Emerging role of immunotherapy for childhood cancers

Rupert Handgretinger, Patrick Schlegel

Children's University Hospital, Department Hematology/Oncology, University of Tuebingen, Hoppe-Seyler-Strasse 1, Tuebingen, Germany

Abstract: Recent developments in cell and gene therapy have a great impact on the new therapeutic approaches in pediatric cancers. Monoclonal antibodies for neuroblastoma and bispecific antibodies for leukemia have induced significant clinical responses for otherwise chemorefractory patients. Moreover, cellular therapeutic approaches including chimeric antigen receptor (CAR) T-cells as well as natural killer (NK) cells have the potential to cure patients with so far incurable malignancies and are the basis for future new therapies for pediatric cancer. Newer generations of cellular therapies, further development and improvement of such new strategies and their earlier use in therapeutic strategies will hopefully allow to significantly reduce the chemotherapeutic burden for children with cancer while increasing the cure rate.

Keywords: Childhood cancer; immunotherapy; monoclonal antibodies; bispecific antibodies; cellular therapy; immunocytokines (ICs); natural killer cells (NK); chimeric antigen receptor (CAR) T cells

Introduction

While adjuvant or neoadjuvant chemotherapy, surgery and radiation are still the major pillars in the treatment of pediatric malignancies, tremendous progress has been made in the treatment of patients who are either refractory or who have relapsed after current standard therapies. Recent developments have fundamentally changed immunotherapeutic approaches to adult patients with cancer, but also to pediatric malignancies. In contrast to adult cancers, the most frequent malignancy in children is acute leukemias, followed by brain tumors, lymphomas, neuroblastoma and various forms of sarcomas. Here, we will review successes, challenges and future perspectives of different immunotherapeutic approaches to the main pediatric malignancies

Hematological malignancies

Acute lymphoblastic leukemia (ALL)

Bispecific anti-CD3/anti-CD19 T-cell engager blinatumomab for B-cell precursor ALL

A major breakthrough in the treatment of refractory B-lineage leukemias was the engineering and clinical application of the single-chain bispecific antibody construct, also named bispecific T-cell engagers (BiTE) (1). The BiTE format consists of two single-chain variable fragments specific for CD3 (expressed on T lymphocytes) and a tumor antigen. Since CD19 is expressed on almost all B-lineage lymphoblastic leukemias, a CD19-specific BiTE has been engineered and has been introduced into the treatment of CD19-positive hematological malignancies (2). The first
clinical application of such an anti-CD3-anti-CD19 BiTE (originally named MT103 and subsequently changed to blinatumomab) was reported in adult patients with CD19-positive lymphomas (3). In this study, an overall response rate of 69% and a median response duration of 13.5 months was demonstrated.

The first three pediatric patients with refractory ALL who received Blinatumomab were treated under a compassionate use approval (4). The first patient was treated at the Children's University Hospital in Tuebingen, Germany. This was a 7-year-old boy diagnosed in 2004 with high-risk B-precursor ALL. After standard chemotherapy for high risk ALL, he experienced a bone marrow relapse. After multiple cycles of chemotherapies, he achieved a second complete remission (CR) and underwent an allogeneic bone marrow transplantation from 9 out of 10 matched unrelated donor. One year post-transplant, he experienced another relapse. Despite multiple cycles of chemotherapy, the patient had persistence of his leukemia. After regulatory approval of a compassionate use application of the CD3xCD19 BiTE, the patient was then treated with MT103 at 15 μg/m²/day for 5 weeks by continuous intravenous infusion. After 2 weeks of treatment, a bone marrow aspiration showed a minimal residual disease (MRD)-negative remission. Two weeks after end of the BiTE treatment, he underwent a haploidentical T-cell depleted stem cell transplantation and as of February 2018, the patient is in continuous CR (own unpublished result). The second patient was treated at the Children’s Hospital of the Charite, Berlin, Germany. This patient had a previous allogeneic bone marrow transplant with a post-transplant relapse. He responded to chemotherapy, but had subsequently a third relapse, which was refractory to a variety of chemotherapeutic agents. At start of 15 μg/m²/day of blinatumomab, he had 90% infiltration of his bone marrow and 23% peripheral blasts. After one day of blinatumomab, the peripheral blood was cleared from blasts, as measured by flow cytometry. The patient became aplastic until day 15 after start of blinatumomab and subsequently showed a complete recovery of donor-derived hematopoiesis. At day 42, he had a normal hematopoiesis and a MRD-negative CR was documented. Unfortunately, the patient was not eligible for a second stem cell transplantation and relapsed a few weeks later. The third patient had a Philadelphia chromosome-positive B-precursor ALL diagnosed in 2001. This patient had multiple relapses and had received two allogeneic bone marrow transplantsations from a human leukocyte antigen (HLA)-identical sibling and an HLA-identical matched unrelated donor, respectively. After the third transplant, the patient remained MRD-positive and received a total of four 4-week courses of 15 μg/m²/day blinatumomab. After this treatment, a bone marrow aspiration showed a MRD-negative CR. However, few weeks later, the patient experienced a relapse in the central nervous system (CNS) and was treated by intrathecal chemotherapy. This clinical observation was an indication, that blinatumomab might not be able to penetrate the CNS at least at the currently used doses. As of February 2018, the patient has another combined BM/CNS relapse and is awaiting experimental therapy.

