Immunotherapy in pediatric hemato-oncology

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Immunotherapy has progressively acquired an important role in the treatment of children with refractory/resistant hemato-oncological diseases. Its ultimate goal is that of increasing the immunological driven anti-cancer effect without causing further immunological complications. Recent experience has made clear some basic principles in the use of immunotherapy: it has a better success rate when applied in the pre-emptive setting and it should be preceded by lymphodepletion. It is, therefore, reasonable to think that the stem cell transplantation setting could be a perfect match for this kind of therapy. The present review summarizes some of the many different strategies that are currently under pre-clinical and clinical evaluation, mostly in the pediatric field, and hints at their possible application in the allogeneic stem cell transplantation setting.

Learning goals

At the conclusion of this activity, participants should know that:

- immunotherapy refers to a complex group of treatments, which all aim, through different strategies, to increase innate or adaptive immune activity against tumor cells;
- immunotherapy can be applied in the allogeneic transplantation setting with the aim of further boosting the graft-versus-leukemia effect, a role already played by the donor cells in the recipient body;
- immunotherapeutic strategies are nowadays highly experimental. Therefore, it is of the utmost importance that patients receiving some kind of immunotherapy be included in clinical studies to allow clinical and immunological readouts to be correctly evaluated.

Introduction

In the last 30 years, the success rate of treatment for most hemato-oncological disease in the pediatric field has substantially increased. The development of risk-tailored chemotherapeutic protocols for children has resulted in greater success of disease treatment, and the improvement in supportive care has translated into less treatment-related mortality. Nowadays, it is estimated that 76-86% of acute lymphoblastic leukemia (ALL) and 49- 63% of acute myeloid leukemia (AML) affected patients can be cured with front-line conventional treatments.[1,2] Second-line treatment for relapsing or resistant patients often includes stem cell transplantation (SCT). Progress in immunology, supportive care and pre-emptive treatments also allowed a progressive increase in overall survival for transplant recipients, disease relapse remaining the most important limiting factor for higher success rate.[3] The use of SCT for patients with aggressive diseases is based on the statement that immunological surveillance and killing of tumor cells from the transplanted immune-system can exert a powerful and long-lasting antitumor action as compared to conventional chemo- and radiotherapy. Nonetheless, so far, a desirable graft-versus-leukemia (GvL) effect cannot be separated from an unwanted graftversus-host disease (GvHD).[4] This explains why stem cell transplantation can be considered a platform for further immunotherapy in transplant recipients.[5] Donor selection, conditioning regimens, GvHD prophylaxis and stem cell processing before infusion already play an important role in determining the immune-reconstitution of the procedure, and therefore also the possible GvL effect. Nevertheless, over the last 20 years, an ever increasing number of possibilities in the field of immunotherapy have been explored. The proof of principle for the use of immunotherapy after stem cell transplantation was set by Kolb in 1990.[6] By infusing unprocessed donor-derived lymphocytes (DLI) he was able to achieve a clinical response in up to 73% of patients with a chronic myeloid leukemia (CML) relapsed after transplantation. Unfortunately, less promising results were obtained with patients affected with acute leukemias, the response rate of whom, in case of relapse, varied between 3% and 30%.[7] A first attempt to improve survival for relapsed AML/ALL patients using DLI was to use them in a pre-emptive setting in order to anticipate morphological relapse. Minimal residual disease (MRD) and chimerism monitoring set the standard for continuous and frequent post-transplantation monitoring in high-risk patients.[8,9] According to this strategy, a relatively simple immunotherapeutic tool allowed good prognostic results to be achieved in the pre-emptive setting for ALL and AML patients after transplantation.[10-13] The drawbacks of DLI administration, however, remain the reduced efficacy in advanced stage of disease, as well as the risk of GvHD and severe immunological complications which can sometimes be life-threatening.[14,15] Immunologists, therefore, tried to elaborate other strategies to try and split the GvL from the GvHD effect of the infused cells. Furthermore, they of course aimed at producing newer tools that could be effective for a wider cohort of patients, and eventually provide a possible therapeutic option also for more advanced stage of disease. This is an updated review of the different immunotherapeutic tools that are currently being evaluated in ongoing clinical studies (**Table 1**).

