potential of CSPG4 as a novel target antigen for CAR T cell therapy of MLL B-ALL using MLL–MLLT1 rearranged B cell precursor leukemia cells as targets. We transfected bulk T cells with a CSPG4-specific CAR and assayed several canonical T cell effector functions upon co-culture with CSGP4-expressing KOPN8 leukemia cells. CSPG4-CAR T cells exhibited antigen-dependent upregulation of activiation markers and antigen-dependent IFNy production response to leukemia cells. Importantly, we could demonstrate that CSPG4-CAR T cells killed KOPN8 leukemia cells in an antigen-dependent fashion. This is the first study reporting on CSPG4-CAR T cells in the context of B-ALL, with special emphasis on the ill-fated MLL B-ALL subtypes. The poster child for CAR T cell therapy of B-ALL has been CD19, which is initially expressed by virtually all B-ALL blasts [5]. Moreover, as part of the B cell receptor, its physiological distribution is exclusively confined to B cells, limiting the potential for on-target/off-tumor toxicity [45]. Given this favorable expression profile, CD19 has emerged as the primary target in B-ALL. Nevertheless, after high initial response rates, many patients relapse with abrogated CD19 expression [2,7,8]. To date, several mechanisms resulting in the shutdown of CD19 expression have been elucidated: (i) alterations

of exon 2, compromising CD19 surface localization [46], (ii) alterations of exon 5, resulting in a truncated form of CD19 [6], and (iii) loss of exon 4, eliminating the binding site of the majority of CD19 CARs [6]. The most obvious strategy to counteract these issues is to target an alternative antigen. However, so far, the arsenal of back-up antigens for CD19 in B-ALL is scant. Promising results have been attained by employing CD22-specific CARs in B-ALL patients including those relapsing after CD19-CAR therapy [9,10]. In a phase 1 clinical trial, 11 out of 15 patients, including 5 patients with prior CD19-CAR T cell treatment, achieved a complete remission following infusion of CD22-CAR T cells [9]. The median remission time, however, did not exceed 6 months, which could be largely traced back to the emergence of leukemia cells with reduced CD22 expression, eluding CD22-CAR T cells. Thus, a more comprehensive arsenal of back-up antigens in ALL is required. This is even more evident against the backdrop of a recent study reporting on the successful deployment of CD19-CAR T cells against 11q23-rearranged MLL B-ALL leukemia [11,12], which is associated with a poor prognosis and preponderance in young patients [22]. All seven patients with MLL B-ALL achieved a complete response upon CD19-CAR infusion [11]. Two patients, however, exhibited a rapid relapse with CD19-negative blasts. Molecular and phenotypic analysis of the relapsing blasts revealed a clonal lineage switch from MLL-B-ALL to MLL-AML conferring negativity for the B-lineage markers CD19 and CD22. This highlights a new immune escape mechanism to CD19-CAR T cell therapy and exposes the dependence on B-lineage antigens as a particular Achilles heel in CAR T cell therapy of MLL-B-ALL. Thus, the creation of a diverse antigen portfolio bears particular relevance for MLL B-ALL. The biology of MLL leukemias is governed by the reactivation of stem cell programs transforming hematopoietic stem cells into highly aggressive leukemic blasts [22]. Intensive combinational chemotherapy regimens have only modestly improved survival at the expense of severe therapy-related morbidity [22]. High-risk hematological malignancies are usually consolidated by hematopoietic stem cell transplantation (HSP) capitalizing on the graft-versus-leukemia effect. In MLL leukemia, however, the majority of studies did not reveal any survival benefit imparted by HSP [47]. Given the paucity of effective treatment options, the advent of CD19-CAR T cells has stirred excitement as a new therapeutic modality against MLL B-ALL with an initial CR rate of 100% in a small cohort [11]. However, as mentioned above, the MLL-leukemia inherent plasticity allowed a lineage switch and concomitant evasion from CD19 targeting, resulting in leukemia recurrence in two patients.