Besides the impressive MRD-negative responses in these very chemotherapy-refractory patients, it was remarkable that none of the patients developed acute nor chronic graft-versus-host disease (GvHD) despite the fact that the BiTE leads to a significant activation and expansion of donor-derived T-cells with a subsequent impressive graft-versus-leukemia (GvL) effect (4). Based on these observations, another cohort of 6 patients was treated with blinatumomab after they experienced multiple relapses after one or two allogeneic stem cell transplantations (5). Again, GvHD was not seen in these heavily pretreated patients. Another important observation was made in two patients in this cohort. These two patients had a high blast load prior to blinatumomab and did not respond to a first cycle and rather showed progressive disease. The progression could be halted by palliative chemotherapy and the patients then again received blinatumomab with a lower tumor load. Surprisingly, both patients experienced an MRD-negative CR. The side effects of the blinatumomab treatment in these patients were acceptable given their previous intensive anti-leukemic therapies including one or more allogeneic stem cell transplantations. Only two of the patients experienced a cytokine-release syndrome (CRS) grade III/IV. Based on these initial promising observations in the compassionate use of blinatumomab in pediatric patients <18 years of age with chemotherapy-refractory B-cell precursor ALL, an open-label phase I/II study was initiated and 49 patients were treated in phase I and 44 patients in phase II (6). The treatment consisted of multiple courses of blinatumomab given as continuous infusion over 4 and 2 weeks rest between the cycles. The primary endpoints were, to determine the maximum-tolerated dose (MTD)
in the phase I part and to monitor the CR rate after two cycles in the phase II part. Four patients had a dose-limiting toxicity in the first cycle in the phase I part and the MTD was determined to be 15 μg/m²/day. Based on the data, the recommended dose was 5 μg/m²/day for 7 days followed by 15 μg/m²/day thereafter. Of the 70 patients who received the recommended dose, 27 (39%) achieved CR after the first two cycles and 14 (52%) of those patients had an MRD-negative CR. The most frequent adverse events ≥ grade 3 were anemia (36%), thrombocytopenia (21%) and hypokalemia (17%). Three patients and one patient had a grade 3 (4%) and grade 4 (1%) CRS, respectively.

These promising data led to the approval of blinatumomab for the treatment of pediatric patients with Philadelphia chromosome-negative (Ph−) refractory ALL. The restriction to Ph− ALL is based on the availability of alternative treatment strategies with tyrosine-kinase inhibitors (TKIs) for patients with Ph+ ALL, but not on the fact, that these patients do not respond to blinatumomab. In adult patients, blinatumomab showed the same efficacy in Ph+ ALL compared to Ph− ALL and 89% of patients with Ph+ ALL achieved a MRD-negative CR, irrespective of resistance to chemotherapy or therapy with TKIs (7). Various ongoing studies are under way, to evaluate the efficacy of blinatumomab in MRD-positive patients or to incorporate blinatumomab into current standard chemotherapeutic regimens [for review see (8)].

The emergence of CD19-negative ALL blasts has been observed in 10–20% of adult patients who relapsed after therapy with Blinatumomab (9,10). This immune escape seems to be rather due to a disrupted CD19 membrane trafficking than to the outgrowth of CD19-negative progenitor cells or myeloid lineage swift (11). However, a CD19-negative relapse with switch to a myeloid phenotype during therapy with Blinatumomab has been observed in a child with infant leukemia (12).

Attempts have been made to identify patients upfront, who will or will not respond to blinatumomab therapy. Recently, the response to blinatumomab was predicted via the level of regulatory T-cells (Tregs) at start of therapy (13). The Tregs were defined by the co-expression of CD4/CD25 FOXP3. Patients who responded to blinatumomab had a mean of 4.8% Tregs compared to 10.3% in non-responders. A cut-off of 8.5% was able to identify all responders to blinatumomab and excluded 70% of non-responders. In addition, a 1.7-fold upregulation of the programmed cell death protein 1 (PD-1) on the Tregs, was observed in vitro after incubation of the cells with blinatumomab. In another study, a correlation between the expression of the PD-1 ligand (PD-L1) on the ALL blasts and the resistance to blinatumomab treatment was hypothesized (14). The expression of PD-L1 was increased in patients, refractory to blinatumomab treatment and exhaustion markers (PD-1, TIM-3) were significantly higher on patients’ T cells compared to physiologic controls. In addition, in vivo treatment of a patient with blinatumomab resulted in the induction of PD-L1 expression on the initially PD-L1 negative blasts. Based on these observations, a 12-year old patient, who relapsed after two allogeneic stem cell transplantations and who was refractory to blinatumomab and in whom the in vivo upregulation of PD-L1 expression during blinatumomab therapy was observed was treated subsequently with a combination of blinatumomab and the anti-PD1 checkpoint inhibitor pembrolizumab (14). The treatment with this combination was without acute toxicities and GvHD was not seen. However, she experienced an inflammatory response with high fever and increase of inflammatory markers. A bone marrow aspiration at day 34 after start of the combined therapy showed a morphological CR. An increase of PD-L1 expression was also observed in an adult patient with B-precursor ALL resistant to blinatumomab (15). Consequently, a clinical trial is currently conducted in adult patients to evaluate the safety and efficacy in patients with refractory ALL (ClinicalTrials.gov Identifier: NCT03160079). Hopefully, similar studies will also be initiated in the near future in pediatric patients with ALL. Since long-term outcome data in pediatric patients are not yet available, it is currently advisable, that patients proceed to allogeneic stem cell transplantation, if they achieve a MRD-negative response after blinatumomab therapy and if they are clinically eligible for a transplant.

Chimeric antigen receptor (CAR) T-cells

CAR T cells are engineered T cells which are transduced with a CAR that couple single-chain Fv domains of antibodies against surface expressed tumor targets to the T cell receptor and thereby redirecting T cells to tumor cells positive for the corresponding target. Various modifications of the T cell activation signals have been introduced and according to the costimulatory domain, first, second and third generation CARs have been engineered [for review...
see (16). Imai et al. first reported the generation of a novel receptor (anti-CD19-BB-ζ) containing the signaling domain of 4-1BB (CD137) and showed a much higher cytotoxicity of these CAR T cells compared to cells lacking the 4-1BB (17). Based on this construct, researchers at the Children’s Hospital Philadelphia, USA, engineered anti-CD19 CAR T cells and treated two patients with recurrent and refractory ALL (18). Both treated patient achieved MRD-negative remission. One of the patients later relapsed with CD19-negative blasts. In a subsequent phase I study, 30 children with ALL were treated and CR was achieved in 27 (90%) patients (19). The 6-month event-free and overall survival was 67% and 78%, respectively. Expanding CD19 CAR T cells were seen in the 27 responding patients.