Peptide- and cell-based cancer vaccine

Autologous T-cell response against leukemia and other solid tumors has been extensively documented and this has paved the way to the possibility of using a vaccine strategy as anti-cancer therapy.[16] The availability of an increasing number of recognized tumor associated antigens (TAAs) has become the starting point for the development of different peptide-based anti-cancer vaccines, as the isolation of cancer-specific proteins gave hope of a possible patient immunization. In this context, hundreds of TAAs have been evaluated as possible efficacious peptides. The first studies were concentrated on BCR-ABL antigenic epitopes. In CML, it had already been demonstrated through the use of DLI that the immunological action could play a substantial role. Therefore, multiple phase I and II studies evaluated a combination of different peptides, associated to adjuvants and eventually to interferon-gamma. Different studies were almost always able to document an increased specific CD4 T-cell immunity, but clinical results could only be seen at molecular level and not for all patients treated.[17-19] Extensive research into TAAs has involved AML, and among the most targeted peptide for this disease, Wilms Tumor Suppressing Gene-1 (WT1), has emerged to be one of the most promising. Tumor regression could be demonstrated

Table 1. Overview of the currently available immunotherapy strategies.

Immunotherapy strategy	Targets	Advantages	Disadvantages	Further development
Vaccine	CML AML Solid tumors	Possible multi-targeted therapy	Short-lasting efficacy because of development of tolerance	Combined adoptive T-cell transfer with vaccination or immunotransplantation
Natural killer (NK) cell infusion	AML Solid tumors	GvT without GvHD	NK isolation and culture is technically challenging. Restricted pool of alloreactive donors available	NK cell line pre-production
Monoclonal antibody	AML NHL CLL ALL	Specific target	Potential toxicity to be evaluated	Combined bispecific targeted therapy with the BiTe technology
Cytokine induced killer cells (CIK)	AML Solid tumors	GvT without GvHD	Repeated administrations needed, possible tolerance induction mechanism	Gene elaboration of CIK cells through the chimeric antigen receptor technique
Chimeric antigen receptors (CARs)	ALL CLL Solid tumors AML?	No HLA restriction, long-lasting expansion in the host	Production is technically challenging, safety profile needs further testing	Wide variety of possible cells to be engineered (NK, CIK) and antibodies to be targeted

CML: chronic myeloid leukemia; AML: acute myeloid leukemia; NHL: non-Hodgkin's lymphoma; CLL: chronic lymphocytic leukemia; GvT: graft-versus-tumor; GvHD: graft-versus-host disease.