Presumably, a major contributor to this plasticity is constituted by the deregulation of promotor methylation and uncontrolled gene activation, which is a repercussion of the 11q23 translocation disrupting the gene coding for the MLL1 methyltransferase [22]. In this study, we intended to exploit this disturbance in gene expression to screen for the presence of CSPG4, which is a well-established CAR-target antigen in melanoma and other solid tumors [35,36,41,42]. The promotor of CSPG4 has been shown to be sensitive to methylation [48], which is reflected by CSPG4 upregulation in several tumor cell lines upon treatment with demethylating agents [48]. Moreover, the global demethylation following MLL1 rearrangement may induce the expression of CSPG4 transcription enhancers or otherwise facilitate its expression. As a model for MLL B-ALL, we selected KOPN8 precursor B cell leukemia cells, characterized by an MLL–MLLT1 translocation (t11;19). Using the anti-CSPG4 9.2.27 antibody, we could demonstrate uniform CSPG4 surface expression by those leukemia cells. The only report on CSPG4-directed targeting of leukemia cell was predicated on the anti-CSPG4 225.28 antibody, the therapeutic efficacy of which against 11q23-rearranged leukemia cells was evaluated in a xenograft mouse model [34]. No detectable impact on survival and tumor growth could be ascertained. Attacking malignant cells with CAR T cells is deemed more powerful than delivering a sole blocking antibody. We have previously shown that T cells equipped with a CAR derived from the 9.2.27 anti-CSPG4 antibody antigen-specifically eliminated melanoma cells [40–42]. In the current study, we could show that those CSPG4-CAR T cells antigen-specifically react also toward MLL leukemia cells, providing the only report on CSPG4-CAR T cells against leukemia to date.

Conspicuously, leukemia cells did not evoke IL-2 secretion from CSPG4-CAR T cells, whereas solid IL-2 secretion could be observed against melanoma cells. Further studies are warranted to

elucidate this interesting finding. One possibility is that KOPN8 cells bind or even consume IL-2, although, to our knowledge, no evidence for such a mechanism has been reported. Alternatively, the lower CSPG4 expression on KOPN8 cells compared to A375M cells may have a larger impact on IL-2 secretion by T cells than on TNF and IFNγ secretion. It might be that the threshold of stimulation needed for IL-2 production is higher than that for TNF and IFNγ production. The role of IL-2 in cancer immunotherapy has become a matter of intense debate. In initial studies on adoptive T-cell therapy, IL-2 was widely applied for its stimulatory impact on T cells, promoting growth and survival of tumor-specific T cells administered to cancer patients [49]. T cells grown ex vivo using IL-2 represented the first efficacious T cell product for cancer immunotherapy [49]. Very recently, it was shown that autocrine IL-2 receptor signaling mediated TGF-β resistance in CAR T cells, making these cells more potent in staying active against TGF-β-positive solid tumors [50]. In the last few years, however, IL-2 consumption by CD25-positive regulatory T cells (Tregs) and subsequent inhibition of tumor-specific T cells has garnered increasing attention [51]. Especially in solid tumors, a high prevalence of Tregs in the tumor microenvironment poses a major obstacle to CAR T cell therapy [52]. Regardingleukemia, low-dose IL-2 administered to 84 patients with AML resulted in a pronounced increase of Tregs in the peripheral blood [53]. These regulatory T cells displayed an augmented expression of CTLA-4 and suppressed the effector functions of conventional T cells in vitro. Hence, the absent IL-2 production by CSPG4-CAR T cells in response to leukemia cells might be beneficial to prevent a surge of regulatory T cells. On the flipside, the deficit in IL-2 production might lead to poor proliferation and a compromised long-term persistence of tumor-specific CAR T cells. This might be of special relevance in the setting of permanently transduced T cells, which are destined to proliferate and form tumor-specific memory cells persisting for months or even years. Our approach relies on transiently equipping T cells with CARs using mRNA electroporation. In this scenario, robust proliferation and persistence are not so important, as the transient receptor expression per se necessitates repetitive injections to maintain therapeutic levels of CAR T cells.