In the subsequent phase 2 multicenter study using CD19 CAR T cells (now named tisagenlecleucel) in children and young adults with ALL, 75 patients received an infusion of tisagenlecleucel (20). The overall remission rate within 3 months was 81% and all patients, who responded, had a MRD-negative CR. The rates of event-free and overall survival were 73% and 90%, respectively, at 6 months. Persistence of tisagenlecleucel was observed as long as 20 months. Grade 3 and 4 adverse events occurred in 73% of patients. Neurologic events were observed in 40%, which could be managed by supportive care. A major side effect was CRS, which occurred in 73% of the patients caused by a systemic inflammatory response with T-cell activation and proliferation. CRS is increasingly seen in patients receiving CAR T cells. A CRS grading system and suggestion for the clinical management of CRS has been established (21). Another phase I escalating study was performed in 21 patients with CD19 CAR T cells. The MTD in this study was defined as 1×10⁶/kg CD19 CAR T cells. In 4 of the 21 patients, severe grade 4 CRS occurred. The CR rate was 70% with 12 of 20 patients achieving MRD-negative response (22). An additional phase I study evaluated the outcome of 45 patients, who received CD19 CAR T cells of a defined CD4/CD8 formulation (23). The overall intent-to-treat analysis of MRD-negative remission rate was 93% and 100% in patients who received a lymphodepletion using fludarabine and cyclophosphamide. Twenty-three percent of patients also experienced reversible severe CRS.

Antigen loss has been reported to be a cause of resistance to CD19 targeted therapies (24,25). Therefore, CD22-targeted CAR T cells have been engineered and given to 21 patients (26). Seventeen patients had received prior CD19-directed therapies, among them 15 who received CD19-targeted CAR T cells. In 10 patients, the lymphoblasts were negative for CD19 or expressed CD19 weakly (CD19°-dim), including 9 after CD19 CAR T cell therapy and 1 after treatment with blinatumomab. CRS occurred in 16 of the 21 patients. A robust expansion and persistence of the cells was seen and 12 patients (57%) achieved CR, and 9 of these patients had a MRD-negative CR. Another approach to prevent antigen escape might be the use of bispecific CARs and CD20/CD19 CARs (27) or CD19/CD22 CARs (28), where T cells express two different CARs (dual CAR) or where T cells express two single chains linked in line using the same signaling domain (tandem CARs) (29). Clinical studies are currently performed in pediatric and young adult patients using CD19/CD22 dual CARs (ClinicalTrials.gov Identifier: NCT03330691) and a clinical study is currently ongoing at the Biotherapeutic Department and Hematology Department at the General Hospital Beijing, China (ClinicalTrials.gov Identifier: NCT03398967). In this phase I/II study, the safety allogenic gene-edited dual specificity CD19 and CD20 or CD22 CAR-T in patients with relapsed or refractory hematological malignancies is evaluated.

In summary, the development of CAR T cell therapy for CD19° and/or CD22° ALL has transformed the treatment of relapsed and refractory disease. However, severe toxicity including neurologic adverse events and mainly CRS have to be acknowledged and new approaches to prevent or ameliorate these adverse events will allow in the future to move this effective strategy into upfront treatment strategies, which might hopefully allow to reduce intensive chemotherapy and first promising results have been reported (30). In this case-controlled study in adult patients, CD19-targeted CAR T cell therapy was compared with reinduction chemotherapy and patients receiving CAR therapy had higher rates of remissions and longer survival. Similar studies are warranted in children, thus hopefully reducing heavy chemotherapy exposure and preventing long-term sequelae from chemotherapy. Whether the responses are long lasting in children and whether the patients still should proceed to allogeneic stem cell transplantation after achieving a MRD-negative remission is still not clear and each patient should be discussed on an individual basis.

**Alloreactive natural killer (NK) cells**

Attempts to exploit autologous NK cells in patients showed limited clinical efficacy, the focus of research was
directed toward the use of NK cells from healthy related or unrelated donors in the context of allogeneic cell therapy [for review see (31)]. NK cell function is regulated by an array of receptors transducing either inhibitory or activatory signals (32). Among these receptors, killer immunoglobulin-like receptors (KIRs) are of great importance. KIRs recognize HLA A, B, and C alleles as their ligands. Alloreactivity is defined as the lack of inhibition, when donor NK cells express an inhibitory KIR, for which the respective ligand is absent on the recipient’s leukemic blasts (33). A strong GvL effect was mediated by alloreactive NK cells in adult patients with acute myeloid leukemia (AML) (34) and in children with ALL undergoing T-cell depleted HLA-haploidentical stem cell transplantation (35,36). Activating KIRs have also been identified and two KIR haplotypes can be distinguished: KIR haplotype A, which has a fixed number of genes encoding inhibitory receptors and KIR haplotype B, which has variable gene content and 1 or more activatory genes. Among individuals with haplotype B, a KIR B-content score can be established based on the number of mainly activatory genes. A reduced risk for relapse was seen in patients with AML given an allogeneic stem cell transplantation from unrelated KIR haplotype B donors (37). While this effect was not seen in adult patients with ALL in the unrelated HSCT setting, KIR B haplotype donors conferred a reduced risk for relapse after haploidentical transplantation in children with ALL (38). Moreover, a high KIR B-content score was associated with a significantly reduced risk for relapse. Therefore, careful donor selection plays an important role in allogeneic transplantation of children with ALL. An approach to overcome the inhibition of NK cells, mediated by the engagement of inhibitory receptors with their ligands, is the activation of NK cells via their FCGR3A receptor CD16. This activation overrides the inhibition and leads to augmentation of the NK activity (39). Based on these findings, an Fc-optimized anti-CD19 antibody was constructed and successfully used post-transplant in selected high risk patients with ALL (40).