in 12 of 20 patients exhibiting MDS by using a WT1 peptide associated to an adjuvant.[20] Positive findings were also reported in a phase II study by Keilholz and colleagues.[21] PR1 was also considered a good peptide target for vaccine delivery since it is a peptide derived from neutral serin protease that is overexpressed in leukemic progenitor cells as well as in CML and AML blasts. A randomized phase II study with 66 leukemia patients demonstrated a tendency towards a better overall survival and event-free survival for those patients receiving PR1-based vaccine plus adjuvant and chemotherapy as compared to chemotherapy alone.[22] The subsequent testing of a combined WT1 and PR3 vaccine made it evident for the first time that the weak point of peptide vaccine strategy relies on the tolerance which is achieved after subsequent inoculations. The same problem was seen in a phase I study of 10 patients treated with a CD168-based vaccine who progressively developed immunological tolerance.[23] To overcome the problem of a weak and fading immunological signal, cellular vaccines and combined adoptive T-cell transfer and vaccination have been developed. The first cellular vaccines were mostly based on dendritic cells, which can be expanded and loaded with a specific peptide. Dendritic cells are specialized antigen presenting cells (APC) that play a critical role in the adaptive immune response. Clinical responses to APC-based vaccines have been reported in pediatric trials for solid tumors.[24] The combination of adoptive T-cell transfer and vaccination on the other hand, relies on vaccinating the patient, collecting lymphocytes before treatment, and reinfusing them with further vaccination after lymphoreductive chemotherapy. Studies in this direction demonstrated at least a clear immunological response to treatment for myeloma patients with no benefit on overall survival.[25] The vaccination strategy has so far been implemented mostly in the autologous setting. There are convincing immunological reasons to think that the early post-allogeneic transplant setting could be an ideal milieu in which to develop vaccine-based strategies.[26] As a matter of fact, not only the tumor burden is limited after SCT, but also the lymphopenic environment would allow a strong expansion of the transferred T cells. The possibility of pursuing this strategy was demonstrated in 1995, when tumor-specific T cells where induced in a stem cell donor and later transferred to the recipient.[27] Nowadays, such an 'immunotransplantation' model is being implemented in a pre-clinical model of lymphoma at Stanford University.[28] Moreover, a novel allogeneic vaccine trial that utilizes WT1 peptide-loaded dendritic cells generated from healthy SCT donors is being conducted at the National Cancer Institute for children and adults with WT1-expressing hematologic malignancies.[29]

Natural killer cells

Natural killer (NK) cells were first identified in

1975.[30] As part of the innate immunity system, they are able to rapidly react towards infected or transformed cells without MHC restriction. Their cytotoxicity develops through perforin and granzyme B as well as through triggering apoptosis pathways. Through complex activating and inhibiting signals, NK cells are endowed with a spontaneous anti-tumor activity.[31] A possible graft-versus-tumor (GvT) effect through alloreactive NK cells was first illustrated in the studies by Ruggeri and colleagues.[32] In HLA haplotype mismatched hematopoietic transplantation, donor versus recipient NK cell alloreactions are associated with enhanced control of AML and ALL relapse and no risk of graft-versus-host disease, through a complex interaction of activation and inhibition signals, the mechanism of which is beyond the intent of this review.[33] To extend this effect, several attempts have been made to boost the NK-cell response in the allogeneic setting, for example through the administration of purified or interleukin-stimulated donor NK cell products. Rubniz and colleagues proposed the isolated infusion of haploidentical donorderived NK cells following a fludarabine and cyclophosphamyde immunosuppressive cycle, as consolidation therapy for children affected with AML. The feasibility and safety of this approach was successfully tested in 10 patients, who also demonstrated an in vivo expansion of the infused cells.[34] In the transplantation setting, however, the simple isolation and reinfusion of NK cells from the donor did not result in a superior outcome in a cohort of haplo-identical transplanted patients. Therefore, different NK-cell expansion protocols have been developed, and these were able to increase the NK cell activity through cytokine stimulation. Though more active, these NK cells are very difficult to expand, are unstable, and need to be strictly depleted from other T cells to avoid risk of GvHD.[35,36] This results in a very expensive and long expansion procedure. To overcome these difficulties, permanent NK-cell lines have been developed under good manufacturing practice (GMP) conditions and are currently being tested in different protocols.[37] NK-cell lines also represent an optimal target for genetic modification to enhance cytotoxic potential.[38]