Finally, our results indicate that CSPG4-CAR T cells are capable of specifically eliminating KOPN8 blasts at low effector-to-target ratios. Generally, a major caveat in CAR T cell therapy arises from potential on-target/off-tumor toxicity due to the accidental killing of non-malignant bystander cells co-expressing the target antigen [54]. With respect to CSPG4, no expression has been detected on hematopoietic stem cells, ruling out therapy-associated myeloablation [20]. Concerns about potential on-target/off-tumor toxicity are stirred by CSPG4 presence on smooth muscle cells and pericytes [38–40]. Nevertheless, the expression level in those cell types is far inferior compared to that in malignant cells. Of note, we detected a pronounced upregulation of activation markers and cytokine secretion by CEA-CAR T cells against A375M melanoma cells, which were found to be CEA-negative. This might indicate CEA-CAR cross-reactivity with surface molecules sharing epitope similarity with CEA, present on A375M melanoma cells [44]. This further highlights the caution necessary in selecting new target antigens for clinical application. In acknowledgment of the power exerted by CAR T cells, we have previously developed a protocol to generate CSPG4-CAR T cells via mRNA electroporation [42]. Using RNA-transfected CAR T cells harbors the advantage that the receptor expression is temporally restricted, rendering potential off-target and on-target/off-tumor toxicity evanescent as well. Unlike solid tumors, such as melanoma, which are usually located extravascularly, leukemia cells primarily reside in the blood and the bone marrow. Thus, early onset cytokine release syndrome upon infusion of CSPG4-CAR T cells might be more frequent in leukemia than in melanoma [55]. To mitigate safety concerns, an initial use of repetitive injections of RNA-transfected CSPG4-CAR T cells may be beneficial to probe for toxicity. In case of no serious side effects, a switch to permanently transfected CSPG4-CAR T cells may be conceivable.

In aggregate, we wish to highlight the therapeutic potential of CSPG4-CAR T cells as a possible back-up modality for B-ALL relapsing to CD19-CAR T cell therapy, with special emphasis on MLL leukemias, which harbor the potential to undergo a lineage switch and shed all B cell-associated antigens. CSPG-4-CAR T cells generated via mRNA electroporation responded to MLL leukemia cells

with antigen-specific upregulation of activation markers, antigen-specific elaboration of Th1 cytokines, and antigen-specific tumor cell lysis. On the basis of these data, we encourage a comprehensive screening of MLL leukemia patients for CSPG4 expression to pave the way for further investigations towards a clinical application of CSPG4-CAR T cells against MLL leukemia. To our knowledge, this is the first study implicating CSPG4-CAR T cells as a potential therapeutic option for MLL leukemia.

4. Materials and Methods

4.1. Cells and Reagents

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood obtained from healthy donors following informed consent and approval by the institutional review board (reference number: 251_16 B, by 14 September 2016), using density centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Following monocyte depletion via 1 h dish adherence, the remaining non-adherent fraction was cryopreserved and stored at - 80 degrees until experimental use.

Target cell lines included the TxB cell hybridoma T2.A1 (kind gift from Prof. Dr. Schulz, Nuremberg), the B-cell precursor leukemia cell line KOPN8 (MLL–MLLT1 translocation; kind gift from Prof. Dr. Slany, Erlangen), and the melanoma cell line A375M (kind gift from Dr. Aarnoudse, Leiden, Netherlands). The Kato III cell line was a kind gift from Dr. Santegoets, Leiden, Netherlands). The cells were maintained in R10 medium containing RPMI 1640 (Lonza, Basel, Switzerland), 2 mM L-glutamine (Lonza), 20 mg/L Gentamicin (Lonza), 2 mM HEPES (PAA, GE healthcare), 2 mM β-mercaptoethanol (Gibco, Life Technologies, Carlsbad, CA, USA), and 10% (*v*/*v*) heat-inactivated fetal calf serum (PAA, GE healthcare, Piscataway, NY, USA).