**AML**

Analogue to blinatumomab, a CD3×CD33 BiTE construct named AMG330 was engineered to target AML (41). This BiTE is very effective in recruiting and activating autologous T cells in preclinical studies (42). Similar to the observation in ALL, the blockade of the PD-1/PD-L1 axis augmented the lysis of AML cells by this BiTE, which might unblocked lead to an immune escape of AML blasts (43). No clinical early phase studies have yet been reported in pediatric patients with refractory AML.

While the depletion of CD33+ blasts and CD33+ normal hematopoietic progenitor with the AMG330 BiTE would only be transient, CAR T cells might persist and lead to a permanent depletion of hematopoietic precursors with subsequent bone marrow aplasia. Currently, no AML-specific antigens have been identified, that might be safely used as targets for the CAR strategy. There are many attempts and numerous strategies to find safe approaches, including gene-editing techniques, antibodies and combination therapies [for review see (44)]. A promising target could be CLL-1, which is expressed on leukemia stem cells, but not in hematopoietic stem cells (HSCs). In a preclinical xenograft model of AML, CAR T cells directed against CLL-1 exerted a strong antileukemic activity, while normal HSCs were not targeted due to the lack of CLL-1 expression (45). Early phase clinical trials using CAR T cells for the treatment of refractory AML in children would be warranted.

**Solid tumors**

**Neuroblastoma**

**Anti-GD2 antibodies**

The disialoganglioside GD2 is highly expressed on almost all neuroblastoma cells and is therefore a suitable target for immunotherapeutic approaches and antibodies against GD2 have been clinically evaluated since the 1980s for the treatment of patients with high-risk neuroblastoma [for review see (46)]. Initially, murine anti-GD2 antibodies m3F8 (47) and murine m14.18 (48) showed promising anti-neuroblastoma activity in vitro and were further developed for clinical applications. The anti-neuroblastoma activity of both antibodies is mediated by human complement-dependent cytotoxicity (CDC) and the antibody-dependent cellular cytotoxicity (ADCC) mediated by Fc-receptor expressing effector cells including NK cells, monocytes and neutrophils. A phase I study with m3F8 showed promising clinical responses in 4 out of 8 patients (49). In six evaluable patients treated with the murine 14.G2a antibody derived from m14.18, a CR was observed in two
patients (50). One of these patients had multiple relapses after autologous and allogeneic stem cell transplantation from an HLA-identical sibling and a CR was induced with the 14.G2a antibody. As of February 2018, this patient is still in remission >27 years after receiving the 14.G2a antibody (Handgretinger, unpublished observation). Common side effects of the m3F8 and 14.G2a were pain, hypotension, fever, urticarial reactions and the induction of human anti-mouse antibodies (HAMAs), which resulted in the inactivation of the antibodies. In order to further exploit and enhance the ADCC activity, clinical studies combining the m3F8 with the granulocyte-macrophage colony-stimulating factor (GM-CSF) were performed and impressive responses were observed (51, 52). In order to augment the ADCC of the 14.G2a antibody, a phase I/IB trial of 14.G2a in combination with Interleukin 2 (IL2) was conducted in 33 patients with refractory neuroblastoma (53). Seven of the 33 patients additionally received GM-CSF. In order to avoid the induction of HAMAs by the murine antibodies, the mouse/human chimeric antibody ch14.18 was developed. This antibody has the variable regions of the 14.G2a and the constant regions of a human IgG1 antibody (54). This chimeric antibody also mediates CDC, but has 50–100-fold higher ADCC activity compared to the 14.G2a antibody. In a first phase I study, 9 patients were treated with 19 courses of ch14.18 at various dose levels. A complete and partial remission was seen in 2 patients each. In contrast to the previous study using the murine 14.G2a antibody, none of the patients developed a HAMA response (55). Based on these initial data, 164 patients, who were treated according to the German neuroblastoma protocol NB97, received at least one cycle of ch14.18 treatment as a consolidation after intensive chemotherapy. The overall survival rate was better after consolidation with ch14.18 compared to no consolidation (56). The Children's Oncology Group (COG) in the US initiated a phase I study of ch14.18 in combination with GM-CSF and IL-2 in patients after autologous bone marrow transplantation (57). Twenty-five patients were enrolled and the MTD was determined to be 25 mg/m²/day for 4 days. It was demonstrated that the addition of IL-2 and GM-CSF was tolerable in the early post-transplant period. Based on these findings, a larger study was performed, which compared in a randomized manner, the treatment with ch14.18, IL-2, GM-CSF and isotretinoin with isotretinoin alone (58). A total of 266 patients were randomized. An interim analysis showed a superiority of the antibody arm to the standard arm with regard to event-free survival (66%±5% vs. 46%±5% at 2 years, P=0.01) and overall survival (86%±4% vs. 75%±5% at 2 years, P=0.02). These results led to the approval of the ch14.18 (then named dinutuximab) by the US Food and Drug Administration (FDA) under the name of Unituxin™ (59). While the American dinutuximab was produced in SP2/0 murine myeloma cells, efforts were made in the SIOPEN study group to evaluate the efficacy of ch14.18 produced in mammalian Chinese hamster ovary (CHO) cells. In a phase I study, 16 patients were treated with ch14.18/CHO (then named dinutuximab-beta) as an 8-hour infusion for 5 consecutive days (60). The dose level of 20 mg/m²/day was confirmed and the side effects were very similar to ch14.18 produced in SP2/0 cells. The results of a phase III study regarding toxicity and outcome in the context of the HR-NBL1/SIOPEN trial were positive (61) and dinutuximab-beta recently received approval by the European Medical Agency (EMA) and is now available under the name of Dinutuximab-beta EUSA.

