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Review Article

Adoptive Cell Therapy in Pediatric Leukemia

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Abstract

Acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) are the two common types of pediatric leukemia. Despite conventional therapy, treatment failure and poor survival are observed in children with leukemia. Adoptive cell therapy needs to get more advanced to overcome high-risk pediatric leukemia. Dendritic Cells and cytokines are two influential factors in natural killer (NK) cell therapy. However, no defined effect of killer-cell immunoglobulin-like receptor (KIR) on NK cells has been obtained. Moreover, a combination of checkpoint fusion protein with chimeric antigen receptor (CAR) T-cell therapy can highly improve the anti-tumor function of T cells. Biomarkers, namely serum cytokines, MicroRNAs (miRs), ADAM6, CD200 and CD123, sGRP78 and CXCR4, and Semaphorin 4D (Sema4D) are helpful in finding patients with a risk of relapse, and an appropriate treatment approach, or act as a potential targetable marker. In this review, the clinical and preclinical/animal studies with the purpose of diagnosis and treatment of relapsed or refractory pediatric leukemia are discussed. Preclinical/animal ACT studies have shown improvements in the treatment of children with high-risk leukemia. However, clinical studies are required to verify the efficacy of these approaches for the treatment of childhood leukemia.

Keywords: Adoptive Cell Therapy; Biomarker; CAR T-Cell Therapy; NK-Cell Therapy, Pediatric Leukemia

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Introduction

The two common types of leukemia in pediatrics include acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). ALL comprises 80% of leukemia cases in children, and AML accounts for 15% (1). Despite immense efforts to improve outcomes over the last decades, new data shows conventional therapy failure in 10-20% of diagnosed cases (2). Survival in children with ALL relapse is approximately 15%, which is a very low rate, and the cure rate in children with AML is 60% despite intensive chemotherapy (3, 4). We can conclude that traditional treatment regimens involving chemotherapy followed by allogeneic hematopoietic stem-cell transplant (HSCT) are unable to cure or achieve relapse-free survival in a relatively large proportion of leukemic children. All in all, the

mentioned points have prompted attempts to develop and apply novel therapeutic approaches in pediatric leukemia. Improving insight into the genetic and epigenetic heterogeneity of pediatric leukemia has resulted in providing novel targeted therapies. Immunogenic target-structures provide the ability to perform a more precise risk assessment and evaluation of the disease course (5). Targeted/multiple targeted-immunotherapy in leukemia imposes less toxicity to normal tissue than conventional chemotherapy (4). It helps in choosing a specified treatment for each patient by implementing of personalized medical approach considering the exclusive molecular features of each tumor (6, 7). Targeted- immunotherapy has various functions, such as inhibiting tumor cells to escape the immune attack, molecular complex aiming cellular metabolism-dependent enzymes (8) and targeting leukemogenesis pathway enzymes (9). Natural killer (NK) cells are the main members of adoptive cell therapy that can be derived from allogeneic donors and haploidentical stem cell transplantation(10). Additionally, NK cells are part of the innate immune system and play the role of effector lymphocytes to identify changed cells either by viruses or by cancer and make them be lysed without prior sensitization (11). Chimeric antigen receptor (CAR) T-cell therapy is another form of adoptive cell therapy and targeted immunotherapy, in which genetically modified autologous T-cells code for an antigen recognition receptor to attack leukemic cells and kill them by cytotoxicity (12, 13). Over time, enhanced recognition of biomarkers has led to earlier diagnosis, better risk stratification, more appropriate targeted therapy selection, improvement in survival estimation, and improved monitoring of the disease course. This review demonstrates new achievements in immunotherapy of pediatric leukemia, including adoptive cell therapy by involvement of natural killer (NK) cell therapy and CAR T-cell therapy. It also discusses biomarkers predicting the outcome of leukemic children.

Adoptive Cell Therapy in Pediatric Leukemia

Adoptive cell therapy (ACT) is a personalized cancer treatment involving administration of immune cells with anticancer behavior in the cancer patients(14). This anti-leukemia role is performed by the graft-versus-leukemia (GVL) effect, mediated by donor-T / NK cells (15). The types of ACT are described below.