4.2. T Cell Expansion

Non-adherent fractions were thawed and rested for one day in R10 medium. Afterwards, bulk T cells were directly activated with 0.1 µg/mL anti-CD3 antibody OKT3 (Orthoclone OKT3; Jannsen-Cilag, Neuss, Germany). Next, T cells were expanded as previously described [56]. In brief, 1000 IU/mL interleukin-2 (Proleukin; Novartis, Nuremberg, Germany) was added on days 0, 2, 3, 5, and 7. On day 3, the culture density was re-adjusted to 0.2×10^6 cells/mL. On day 7, the total cell culture volume was first doubled and subsequently equally distributed to two culture flasks. After 10 days, T cells were harvested for further experiments.

4.3. In Vitro Transcription of mRNA

A second-generation CAR ($CSPG4_{HL}$-CD28/CD3ζ-CAR) directed against CSPG4 (chondroitin sulfate proteoglycan 4) and a second-generation CAR specific for CEA (CEA-CD28/CD3ζ-CAR) were transferred into T cells. The detailed structures of both chimeric antigen receptors were previously reported [42,57]. T7 RNA polymerase (mMESSAGE mMACHINE T7 Ultra kit; Life Technologies, Carlsbad, CA, USA) was employed for in vitro generation of receptor-encoding mRNA, according to the manufacturer's instructions. Finally, mRNA was purified on RNeasy columns (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. Before electroporation, mRNA integrity was evaluated by agarose gel electrophoresis.

4.4. RNA Electroporation

RNA transfection was performed as detailed elsewhere [58]. In short, following expansion, T cells were washed in OptiMem (Life technologies, Carlsbad, CA, USA,) and transferred to 4 mm-gap electroporation cuvettes (Biolab Products, Bebensee, Germany). The cells were either mock-electroporated (no RNA) or transfected with 15 µg of RNA coding for CSPG4-specific CAR ($CSPG4_{HL}$-CD28-CD3ζ) or for CEA-specific CAR, using a Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA) at 500 V (square wave pulse) for 5 min. After transfection, T cells were cultured in R10 medium.

4.5. Flow Cytometry

CSPG4 expression on T2.A1, KOPN8, and A375M cells was detected using an anti-human CSPG4 antibody (BD Biosciences, USA, clone: 9.2.27). IgG2a isotype-stained cells served as controls. Cellular composition after expansion was analyzed on day 10 with anti-CD3 (BD Biosciences, USA, clone: UCHT1) and anti-CD8 (BD Biosciences, USA, clone: SK1) antibodies. IgG1 isotype-stained cells served as controls. CEA expression on T2.A1, KOPN8, A375M, and Kato III cells was analyzed using an anti-human CEA antibody (BD Biosciences, USA, clone: B1.1/CD66). IgG2a isotype-stained cells served as controls.

Surface expression of the introduced receptors was analyzed flow cytometrically 24 h after electroporation. CARs were stained with goat-F(ab')2 anti-human IgG antibodies (Southern Biotech, Birmingham, AL, USA) directed against the extracellular IgG1 CH2CH3 (Fc-spacer) CAR domain.

Analysis of T cell activation markers was conducted 24 h after electroporation, upon co-culture (4 h for CD69 and 20 h for CD25) of CSPG4-CAR T cells with T2.A1, KOPN8, and A375M target cells at a 1:1 ratio. T cells, either mock-electroporated or transfected with a CEA-specific CAR, served as controls. T cells were stained with either anti-CD69 (BD Biosciences, USA, clone: FN50) or anti-CD25 antibodies (BD Biosciences, USA, clone: M-A251). The specific mean fluorescence intensity (MFI) was calculated by subtraction of the background MFI obtained with mouse IgG1 isotype control antibodies.

Immunofluorescence was measured using a FACScan cytofluorometer (BD Biosciences, Heidelberg, Germany) equipped with CellQuest software (BD Biosciences). Data were analyzed using FCS Express 5 (De Novo Software, Glendale, CA, USA).