A major side effect of both ch14.18 antibodies is the induction of neuropathic pain, which may partially be attributed to the complement activation. Therefore, a humanized version of the ch14.18 has been engineered to diminish complement activation by a point mutation in the Fc region of the antibody (hu14.18K332A). In a rat model of allodynia, hu14.18K332A elicited less allodynia than ch14.18 due to a reduced complement activation (62). In a preclinical model, the antibody was still active against neuroblastoma despite the decreased CDC (63). In a phase I study, 38 patients received a median of 2 cycles of hu14.18K332A (64). The MTD and recommended dose for phase II studies was 60 mg/m²/day for 4 days. In a retrospective analysis, hu14.18K332A induced less pain than ch14.18 based on the opioid requirements (65). Further studies are currently evaluating hu14.18K332A in combination with IL-2, GM-CSF and haploidentical NK cells (66) or in combination with chemotherapy and NK cells (67). In parallel, a humanized version of the m3F8 was engineered to enhance ADCC and diminish CDC (68) and initial phase I trials with the hu3F8 showed that the adverse events in monotherapy were similar to m3F8 (69) and that the combination of hu3F8 and GM-CSF is feasible and safe (70). Another strategy to diminish pain is the reduction of the infusion rate. While the ch14.18 is normally infused over an 8-hour period, a 24-hour long-term infusion of 10 mg/m²/day over 10 days has been investigated in 53 patients in combination with IL-2 (71,72).
Immunocytokines (ICs)

Another way to concomitantly use antibodies and cytokines, is the use of ICs. These are monoclonal antibodies with a cytokine linked to the Fc part of the antibody [for review see (73)]. Higher concentrations of the cytokines at the tumor environment should induce an effective immune response, while minimizing the systemic side effects of the cytokine. In preclinical studies, the hu14.18-IL-2 IC was highly effective (74). In early clinical studies, the toxicity profile of the ICs was similar to the ones reported in patients receiving ch14.18 (75) and responses restricted to patients with low burden osteo medullary disease were reported (76).

CAR T cells

CAR T cells have been generated targeting GD2 and first generation CAR T cells were given to patients (77). Interestingly, neuropathic pain was not elicited by the CAR T cells and promising anti-neuroblastoma activity was seen. The CAR T cells persisted up to 192 weeks after injection (77). In a phase I study, patients were treated with a third-generation GD2-CAR. In order to enhance the CAR expansion, lymphodepleting chemotherapy consisting of fludarabine, cyclophosphamide and a PD-1 immune checkpoint inhibitor were concomitantly given (78). In the cohort treated only with CAR T cells, the cells were detectable whereas in the cohort receiving CAR T cells and lymphodepletion, an increase of CAR T expansion by up to 3 logs was observed. Additional PD-1 inhibition in a third cohort did not further enhance expansion or persistence of the CAR T cells. The antitumor response at week 6 was modest, which might be explained by the immunosuppressive tumor microenvironment and, which might prevent the infiltration of the tumor by the CAR T cells. A strategy to improve tumor infiltration and anti-tumor efficacy was evaluated in a preclinical orthotopic xenograft model of neuroblastoma. GD2-CAR T cells were given together with the inhibitor of angiogenesis bevacizumab, which led to a tumor vasculature remodelling and a massive infiltration of the tumors by the CAR T cells (79). The infiltrating CAR T cells produced large amounts of interferon-γ (IFN-γ), which resulted in the upregulation of PD-L1 on tumor cells and PD-1 on the T cells. Therefore, checkpoint inhibitors might further enhance the antitumor effect and clinical studies using this approach are warranted. However, in another human neuroblastoma xenograft model, high affinity GD2-CAR T cells induced fatal encephalitis with CAR T cell infiltration of the brain and neural destruction (80). Therefore, studies using GD2 CAR T cells should be conducted cautiously and should be well-monitored for such adverse events.

NK cells

NK cells and IL-2 activated NK cells can target and lyse neuroblastoma cells either spontaneously or more effectively via ADCC [for review see (81)]. It has been shown that the KIR mediated inhibition by certain HLA antigens can be overcome by activation of the NK cells via their Fc receptor with ch14.18 (82). In addition, the genotypes of KIR receptors, their HLA ligands and of the Fc receptors influenced the response to immunotherapy with the hu14.18-IL-2 IC (83) and KIR3DL1 allelic polymorphisms and HLA-B epitopes modulated the in vivo response to 3F8 and patients with lack of inhibition had a survival advantage (84). Patients’ KIR and KIR-ligand genotypes influenced also the outcome of patients receiving dinutuximab (85). Patients with Fc-gamma-receptor polymorphism 2A and 3A (FCGR2A and -3A) and stimulatory KIR2DS2 genotypes showed a higher ADCC and had an improved survival after treatment with ch14.18/CHO (86). The concomitant use of haploidentical NK cells selected for non-inhibition based on their KIR-HLA interaction is tested in phase I trials with 3F8 (87) and with hu14.18 (86). The safety of in vitro amplified HLA-haploidentical donor immune cell infusions was demonstrated in patients with neuroblastoma (88). It is noteworthy that none of the patients experienced acute nor chronic GvHD. Another approach exploiting haploidentical NK cells is the combination of myeloablative conditioning regimen followed by the transplantation of CD3/CD19-depleted peripheral mobilized stem cell grafts enriched with NK cells and the post-transplant treatment with ch14.18/CHO (ClinicalTrials.gov Identifier: NCT01919866). This study has reached the accrual goal of 60 patients and the preliminary results are promising (89). Similar to CAR T cells, NK CAR cells can also be engineered and be used in the treatment of tumors [for review see (90)]. The human NK leukemia cell line NK92 was engineered to express a GD2-specific CAR, which markedly enhanced the killing of neuroblastoma cells (91).