Natural Killer Cell Therapy

NK cells are able to play an anti-cancer role by recognizing molecular structures expressed on neoplastic cells (16). This recognition is maintained through the balance between signals triggered by NK cells' surface receptors that are responsible for activating and inhibitory responses (17, 18). In order to prevent post-alloHSCT relapse, activation/expansion of donor NK cells should happen as described below.

Effects of Blood Dendritic Cells (DCs) on NK Cells

The cDCs help the immune system in defeating infection by secreting pro-inflammatory cytokines and improving T helper 1 response and T cell cytotoxicity. On the other hand, pDCs perform an anti-viral role in immunity by producing type I interferons (INFs) to improve innate and adaptive immunity (19). There are two major subsets of DCs in human blood: conventional (cDCs) and plasmacytoid (pDCs)(20). Ex-vivo-generated monocyte-derived DCs have been employed as vaccines in some studies. It has been shown that this vaccine is potent and tolerable but has limited clinical response when used for cancer patients (21). Therefore, a clinically applicable culture protocol was developed that was able to produce high numbers of ex vivo-generated DC subsets from donor-derived $CD34^{+}$ hematopoietic progenitor cells (HPCs). These DCs showed similar functions and phenotypic features with their in vivo blood counterparts; they secreted TNF-α and IL-12 and promoted T helper-1 and cytotoxic T lymphocytes, and strongly cross-presented antigens. These DC subsets were able to boost anti-tumor T cell functions as well as to improve NK cell activation in alloHSCT patients (22).

Effects of Cytokines

Insufficient primary efficacy of donor NK cells is due to a lack of antigen specificity and incomplete expansion and persistence of alloreactive NK cells. Therefore, one of the most important challenges in NK cell therapy is in vivo durable

proliferation and effector functions of donor NK cells (23). Studies have shown that it is possible to produce in vitro human memory-like NK cells through activation with certain cytokines (24). The murine in vitro-generated memory-like NK cells that were infused post-alloHSCT displayed GVL effect and reduced GVHD (25). In one human phase I study, allogenic memory-like NK cells infusion upon chemotherapy, in order to improve engraftment of alloHSCT led to limited toxicity and complete remission of AML in adult patients (26). In a murine study, Tanzi *et al.* described the best experimental settings to acquire high numbers of durable memory-like NK cells. This clinically applicable method provided alloreactive memory-like NK cells with modest activity against non-malignant cells and potent anti-tumor activity against leukemia blasts or autologous cancerous cells in patients with solid tumors (27).

Effects of *KIR* Genotype

NK cells play their immune role (cytotoxicity and cytokine secretion) based on a type of NK cell receptor named killer-cell immunoglobulin-like receptor (KIR) (28). These receptors are encoded by *KIR* gene cluster that includes 15 genes on chromosome 19. There is high heterogeneity in the expression of these genes (29). Based on the content of *KIR* genes, there are two haplotypes A or B in donors and recipients. The haplotype A gene content (overall up to 8 genes) is fixed and includes inhibitory genes except *KIR2DS4*. The haplotype B contains 1 or more activating genes, *KIR2DS1/2/3/5, KIR3DS1*, and inhibitory genes *KIR2DL5A/B, KIR2DL2, KIR3DP1,* and *KIR2DL4* (30, 31).

The analysis of *KIR* genotype and haplotype identified in children treated for ALL showed that *KIR* genes do not affect the pathogenesis of ALL significantly. Moreover, it has been proposed that higher accumulation of activating *KIR* genes might be a risk factor for ALL (32). Assessment outcomes of alloHSCT in children with ALL and AML while considering KIR alloreactivity and donor *KIR* gene content showed that none of them were significantly associated with relapse or disease-free survival in those patients. Furthermore, the findings of this study do not support selection of alloHSCT donors based on KIR in children with leukemia (33).

Chimeric Antigen Receptor (CAR) T-cell Therapy

CAR T-cell therapy seems to be the most effective treatment approach in pediatric leukemia. In CAR T-cell therapy, autologous T cells are attained through apheresis from a patient, then they are introduced with a gene encoding for an antigen recognition receptor. These ex vivo modified cells potentially express a single chain variable fragment (scFv) from an antibody that is able to merge into T cell costimulatory domain after being transfused back into the patient. These modified T cells can eliminate malignant cells (13, 34).