4.6. Cytokine Secretion

Cytokine secretion by CSPG4-CAR T cells in response to leukemia cells was assayed as described before [59]. T cells either mock-electroporated or transfected with a CEA-specific CAR served as controls. In short, 24 h after transfection, T cells were stimulated overnight at a 1:1 ratio with T2.A1, KOPN8, and A375M cells. The supernatants were recovered, and the concentrations of the indicated cytokines were quantified using the Th1/Th2 Cytometric Bead Array Kit II (BD Biosciences), in accordance with the manufacturer's instructions. Immunofluorescence was measured with the FACSCanto (BD Biosciences, Franklin Lakes, NJ, USA) operating with FACSDiva software (BD Biosciences). Data analysis was carried out using FCS Express 5.

4.7. Degranulation

Degranulation of CSPG4-CAR T cells towards leukemia cells was measured using conventional CD107a staining. T cells, either mock-electroporated or transfected with a CEA-specific CAR, served as controls. Twenty-four hours after electroporation, T cells were stimulated at a 1:1 ratio with T2.A1, KOPN8, and A375M target cells. Monensin (eBioscience, San Diego, CA, USA) at a final concentration of 1 µM and an anti-CD107a antibody (BD Biosciences, San Jose, CA, USA, clone: H4A3) were added right at the beginning of co-culture. After 4 h, the cells were stained with an anti-CD8 antibody (BD Biosciences, San Jose, CA, USA, clone: SK1) and analyzed via flow cytometry. Degranulation of CD8-positive T cells was calculated by dividing the portion of CD107a-positive/CD8-positive cells by the portion of CD8-positive cells.

4.8. Chromium Release Assay

Specific cytotoxicity of CSPG4-CAR T cells towards leukemia cells was analyzed with a standard 4–6 h ^{51}chromium-release assay, 24 h after electroporation, as previously described [59]. T cells, either mock-electroporated or transfected with a CEA-specific CAR, served as controls. In brief, human tumor cell lines T2.A1 and KOPN8 were labeled with 20 µCi of $Na_2{}^{51}CrO^4/10^6$ (PerkinElmer, Waltham, MA, USA) for 1 h. Next, the targets were plated on 96-well plates and co-cultured with the effectors at the indicated effector-to-target ratios. The supernatants were recovered after 4–6 h, and chromium-release

was measured with the Wallac 1450 MicroBeta plus Scintillation Counter (Wallac, Turku, Finnland). The following equation was used to determine the percentage of cytolysis: 100% x [(measured release - background release)]/[(maximum release - background release)].

4.9. Figure Preparation and Statistical Analysis

Graphs were created, and statistical analysis was performed using GraphPad Prism, Version 7 (GraphPad Software, San Diego, CA, USA); p values were calculated by paired t test, * indicates $p \leq 0.05$, ** indicates $p \leq 0.01$, and *** indicates $p \leq 0.001$.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/11/2764/s1.

Author Contributions: Conceptualization, D.C.H., J.D., G.S., and N.S.; methodology, D.C.H., J.D., and N.S.; validation, D.C.H., J.D., and N.S.; formal analysis, D.C.H.; investigation, D.C.H.; data curation, J.D. and N.S.; writing—original draft preparation, D.C.H.; writing—review and editing, J.D., G.S., and N.S.; supervision, J.D., G.S., and N.S.; project administration, N.S.; funding acquisition, J.D., G.S., and N.S.

Funding: This research was funded by the Deutsche Forschungsgemeinschaft (German Research Foundation), trilateral grant SCHA 1247/3-1.