Other neuroblastoma target antigens

Other antigens highly expressed on neuroblastoma cells are B7-H3 (CD276) (92) and the recently reported expression of the GPC2 oncoprotein as a candidate for an
immunotherapeutic target in neuroblastoma (93). Glypicans are a family of six (GPC1-6) glycosylphosphatidylinositol (GPI)-anchored, extracellular proteoglycan signaling co-receptors, which play different roles in growth factor signaling and cancer cell growth (94). In a preclinical model of disseminated neuroblastoma mouse model with metastases to clinical relevant sites such as spine, skull, legs and pelvis, CAR T cells targeting GPC2 were able to effectively eliminate neuroblastoma cells (95) and GPC2 might be a promising target for further immunotherapeutic approaches. The B7-H3 antigen is also a promising target for NK cells via their ADCC (96) or for CAR T-cells (97). Another promising antigen expressed on neuroblastoma is L1-CAM (CD171), which is recognized by the CE7 monoclonal antibody. In preclinical studies, CD171-directed CAR T cells were generated and the safety was demonstrated in rhesus macaques and the feasibility of generating CD171-directed CAR T cells was assessed in heavily pretreated patients with neuroblastoma (98).

In summary, neuroblastoma is the first pediatric solid tumor, for which immunotherapy has been shown to be effective. It took almost 30 years from the first applications of the murine anti-GD2 antibody until approval and anti-GD2 antibodies are now a component of multimodality therapy for patients with high-risk neuroblastoma. Early phase clinical studies using other immunotherapeutic approaches are being conducted or are in planning phases and it can be expected that further improvements will be made in the future, most likely by combinations of different immunotherapeutic strategies.

**Brain tumors**

The main pillars of therapy are maximal surgical resection, adjuvant radiotherapy and chemotherapy. Despite improvements in outcomes, survivors often suffer long-term toxicities of these treatments including neurological deficits and cognitive disorders, thus new therapies must be evaluated. A major barrier for better strategies is the blood brain barrier (BBB) and chemotherapeutic agents show limited penetration of the BBB. New immunotherapeutic approaches should overcome the BBB. The immune checkpoint inhibitor pembrolizumab is currently being evaluated in children with refractory/progressive high grade glioma, diffuse pontine gliomas (DIPGs) or hypermutated brain tumors (ClinicalTrials.gov Identifier: NCT02359565). Especially patients with hypermutant glioblastoma multiforme, caused by a biallelic germline mismatch repair deficiency, might benefit from the treatment with checkpoint inhibitors (99). Other strategies include cancer vaccines, oncolytic viral therapy and CAR T cells. Glioma associated peptide vaccines have been evaluated in HLA-A2—positive patients with HGGs and DIPGs (100). Thirteen of 21 children had an immune response to the targeted antigens. However, this approach is restricted to HLA-A2-positive patients. Another approach is the use of ex vivo activated autologous dendritic cells loaded with tumor antigen mRNA in patients with medulloblastoma (101) and a clinical study is currently being performed using this approach (ClinicalTrials.gov Identifier: NCT01326104). Oncolytic viral therapy is also currently being evaluated in pediatric phase I studies in patients with recurrent supratentorial tumors (ClinicalTrials.gov Identifier: NCT02457845) (102). CAR T cells have also been demonstrated to exert potent antitumor activity against adult glioblastomas by targeting human epidermal growth factor receptor 2 (HER2) or IL13Ra2 and a dramatic response has been reported in an adult patient with glioblastoma after intracavitary infusion of CAR T cells targeting IL13Ra2 (103). Other approaches comprise phase I studies of adoptive cell therapies in children with posterior fossa tumors by local injection of ex vivo expanded autologous NK cells via an ommaya reservoir (ClinicalTrials.gov Identifier: NCT02271711), or the use of allogeneic T or NK cells in the context of non-myeloablative haploidentical stem cell transplantation in phase II trials (ClinicalTrials.gov Identifier: NCT01804634 and ClinicalTrials.gov Identifier: NCT02100891). These trials are also open for patients with advanced sarcomas.

The BBB remains a significant barrier for effective treatment strategies, but activated T cells can cross this barrier and it is anticipated that CAR T cell based immunotherapeutic strategies will hopefully show efficacy and improve outcome of patients with recurrent/refractory brain tumors.

**Sarcomas**

While progress has been made in the treatment of localized sarcomas, the clinical outcome of disseminated soft tissue sarcomas, such as rhabdomyosarcoma, Ewing's sarcoma, osteosarcoma and others is still dismal (104)
and new therapeutic approaches are urgently needed. Immunotherapeutic approaches comprise antibodies against targets expressed on sarcoma cells, CAR T cells, immune checkpoint inhibitors and their combinations.

**Monoclonal antibodies**

Various antibodies have been and are explored in early phase clinical studies including antibodies directed against insulin growth factor 1 receptor (IGF-1R) (105) or directed against the HER2 (106) with only modest efficacies and no apparent survival benefit. A promising target antigen broadly expressed on almost all pediatric sarcomas is B7-H3 (CD276) (107). The function of B7-H3 is not fully understood, but recent studies support its role in inhibiting T-cell activation, proliferation and cytokine secretion indicating that B7-H3 might function as an immune checkpoint (108). Unfortunately, the receptor of the B7-H3 ligand has not yet been clearly identified, which would lead to a better understanding of the function of this interaction. A humanized Fc-optimized antibody against B7-H3 has been generated and is tested in adult patients with CD276+ neoplasms (109). This MGA271 antibody, also named enoblituzumab, is currently being tested in pediatric patients with solid tumors (ClinicalTrials.gov Identifier: NCT02982941). Another anti-CD276 antibody is the humanized 8H9 antibody, which has in vitro potent antitumor activity (110). This antibody has been used for intrathecal radioimmunotherapy in metastatic CNS neuroblastoma (111) and the radiolabeled 8H9 is currently evaluated in early phase studies in the treatment of refractory, recurrent, or advanced CNS or leptomeningeal cancer (ClinicalTrials.gov Identifier: NCT00089245) and in patients with desmoplastic small round cell tumors and other solid tumors involving the peritoneum (ClinicalTrials.gov Identifier: NCT0109964). Other anti-CD276 chimeric Fc-optimized antibodies and fusion proteins are currently being developed preclinically (own unpublished data).