Checkpoint Fusion Proteins

Despite substantial responses upon CAR T-cell therapy in patients affected by ALL and B-cell lymphoma (35-37), risk of relapse remains 40-60% in the course of disease (36, 38). It seems that malignant cells are able to escape the immune system attack. To do this, tumor cells redirect immune checkpoints. Immune checkpoints in T cells are responsible for balancing between activation and inhibitory responses in order to effectively control infections and inhibit autoimmunity (39). Based on this physiologic phenomenon, the blockade of the immune checkpoint leads to reactivating and redirecting antitumor T cells (40). T cell immunoglobulin and mucin-containing protein 3 (TIM-3) as a transmembrane protein and member of TIM family proteins is expressed both on activated T cells and other immune cell types like NK cells, myeloid cells, and regulatory T cells. TIM-3 ligands include galectin-9, high mobility group box protein 1 (HMGB1), phosphatidylserine, and CEA cell adhesion molecule 1 (CEACAM1). CEACAM1, as a membrane protein, is expressed on T cells, other immune cells, and tumor cells like melanoma. It has been shown that expression of TIM-3 on T-cells in pediatric patients affected by ALL results in poor prognosis. Moreover, TIM-3 overexpression may decrease T cell anti-leukemia responses (41). Finally, checkpoint axes blockade on tumor-specific T cells has led to promising results, especially when combined with CAR T-cells to avoid systemic side effects. In this regard, checkpoint fusion proteins like TIM-3-CD28 protein have been developed. This synthetic fusion protein is able to transform tumor cells' inhibitory signals to stimulatory ones

through extracellular inhibitory TIM-3 receptors fused to the intracellular stimulatory domain of the CD-28 molecule. Additionally, combination of checkpoint fusion protein with anti-CD19 CAR T-cells highly improves T cell expansion alongside with ability to eliminate malignant cells by overcoming inhibitory signals (42).

Relapsed or Refractory Extramedullary Leukemia

CAR T cells have been broadly and successfully implemented for the treatment of patients with refractory and multiply relapsed B cell precursor ALL (B-ALL). The most extramedullary (EM) relapses occur in testicles and central nervous system (CNS), approximately 5% and 20% of total relapses, respectively (43, 44). It appears that CAR T-cells are able to traffic to EM disease sites and kill malignant cells (45-48). It has been found that the rate of CAR T-cell related toxicities in a small subgroup of adult patients with EM leukemia who had undergone CAR T-cell therapy was high (49). Children with CNS relapse have mostly been excluded from pediatric investigation due to concerns for high risk of neurotoxicity. In a case series of seven child patients with relapsed or refractory B-ALL and isolated EM relapse, all seven patients reached complete remission by a CD19-directed CAR T-cell therapy. Moreover, this treatment has been followed by acceptable short-term remission and the least CNS toxicity. To reach more accurate results, a longer follow-up duration is needed, and additionally larger patient population with isolated EM should be involved in prospective studies (50).

Biomarkers

To improve patient outcomes, the early diagnosis of malignancies by utilization of highly advanced molecular techniques is necessary. Biomarkers can provide exact data on patient disease status, prognosis, and cancer type.

Serum Cytokines Level can Predict the Prognosis of Children with Leukemia after alloHST

AlloHSCT in leukemic patients may fail due to relapse or transplant-related complications following immune dysregulation or uncontrolled cytokine secretion (51-59). Almeida *et al.* have demonstrated that high levels of soluble human leukocyte antigen-G (sHLA-G) by leukemic cells can lead to relapse (59). On the other hand, Deschaseaux *et al.* have shown that a high level of sHLA-G may constrain transplant complications post-alloHSCT (54). In healthy subjects, the proinflammatory and anti-inflammatory cytokines cause a balance between hematopoietic stem cells and lineage-specific cells, whereas this balance is interrupted in a leukemic state. Furthermore, HLA-G expression in leukemic cells helps in their over-proliferation and also enables them to escape NK cells (60, 61). Finally, investigation of pro-inflammatory cytokines (interleukin [IL]-1, IL-2, IL-6, Tumor necrosis factor [TNF]-α) and anti-inflammatory cytokines (IL-4, IL-10) and sHLA-G in pediatric leukemia patients showed that their levels were high at diagnosis time and post-transplantation period. Increased levels of IL-4, IL-10, and/or sHLA-G at diagnosis time (specifically in ALL) and at post-transplantation period, led to increased post-transplant relapse, and high levels of IL-2 and TNF-α on the day of transplantation resulted in lower survival rates. Therefore, measurement of serum cytokines and sHLA-G may be effective in predicting survival and relapse in pediatric leukemia patients after alloHSCT (62).