Acknowledgments: We would like to thank Matthias Peipp and Georg Fey for preliminary work on the CSPG4 single-chain fragment variable, Kris Thielemans for providing the pGEM4Z RNA-production vector, and Hinrich Abken for the CAR backbone. We also express our gratitude to Robert Slany for providing KOPN8 cells. We acknowledge support by the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding program Open Access Publishing.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

CAR	Chimeric antigen receptor
CSPG4	Chondroitin sulfate proteoglycan 4
CEA	Carcinoembryonic antigen
PBMC	Peripheral blood mononuclear cells
MLL	Mixed-lineage leukemia
ALL	Acute lymphoblastic leukemia
CAR	Cfigurehimeric antigen receptor

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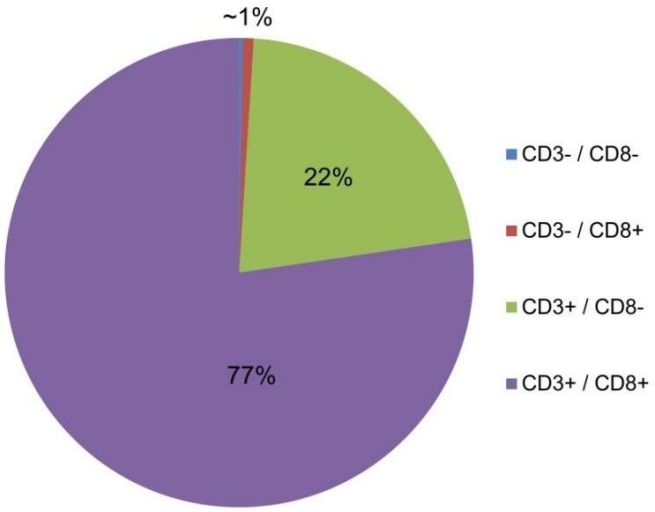
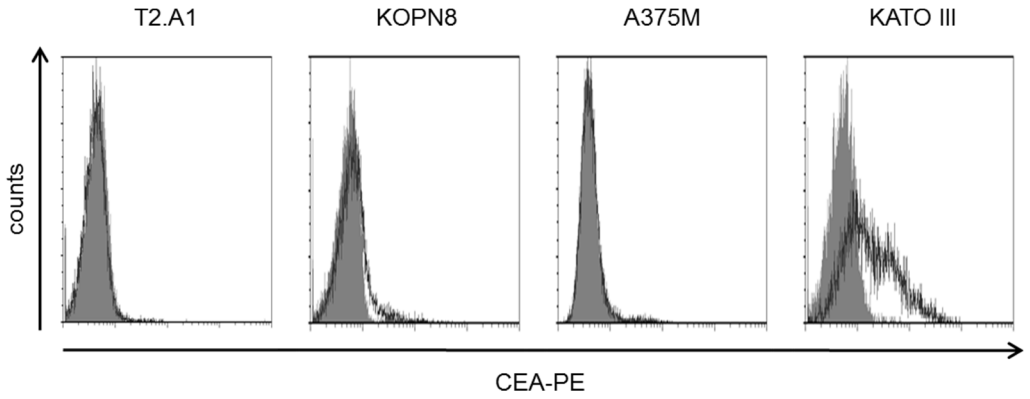
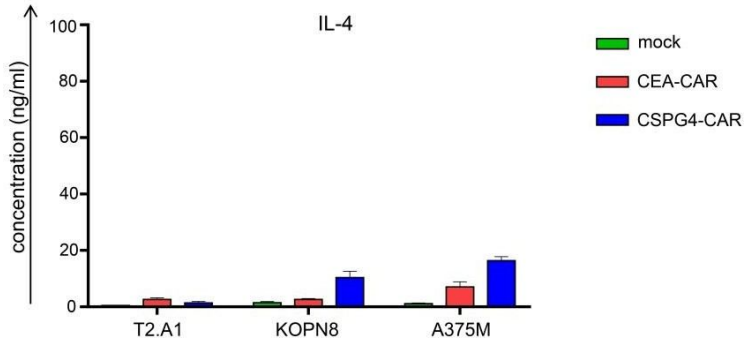


Supplemental figure S1: Cellular composition after T-cell expansion.
Healthy donor-derived bulk T cells were expanded using OKT3 and IL-2 as described in the Materials & Methods section. After 10 days, cellular composition was analyzed using double-staining for CD3 and CD8. Data are presented as means of 3 independent experiments.