**ICs**

ICs might be able to overcome the immunosuppressive effect of the tumor microenvironment by the targeted delivery of proinflammatory cytokines, to locally stimulate immune effector cells and anti-CD276-IL-2 or –IL15 ICs are currently being developed and have so far shown impressive antitumor activity in vitro (own unpublished data). A promising IC is NHS-IL-12 (112). Interleukin 12 (IL-12) is a pleiotropic proinflammatory cytokine produced by activated dendritic cells and promotes the differentiation of Th1 T cells (113). It also increases the proliferation and lytic activity of T and NK cells (114). The NHS antibody is directed against necrosis-associated antigens, which are abundantly expressed in tumors, but to a much lesser extent on normal tissue. The necrosis-targeted IL-12 immunocytokine NHS-IL-12 was engineered by genetically fusing two human IL-12 heterodimers to the C-termini of the heavy chains of the NHS76 antibody, which was selected for its specific ability to bind to necrotic regions of tumors in vivo (115). In a humanized mouse model of human rhabdomyosarcoma, it was demonstrated that NHS-IL-12 induced senescence and myogenic differentiation, most likely induced by the local induction of IFN-γ and tumor necrosis factor-α (116). In order to increase the local concentration of IL-12, low dose local tumor irradiation increased the uptake of radiolabeled NHS-IL-12 up to 8-fold in a xenograft model of rhabdomyosarcoma (117) and NHS-12 in combination with local irradiation induced an abscopal effect in vivo in a humanized rhabdomyosarcoma mouse model (118). A clinical study in adult patients with solid tumors and NHS-IL12 alone is currently recruiting (ClinicalTrials.gov Identifier: NCT01417546) and another study is evaluating the safety, tolerability and pharmacokinetics of the checkpoint inhibitor avelumab in combination with NHS-IL12 (ClinicalTrials.gov Identifier: NCT02994953). Similar studies are warranted for pediatric disseminated sarcomas. In a phase IB trial, the IC NHS-IL-2 was combined with radiotherapy in patients with metastatic non-small cell lung cancer following first line chemotherapy in adult patients. This combination was well-tolerated and 2 of 13 patients achieved long-term survival (119). Together with the preclinical data of Eckert et al. (118) in the humanized mouse model of rhabdomyosarcoma, combinations of NHS-12 or NHS-IL2 together with local low-dose irradiation and/or with checkpoint inhibitors are warranted for pediatric patients with sarcomas.

**CARs and TRUCKs**

While CAR T cells have shown exciting results in leukemia, there are numerous challenges in solid tumors, namely (I) to overcome the microenvironment and to increase the migration and infiltration of CAR T cells into solid tumors, bone metastases or the core of solid tumors (120), (II) to overcome the immunosuppressive microenvironment...
encountered in solid tumors (121,122) (III) to avoid off-target tumor toxicity, since most of the tumor-associated antigens are not tumor specific and might also be expressed on normal cells (123) and (IV) to avoid tumor escape mechanism by antigen loss (124). While many preclinical studies for the generation of anti-sarcoma CAR T cells are currently underway, only few clinical studies have been reported in pediatric sarcomas. A phase I/II study was conducted in patients with refractory HER2—positive sarcomas using CAR T cells expressing an HER2 specific receptor (125). Nineteen patients were enrolled at various dose levels. At doses of 1×10⁶/m² or higher, HER2-positive CAR T cells were detected 3 hours after infusion in 14 of 16 patients. The cells persisted for at least 6 weeks in 7 of 9 evaluable patients who received more than 1×10⁶/m² CAR T cells. Of 17 evaluable patients, 4 had stable disease for 12 weeks up to 14 months. In a resected tumor of one patient, ≥90% necrosis was found. The median survival of all 19 infused patients was 10.3 months (range, 5.1 to 29.1 months). Most importantly, no significant toxicity was seen, while a severe fatal adverse event was reported in an adult patient who received a total of 1×10⁹ HER2/neu targeting CAR T cells (126). Therefore, careful dose-escalating studies in pediatric sarcoma patients using other target antigens, such as CD276 or others are warranted.

An interesting approach to overcome the immunosuppressive effect of the tumor microenvironment is the use of TRUCKs (127). TRUCK stands for T Cell Redirected Cytokine Killing and describes the use of CAR T cells as a vehicle to produce and release large amounts of cytokines that accumulate in the solid tumor microenvironment and induce an inflammatory immune response. This strategy is similar to the described NHS-IL2 or –IL12 ICs, but might be more specific due to the CAR receptor. The feasibility of the TRUCK strategy was demonstrated by the local accumulation of IL-12 and T cells redirected by a tumor-targeting CAR and additionally transduced with a CAR-inducible IL-12 cassette, with secretion of IL-12 upon CAR recognition of the tumor antigen (128). More recently, CAR T cells, releasing the proinflammatory cytokine IL-18, have been engineered and augmented cytotoxic activity against advanced solid tumors has been demonstrated (129,130). Future clinical trials should show whether these cells hold their preclinical promises in the treatment of solid tumors (131).