MicroRNAs (miRs), the Novel Biomarker for Monitoring CNS Involvement in Pediatric ALL

Most of the pediatric EM leukemia relapses occur in CNS (approximately 20% of total relapses) (44), and it seems that CNS leukemic involvement is due to therapeutic failure (63). In this regard, finding a sensitive method to follow up CNS leukemia is crucial. Some biomarkers such as MicroRNAs (miRs) seem to be effective in diagnosing CNS leukemia as initial or relapsing disease (64). MiR is a member of highly conserved, non-coding and small RNAs that play pivotal role in regulation of some biological processes such as proliferation, survival and differentiation (65). The expression patterns of miRs change during cancer progression (66). Human cells routinely release lots of vesicles in extracellular space. These extracellular vesicles (EVs) include RNAs, proteins and lipids (67). Analysis of miR as a biomarker, was done on CSF, bone marrow (BM) and peripheral Sblood (PB) samples in pediatric

patients with acute leukemia. The expression level of miRs in the CSF of patients with CNS leukemia was considerably higher than those without the condition. Similarly, an apparent difference was found between density of EVs in the CSF samples of leukemia patients with CNS involvement and those lacking meningeal involvement. Eventually, measurement of miR as a novel liquid biomarker might be employed to monitor CNS involvement in pediatric ALL (64).

ADAM6 Might Provide Insights into the Progression of BCP-ALL in Children

Some biologically aggressive ALL cases are detected in children under one year old (68). In addition, 60% of ALL cases are identified under the age of 20 years (69, 70). Evidently, pediatric leukemia is a major health problem that leads to childhood mortality and imposes disease burden on children and young adults (71, 72). In B-cell precursor (BCP)-ALL patients, due to chromosomal alteration and chimeric gene organization, different genetic subtypes emerge (73). Therefore, ALL generally and BCP-ALL specifically include a repository of biomarkers. In this regard, ADAM6 as a potential genetic biomarker, helps in early detection, risk stratification and improvement of the prognosis of pediatric BCP-ALL and finally improvement of personalized medicine. It has been found that in BCP-ALL patients, homozygous deletion (HOM:DEL) of *ADAM6* gene is associated with poor 10-year survival. Moreover, *ADAM6* HOM:DEL is related to significant overexpression of MIR-574/3P. Overall, relapse in patients with *ADAM6* HOM:DEL occurs more in CNS. *ADAM6*, as a novel biomarker, might provide insight into the development and progression of BCP-ALL (74).

CD200 and CD123: Two Determinant Factors in the Clinical Course of Children AML

Pediatric AML is widely different in genotype, phenotype and epigenetic, which are influential factors in patient response to treatment approaches (75). In spite of risk stratification and complete remission in patients with AML, relapse and treatment failure happen in some patients (75). Therefore, following identification of influential factors in leukemic cells, new strategies might be considered (76). CD200, a membranous glycoprotein, is involved in some hematological malignancies and is normally expressed in different cell types (77). CD200 down regulates immune system through expansion of regulatory T cells (Treg) by binding to CD200R (CD200 receptor) that eventually leads to immune evasion and leukemia progression (78, 79). CD123 (interleukin (IL)-3 receptor) involves in different biological functions as immunity, inflammatory responses, and control of hematopoiesis in normal or malignant subjects (80). CD123 is assumed to be a promising marker for detection of minimal residual disease (MRD) in AML patients.