Supplemental figure S2: CEA expression on tumor cell lines.
Surface expression of CEA on T2.A1 cells, KOPN8 cells, and A375M cells in comparison to the CSPG4-positive Kato III cell line. One representative staining out of four independent experiments is presented.



Supplemental figure S3: CSPG4-CAR T cells produce negligible quantities of IL-4.
The different T-cell conditions were generated as mentioned above (Fig. 2). Mock (no RNA) electroporated T cells and CEA-CAR T cells served as controls. Twenty-four hours after electroporation, T cells were co-incubated over- night at a 1.1 ratio with T2.A1 cells, KOPN8 cells, and A375M cells. Induced cytokine secretion was quantified in the supernatant with a cytometric bead array (CBA). Concentration of IL-4 is shown [pg/ml]. Data represent means ± SEM from 3 independent experiments.

5. Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATT	Adoptive T cell therapy
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CRS	Cytokine release syndrome
CSPG4	Chondroitin sulfate proteoglycan 4
EMA	European Medicines Agency
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FLT-3	Fms-like receptor tyrosine kinase-3
GMP	Good manufacturing practice
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HMW-MAA	High molecular weight melanoma-associated antigen
HSC	Hematopoietic stem cell
IL	Interleukin
MAPK	Mitogen-activated-protein-kinase
MLL	Mixed lineage leukemia
MSKCC	Memorial Sloan Kettering Cancer Center
NCI	National cancer institute
NG2	Neuron-Glia Protein 2
r-MLL	Mixed lineage rearranged leukemia
r/r	Relapsed/refractory
PBMCs	Peripheral blood mononuclear lymphocytes
PDGF	Platelet derived growth factor

scFv	Single chain Fragment variable
TCR	T-cell receptor
TIL	Tumor infiltrating lymphocyte
TRUCK	T cell redirected to universal cytokine signaling

6. List of publications

A Research articles

1. Simon B, Harrer DC, Thirion C, et al. **Enhancing lentiviral transduction to generate melanoma-specific human T cells for cancer immunotherapy.** J Immunol Methods 2019.

2. Harrer DC, Schuler G, Dörrie J et al. **CSPG4-Specific CAR T cells for High-Risk Childhood B cell precursor leukemia**. Int J Mol Sci 2019.

3. Simon B, Harrer DC, Schuler-Thurner B, et al. **Arming T cells with a gp100-Specific TCR and a CSPG4-Specific CAR Using combined DNA- and RNA-Based Receptor Transfer** . Cancers (Basel) 2019.

4. Simon B, Harrer DC, Schuler-Thurner B, et al. **The siRNA-mediated downregulation of PD-1 alone or simultaneously with CTLA-4 shows enhanced in-vitro CAR-T cell functionality for further clinical development towards the potential use in immunotherapy of melanoma**. Exp Dermatol 2018.

5. Harrer DC*, Simon B*, Fujii SI, et al. **RNA-transfection of gamma/delta T cells with a chimeric antigen receptor or an alpha/beta T-cell receptor: a safer alternative to genetically engineered alpha/beta T cells for the immunotherapy of melanoma**. BMC Cancer 2017. *share first authorship

B Review articles

1. Harrer DC, Dörrie J, Schaft N. **Chimeric Antigen Receptors in Different Cell Types: New Vehicles Join the Race**. Hum Gene Ther 2018.

7. Acknowledgements

I would like to thank my supervisors PD Dr. Niels Schaft and PD Dr. Jan Dörrie for excellent scientific and practical guidance as well as continuous encouragement. Moreover, I would like to express my gratitude to Prof. Schuler for providing a magnificent environment for research in cellular therapy. Finally, I would like to thank all present and former members of the Kussmaul research Campus for excellent support and a great time.