## Checkpoint inhibitors

Few studies have interrogated the efficacy of immune checkpoint inhibitors in pediatric tumors. While impressive responses have been observed in adults with solid tumors, less promising results have been seen in children. In a phase I clinical trial, the CTLA4 checkpoint inhibitor ipilimumab was evaluated in 33 patients with recurrent of progressive solid tumors (132). The toxicity profile was very similar to the one seen in adult patients, but no objective tumor response was observed. A clinical study evaluating the combination of nivolumab (PD-1 blocking antibody) and ipilimumab is ongoing (ClinicalTrials.gov Identifier: NCT02304458), but so far no objective response was seen in the nivolumab alone arm (133). A study evaluating pembrolizumab (anti-PD-1 antibody) showed a response rate of 6.1% (134) and in a study with atezolizumab (PD-L1 antibody), partial responses were seen in some patients with Hodgkin lymphoma. The induction of a tumor-specific T cell response is driven by the mutational landscape and the neo-antigen load of the tumors (135) and adult tumors with microsatellite instability have a very high mutational load and have been proven very sensitive to checkpoint inhibitors (136). Pediatric patients harboring homozygous germline mutations of mismatch repair genes have also a high mutational load (137) and this subgroup of patients might benefit from checkpoint inhibitors (99).

## Conclusions

The benefit of immunotherapeutic strategies has been demonstrated in pediatric leukemia with blinatumomab, CD19-CAR T cells and to some extent with anti-GD2 antibodies in neuroblastoma. Many other immunotherapeutic strategies are currently being developed and evaluated in early phase clinical studies. A list of actively recruiting immunotherapeutic clinical trials is depicted in Table 1.

Compared to the “soluble” hematologic malignancies, solid tumors are a major challenge for immunotherapy due to the microenvironment of solid tumors, which protects tumor cells from immunotherapeutic attacks. New strategies will be necessary to overcome the immune suppressive effects of the tumor microenvironment (Figure 1) and rational combination of different strategies will be necessary in the future to cure pediatric patients, who cannot be cured currently with standard treatments. Much more research and close
## Table 1

Current actively recruiting immunotherapeutic clinical trials in pediatric patients <18 years of age as listed in www.clinicaltrials.gov

<table>
<thead>
<tr>
<th>Targeted malignancy</th>
<th>Target antigen</th>
<th>Immunotherapeutic</th>
<th>Clinical trial recruiting in children</th>
<th>FDA approval status</th>
<th>Year</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19 B-lineage derived</td>
<td>CD19</td>
<td>Fc-optimized mAb</td>
<td>0</td>
<td>Breakthrough status</td>
<td>2017</td>
<td>MOR208</td>
</tr>
<tr>
<td>CD19</td>
<td>Bispecific T cell engager (BiTE\textsuperscript{®}), blinatumomab</td>
<td>6</td>
<td>Yes</td>
<td>2014</td>
<td>Blincyto\textsuperscript{®}</td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td>CAR T cell</td>
<td>37</td>
<td>Yes</td>
<td>2017</td>
<td>Kymriah\textsuperscript{®}</td>
<td></td>
</tr>
<tr>
<td>CD22</td>
<td>CAR T cell</td>
<td>3</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CD19/CD22</td>
<td>CAR T cell</td>
<td>7</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Immune checkpoint inhibitor, ipilimumab</td>
<td>9</td>
<td>Yes</td>
<td>2011</td>
<td>Yervoy\textsuperscript{®}</td>
<td></td>
</tr>
<tr>
<td>Induced self NK cells</td>
<td>3</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloid Leukemia</td>
<td>Induced self NK cells</td>
<td>9</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CD33</td>
<td>Bispecific T cell engager (BiTE\textsuperscript{®}), AMG330</td>
<td>0</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CD33</td>
<td>CAR T cell</td>
<td>5</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>GD2</td>
<td>mAb (chimeric and humanized)</td>
<td>3</td>
<td>Yes</td>
<td>2015</td>
<td>Dinutuximab\textsuperscript{®}</td>
</tr>
<tr>
<td>GD2</td>
<td>Bispecific antibody construct</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>GD2</td>
<td>Immunocytokine Hu14.18-IL2</td>
<td>0</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>GD2</td>
<td>CAR T cell</td>
<td>3</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CD171</td>
<td>CAR T cell</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>GD2</td>
<td>NK CAR (NK92)</td>
<td>0</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>CD276</td>
<td>Fc-optimized mAb, enoblituzumab</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>GD2</td>
<td>mAb</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD276</td>
<td>Fc-optimized mAb, enoblituzumab</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Brain tumors</td>
<td>PD1</td>
<td>Immune checkpoint inhibitor, pembrolizumab</td>
<td>13 (not solely brain tumors)</td>
<td>Yes</td>
<td>2017</td>
<td>Keytruda\textsuperscript{®}</td>
</tr>
<tr>
<td>IL13Ra2</td>
<td>CAR T cell</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Sarcomas, solid tumors</td>
<td>PD1</td>
<td>Immune checkpoint inhibitor, pembrolizumab</td>
<td>See above</td>
<td>See above</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD276</td>
<td>Fc-optimized mAb, enoblituzumab</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>PD1</td>
<td>Immune checkpoint inhibitor, nivolumab</td>
<td>18</td>
<td>Yes</td>
<td>2014</td>
<td>Opdivo\textsuperscript{®}</td>
<td></td>
</tr>
<tr>
<td>Her2</td>
<td>CAR T cell</td>
<td>1</td>
<td>No</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

CAR, chimeric antigen receptor; NK, natural killer.
interaction between researchers and clinicians will hopefully bring us to our final aim, namely to cure every child with cancer.

Acknowledgements

This work was supported from the “Stiftung für krebskranke Kinder Tübingen e.V.”, the Jose-Carreras Leukemia foundation and the “Stefan-Morsch-Stiftung”. We thank Peter Weber for the graphic art.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References


activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. J Immunol 2004;173:7647-53.