Antibodies

Since the discovery of hybridoma technology by Kohler and Milstein in 1975, the availability of monoclonal antibodies (mAbs) has continued to increase. mAbs targeting cell clusters of differentiation (CD) today represent a potential targeted therapy for several malignancies. MoAbs can kill cancer cells by means of direct and indirect pathways. Specific antibody-receptor binding can directly cause apoptosis through intracellular signaling. Indirect killing can occur by complement-activation, antibody-dependent cellular cytotoxicity (ADCC), complement-dependant cytotoxicity (CDC) or cell-mediated cytokine release.[39] The first mAbs to receive approval from the Food and Dug Administration (FDA) for clinical purpose was anti-CD20 rituximab in 1997. Its mechanisms of action include inhibition of B-cell proliferation, ADCC, CDC and possible induction of apoptosis. This drug now represents a consolidated treatment, in combination with chemotherapy, for CD20+ non-Hodgkin's lymphomas (NHL). Around 30% of adults and 48% of children with B-lymphoblastic ALL also express CD2040, the upregulation of which has been demonstrated in resistant blasts.[41] Based on these findings, rituximab has been studied in patients with de novo Philadelphia chromosome negative, precursor B-lineage, CD20+ ALL in combination with hyper-CVAD regimen (fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone). Among patients under 60 years of age, those who received rituximab had a statistically significant improvement in 3- year overall survival (75% vs. 47%; P=0.03).[42] Alemtuzumab targets anti-CD52 positive cells. Its use has been widely explored in the transplantation setting, as part of the preparatory regimen and anti-GvHD prophylaxis. It mediates ADCC, and induces apoptosis of tumor cells. Alemtuzumab is officially approved for the treatment of adults with B-cell chronic lymphocytic leukemia (CLL). This drug has also been studied in combination with chemotherapy in adult and pediatric patients with relapsed ALL. A phase II study was conducted within the Children Oncology Group for the treatment of relapsed pediatric ALL. Only one of 13 patients showed a complete response to single-agent treatment.[43] The most important drawback in its use of alemtuzumab is the high risk of toxicity and infectious complications that it causes.[44] Epratuzumab targets the extracellular domain of CD22, an antigen expressed in over 95% of pediatric B-lymphoblastic ALL.45 Its proposed mechanisms of action include ADCC, CD22 phosphorylation and the inhibition

of cell proliferation. Epratuzumab was first studied in adults with indolent and aggressive B-NHL, displaying good safety and efficacy. Epratuzumab alone and in association with chemotherapy for CD22+ ALL with first or subsequent relapse was tested in a pediatric cohort of patients. While the overall remission rate did not differ from historical controls, the MRD negative rate was better for the patients receiving epratuzumab (42% vs. 25% MRD-negative P<0.01%).[46] The safety profile of the drug was confirmed. Blinatumomab is a bi-specific antibody that binds CD19 and CD3. Its function is to attract CD3+ cytotoxic T cells to CD19-expressing leukemic blasts with a so-called BiTE (bi-specific T-cell engagers). Blinatumomab was first tested in adults with relapsed/refractory B-NHL and B-lymphoblastic ALL. A recent update of an ongoing German multicenter trial documented a high rate of clinical and molecular response (67%).[47] Though concerns remain about the toxicity of this drug, its promising efficacy has led to the opening of a pediatric study for relapsed/resistant ALL in Europe as well as in the USA. In the field of AML malignancies, the availability of antibodies has not so far been so extensive, especially after the official withdrawal of gemtuzumab ozogamicin from the market due to increased fatal infectious events. However, some promising tools are being tested in the pre-clinical and early clinical phase. A promising 3rd generation antibody with improved capability to recruit Fc receptor-bearing effector cells has been created against CD135, an antigen often expressed in AML blasts.[48] Moreover, the same BiTE technology used with blinatumomab is now being applied to an anti-CD33/CD3 double antibody, which could potentially have a high impact on the treatment of relapsed/refractory AML.[49] It is worth adding that antibodies can often improve their efficacy by being combined either with radioisothops or with chemotherapy/toxins. Among the combinations that are now undergoing clinical trials, gemtuzumab ozogamicin has been confirmed as an innovative anti-AML treatment. Its combination of anti-CD33 and calicheamicin demonstrated its activity in relapsed adult and pediatric AML even as single agent therapy (30% remission rate).[50,51] However, the high toxicity profile (myelosuppression, systemic infections, transaminitis, veno-occlusive disease) led to its withdrawal from the market in 2010. A recent detailed review about the use of gemtuzumab ozogamicin in the treatment of adult AML has, however, advocated the re-introduction of this drug in combination with cytarabine and anthracycline for the treatment of patients with a favorable cytogenetic profile on the bases of five reported randomized studies that suggested an overall survival benefit for this subgroup of patients.[52] Conjugated anti-CD22 antibodies have also been developed. Inotuzumab ozogamicin (anti-CD22 plus calicheamicin), BL22 and moxetumomab pasudotox (both anti-CD22 combined with Pseudomonas exotoxin A) have begun tests in clinical trials. The results seem promising, but the number of pediatric patients treated is so far too small to allow conclusions to be drawn.[53-55] At the present time, there are very studies involving antibodies combined to radioactive isotopes in the pediatric population, and these are mostly feasibility phase studies. In the adult setting, radioisotope-antibody conjugates directed against surface markers of leukemia cells (CD33, CD45) are available for routine clinical use. These agents concentrate in the bone marrow, generating a severe myelosuppression. Given as an adjunct to TBI, no increased side effects were observed.