It has been shown that CD200 and CD123 overexpression, alone or together, could play a negative role in clinical presentation and treatment outcome of pediatric patients with AML. The CD200 overexpression could result in MRD and lymphadenopathy. In addition, CD123 expression might lead to MRD, lymphadenopathy, and low platelet count. Also, co-expression of CD200 and CD123 has been accompanied by adverse cytogenetic karyotypes and high total leucocyte count (TLC). The complete remission, MRD and overall survival have been shown unfavorably influenced by the expression of CD200 and/or CD123. Therefore, CD200 and CD123 can be utilized as biomarkers of MRD in AML patients as well as being considered as therapeutic factors (76).

Cell Surface Expression of GRP78 and CXCR4 Can Discriminate High Risk Children with Leukemia

The 78-kDa glucose regulated protein (GRP78) is an immunoglobulin heavy chain binding protein that plays an important part in regulation of endoplasmic reticulum (ER) function because of its effective role in unfolded protein response (UPR) pathway (81-83). GPR78 normally binds to inositol requiring enzyme 1 (IRE1), PKR-like ER kinase, and activating transcription factor 6 (ATF 6). In term of defective protein aggregation in ER, GRP78 segregates these sensors and unbinds them to start the UPR pathway (84).

The UPR cascade helps the integrity of hematopoietic stem cells be maintained by inhibiting damaged cells propagation and reduction of malignancy risk (85). Moreover, UPR cascade serves the preleukemic stem cells to enhance their clonal dominance (86). In addition, it has been found

that role of GRP78 is significant for survival of stem cells (87) and sGRP78 represents cellular stem-origin in cancer (88). The cell surface GRP78 (sGRP78), as a multifunctional receptor on tumor cells, triggers different responses that lead to immunity, proliferation, apoptosis, invasion, and inflammation (89-93). GRP78 overexpresses in the leukemic blasts of adult patients and therefore its evaluation at diagnosis can help in detection of high-risk leukemia (94-97). Investigation of sGPR78 in childhood leukemia has shown that presence of SGPR78, CD10, CD19, and CXCR4 in samples obtained from bone marrow is associated with high-risk leukemia while these biomarkers were absent in the similar compartment of standard-risk leukemic patients. In addition, two biomarkers, $sGPR78^{+}$ $CXCR4^{+}$, were found abundant in peripheral blood cells of high-risk patients. Therefore, analysing the presence of standard biomarkers with both sGRP78 and CXCR4 may help to find high-risk patients and choose better treatment approach (98).

Semaphorin 4D a Potential Marker at Diagnosis and Development of Leukemia

Semaphorin 4D (Sema4D) or CD100 was first found in the immune system in 1992 (99). The Sema4D is a member of the IV subfamily of the semaphorin superfamily. There are two forms of Sema4D: membrane-bounds and soluble forms. The membrane Sema4D binds to calmodulin via its C-terminal exodomain, leading to proteolytic cleavage of Sema4D and eventually the release of soluble Sema4D into circulation (100, 101). It has been demonstrated that Sema4D plays an important role in the immune response regulation (102). In chronic lymphocytic leukemia (CLL), Sema4D helps in viability and proliferation of cancer cells (103). Moreover, binding of Sema4D to Plexin-B1, one of its receptors, has led to improvement of survival and growth in B-CLL cells and inhibition of malignant cell apoptosis (100, 104). High serum levels of soluble Sema4D were detected in B-ALL and non-B-ALL, mainly AML and T cell-ALL patients. Sema4D overexpression triggers proliferation and decreases apoptosis of leukemic cells. In addition, Sema4D plays a significant role in migratory capacity, invasion, and development of leukemia through activating PI3K/AKT and ERK signaling pathway via increasing their phosphorylation. All in all, Sema4D might be applied as a potential targetable marker at diagnosis and treatment of leukemia (105).