Cytokine-induced killer cells

Cytokine-induced killer (CIK) cells are ex vivo expanded T lymphocytes (CD3+) that share a natural killer (NK) phenotype (CD56+). CIK cells display a high antileukemic activity, independently of MHC restriction while having negligible alloreactive potential. They can kill a broad array of tumor targets, including hematologic and solid malignancies. In this way, cell-cell interaction is mediated via TNF-related apoptosis-inducing ligand (TRAIL) on CIK cells and death receptors on tumor targets (**Figure 1A and B**) that results in an activation of the caspase cascade enrolling the intrinsic apoptotic pathway. But the molecule that probably plays the most important role in CIK cell-mediated killing, as shown by blocking experiments (**Figure 1C**), is the NKG2D receptor, which is an activating NK-cell receptor. The ligands of this receptor known so far are relatively restricted to tumor cells. However, the NKG2D only mediates the interaction between CIK cells and tumor targets while the final execution of apoptosis is mediated via a perforin and granzyme release (**Figure 1A**). CIK cells can be expanded from peripheral blood, from cord blood, and also from washout of

leftover mononuclear cells from cord blood unit bags.[56] One of their hallmarks is that they can be easily produced under GMP conditions through different cytokine protocols, some of which only require ten days of expansion before harvesting.[57] In the allogeneic setting, these cells have been tested in 3 different clinical trials. All of them included adult patients who had relapsed from hematologic malignancies after stem cell transplantation. All of them showed a good safety profile, with only a few GvHD cases and no severe toxicity. In all studies, a clinical transient response of the disease could be observed in 30-50% of the patients treated.[58-60] These trials therefore, suggest a true activity of CIK cells in hematologic malignancies, but also underline the absence of long-lasting efficacy, thus questioning possible resistance mechanisms developed by the target cells. Interestingly, the rate of immunological complications (GvHD) for patients receiving those cells is low, and this holds true even when those cells are applied to haplo-identical settings (P Bader, personal communication, 2012). Moreover, Introna and colleagues demonstrated that these cells retain a dual function, being active both as CD8-specific effector T and NK cells. In the posttransplantation setting, this would allow their use not only as cancer specific treatment, but also in the treatment of lifethreatening viral reactivation.[61]

Anti-tumor cytotoxic T cells

After the first attempt at infusing donor-derived lymphocytes or specific T lymphocytes in relapsing recipients, the first real documentation of specific T-cell production was linked to anti-viral treatment. Riddel was the first to produce clinical grade cytotoxic T cells,[62] followed by Rooney and Heslop, who developed EBV-specific T cells and even proved their activity against posttransplant lympho-proliferative disease (PTLD).[63] In the same era, Rosenberg was attempting to expand T cells from tumor mass and re-infusing them to patients affected with metastatic melanoma, obtaining only an occasional response.[64] As the experience with T-cell production grew, a number of factors presented themselves and had to be considered. First of all, it was made clear that anti-viral immunity could only be delivered if both CD4 and CD8 cells were expanded, a lympho-depleted setting was necessary to achieve cell expansion, central memory T cells represented a better cell population as compared to effector T cells as they could expand better. Moreover, it became clear that, when elaborating possible anti-tumor specific T cells, a variety of means had to be used to take into account the possibility of tumor escape. Last but not least, the production system evolved so as to allow GMP manipulation of cells. In general, it has also become clear that targeting tumor cells with T cells was more challenging than targeting virus,