Expert Opinion

Adoptive cell therapy (ACT) has been developed because traditional treatment regimens, chemotherapy followed by HSCT, is not able to cure or provide relapse-free survival in pediatric leukemia. ACT includes NK/CAR T – cell Therapy, and biomarkers; that help outcome prediction in children with leukemia. Ex vivo generated dendritic cells (DCs) were able to highly improve anti-tumor T and NK cells properties. More studies are required to evaluate potency of these ex vivo generated DCs to coordinate in vivo anti-tumor T and NK activity. Thereafter, an effective vaccine composition and vaccination strategy might be designed which would induce potent in vivo anti-tumor T and NK cells responses. Additionally, these ex vivo generated DCs are impure, then it is necessary those non-DCs are biologically and functionally recognized and also their potential effect on DCs function does. Finally, some adjustment of culture protocol may be done to enhance these DCs purity for vaccination usage. On the other hand, ex vivo murine model generation of memory-like NK cells has been very promising because sufficient cell number is produced at the end of culture, anti-tumor activity of these cells increases and at last, risk of GVHD decreases. This method is very feasible and it can be simply translated to clinical trial for adoptive cell therapy in hematologic malignancies or solid tumors.

There are encouraging results on CAR T-cell therapy in field of leukemia but for improvement of CAR T-cell therapy, combination of checkpoint fusion proteins with CAR T-cell, were developed. Therefore, these modified CAR T-cells showed less systemic side effects and persisted longer. TIM3-CD28, as one of these modified CAR T-cells, might be more analyzed in future researches for even better expansion and persistence.

Investigation of biomarkers in patient with leukemia provides valuable data about the prognosis and treatment planning but these biomarkers need to be investigated further. For example, serum cytokine should be studied in larger patient population, and including more cytokine items to be compared within patients and normal con-

trols. Because CNS involvement in pediatric patients with leukemia is rare therefore studies related to this issue are affected with low number of patient population, resulting in the need for large cohort studies, verifying previous results. However, these results redirect researchers to find clues for targeted therapy of EM leukemia. Also, risk stratification of childhood leukemia, to High and Standard-risk, is better done by application of biomarkers. Biomarkers can be a potential target for personalized therapy, too. It seems that future will be full of diagnostic and treatment approaches for patients with cancer like leukemia. Because new and effective generations of ACT e.g., CAR T-cell therapy will appear that it will be based on current or prospective-known biomarkers and fusion checkpoint proteins. Another improvement may include DCs vaccination for patients with cancer or in vivo NK cell therapy to increase OS or RFS.

Undoubtedly, there will be changes in current norms of ACT through time. Maybe, we will witness that some standard procedures might have gained or lost due to progress in our knowledge of leukemia heterogeneity, tumor-associated antigens, cytokines and etc. Patients' outcome would make progress following effective DC/NK vaccination or CAR T-cell through their de novo co-stimulatory molecules.

Conclusion

As immunotherapy demonstrates dramatic success in leukemia, our next step can be finding pediatric patients bearing risk factors that lead to relapse/refractory settings. Immunotherapy by implementation of adoptive cells as NK cell and CAR T-cell therapy has met failure. Therefore, preclinical studies seek to enhance anti-tumor activity of adoptive cells. The anti-tumor T and NK cell responses might be boosted through ex vivo generation of blood DCs, resembling phenotypically and functionally their in vivo counterparts. Also, adoptive NK cell therapy may be improved with ex vivo NK cell expansion upon cytokine induction. On the other hand, a distinctive relation between NK cells' KIR genes and ALL pathogenesis has not been discovered yet and needs further investigations. CAR T-cell therapy has improved by development of synthetic checkpoint fusion protein and its combination with anti-CD19 CAR T-cell, leading to increased T cell expansion and functionality. It has been suggested that CAR T-cell therapy may be effective in pediatric EM leukemia; however, more studies should be conducted in this field.

In leukemic patients, at-diagnosis and/or post-alloHSCT biomarker consideration might be very helpful in determining patient disease status, prognosis, and cancer type. In this regard, some biomarkers have been investigated as follow: investigation of serum pro/anti-inflammatory cytokines and sHLA-G, body fluid (CSF, bone marrow, peripheral blood) miR, leukemic-cell surface-expression of GRP78 and CXCR4 together with standard markers such as CD10 and CD19, presence of *ADAM6* gene in B cell leukemia, CD200 and CD123 in bone marrow samples of AML patients and finally, serum level consideration of Semaphorin 4D (Sema4D). Further clinical investigations should be done to determine the most effective immunotherapeutic methods and associated biomarkers in leukemia.

Conflict of Interest

The authors report there are no competing interests to declare.

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