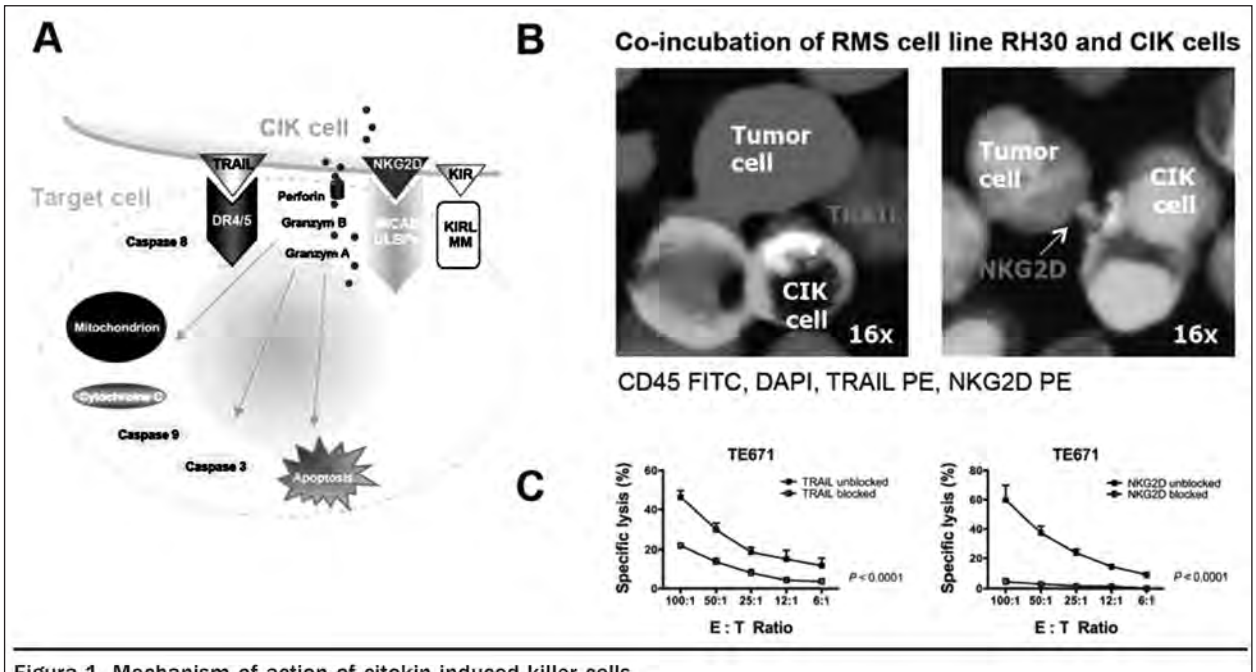

Figura 1. Mechanism of action of citokin induced killer cells.

first of all because of their immunological escape capability, and because T-cell therapy could only address residual tumor mass, being insufficient to treat overt relapses.[65] The first technique which was used to overcome tumor escaping was to elaborate multitumor-specific or gene-modified T cells, the construction of which could be one of the next clinical achievements.[66] Moreover, combining multitumor specific T cells with demethylating agents or T-cell activators or with proliferative stimuli could also be considered a further evolution of this technique.[67-69]

Chimeric antigen receptors

Chimeric antigen receptors (CARs) have been developed through advanced gene-transfer technology in order to overcome HLA restriction limitation of conventional Tcell therapy. Through genetic reprogramming, immune effector cells can be redirected to target antigens expressed by leukemic cells. In most clinical applications to date, a patient's own T cells may be reprogrammed to express these tumor-specific receptors, largely minimizing the potential immunological risks. CARs are composed of an Ag-specific binding domain (most commonly a singlechain variable fragment derived from the fused variable heavy- and light-chain domains of a tumor-targeted mAb) fused to a transmembrane domain followed by one or more cytoplasmic signaling domains. The evolution of CARs from 1st to 3rd generation has progressively combined activating to co-stimulatory signaling domain, so as to achieve not only T-cell activation, but also T-cell expansion on long-lasting antigen exposure and, therefore, Tcell persistence. Nowadays, Cars are designed to recognize several surface antigens, and more than 30 phase I trials are ongoing in the field of hemato-oncology and solid tumors. An elegant review by Davila and colleagues has recently analyzed the results of the first 28 patients treated at 5 different clinical centers with CARs-based protocols.[70] These studies presented several differences in terms of treated disease, stage of disease, type of CARs used, gene technology used, and number of infused T cells, making a direct comparison of the obtained results inappropriate. However, the overall consideration that can be derived from these studies is that anti CD19 CARs have shown some degree of clinical activity in patients affected with CLL and lymphomas. Tumor burden was directly related to the degree of response shown by these patients even if a high tumor burden did not prevent some degree of clinical response. The use of lymphodepletion before CARs infusion proved to be fundamental, whereas the number of infused T cells was not shown to have a great impact on outcome. In all the clinical studies, a number of acute reactions were associated to CARs infusion but no lethal complication was observed, producing reassuring results on the safety of these products. Most recently, very promising clinical results were reported at the ASH meeting in Atlanta 2012 by Carl June and colleagues. A 7-year girl affected with relapsed refractory ALL received autologous CART19
after chemotherapy. The cells where transduced with a lentivirus encoding CD-19 scFv linked to 4-1BB and CD3-z signaling domains. CART19 were documented in the girl's bone marrow as well as in her central nervous system on Day +23 after infusion; the maximal expansion of CART cells occurred on Day +11 after infusion. The treated child achieved a complete morphological and molecular remission of the disease and this was maintained at a 4-month follow up, with stable levels of CAR+CD3 cells in peripheral blood as well as in bone marrow. Notably, the girl displayed a severe cytokine release syndrome (CRS) which required admission to the intensive care unit and respiratory support, and which was successfully treated with IL-6 inhibitor and steroid. Another 9 adult patients affected with relapsed refractory CLL were treated with the same CAR cells (3 of them have already been reported in the review by Davila and colleagues70) and 4 of them achieved a complete remission at a median follow up of 5.6 months. All responding patients developed a mild to moderate CRS, which temporally always correlated with the peak of T-cell expansion in peripheral blood.[71] While the pioneering centers for the development of CARs try to set up common standard criteria and evaluation tools to be able to perform comparable clinical evaluations, other pre-clinical studies are reaching out to new possible CARs targets. In AML, for example, Marin et al. developed a 3rd generation CAR complexing a CD33-specific CAR with CD28 and OX-40 co-stimulatory signaling. The study was able to show that cytokine-induced killer cells inherited increased proliferative, migratory, and lytic functions at a variety of leukemic cell lines.[72] A further application of this technique involved the targeting of CD123 and this is being developed by the same group.[73]

Conclusion

The range of immunotherapeutic strategies under development to address relapsed or resistant leukemia is steadily increasing, and the ultimate goal of achieving clinical success for high-risk patients may be closer. As far as published reports show, these techniques have an adequate safety profile as referred to the high-risk patients in which they need to be used. It is possible that different techniques will emerge and will prove to be more advantageous for different diseases at different time points, and it is not to be excluded that some of the described techniques will be fused and combined to achieve better results. In such an ample repertoire of possible new therapies, it is of the utmost importance that all the patients treated in predefined protocols are included in results analysis according to preset end points.

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