



Synthetic Biology Technologies And Genetically Engineering Strategies For Enhanced Cell Therapeutics

Siyu Li¹ · Hao Tang² · Cheng Li¹ · Jiajia Ma¹ · Maqsood Ali¹ · Qi Dong¹ · Jiajia Wu¹ · Yang Hui^{1,3}  · Chongran Sun⁴

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Abstract

Stem cell therapy mainly uses natural stem cells for transplantation, and the use of genetic engineering to optimize stem cell products is a very important process. This article reviews successful gene modification methods in the field of immune cell therapy and summarizes some attempts at stem cell gene editing in current research. Cell bridging is an innovative cutting-edge strategy that includes the specific recognition and signal transduction of artificial receptors. The “off-the-shelf” cell strategies mainly introduce the advantages of allogeneic cell therapy and how to overcome issues such as immunogenicity. Gene regulatory systems allow us to manipulate cells with small molecules to control cellular phenotypes. In addition, we also summarize some important genes that can provide a reference for cell genetic engineering. In conclusion, we summarize a variety of technical strategies for gene editing cells to provide useful ideas and experiences for future stem cell therapy research.

Keywords Genetic modification · Stem cell · Immune cell · Cell engineering · Cell therapy

Background

As a precision and personalized treatment, cellular therapy has become a hot topic in the recent medical industry. At present, many cellular products have been used in the clinic; for example, hematopoietic stem cell transplantation is a well-known treatment method, and there are currently 10 internationally approved hematopoietic stem

cell therapies, including 8 cord blood products, all for the treatment of blood disorders and immune deficiencies [1]. In addition, there are 10 mesenchymal stem cell products approved globally. There are also many more cell therapy types in different research stages, and the statistics indicate that cancer research represents more than half of all cytotherapy studies [2, 3].

Immune cell therapy covering T cells, dendritic cells (DCs), natural killer cells (NKs) and other cell types has gone through stages such as cytokine-induced killer (CIK), DC-CIK and tumor-infiltrating lymphocytes (TILs). Stem cells come from a wide variety of sources, such as embryos, bone marrow, neural tissue and fat, and can even be induced in vitro by reprogramming. Many types of stem cells are widely used to treat diseases of various organs or systems, such as the cardiovascular system, liver, nervous system and kidneys, but some do not meet clinical standards, including induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs) [4–7]. Stem cells can differentiate in situ and secrete cytokines or nutrients after transplantation to repair tissue damage and exert anti-inflammatory effects. Current research also indicates that mesenchymal stem cells are used to treat pneumonia caused by COVID-19 [8]. Stem cell therapy is limited by numerous problems, including immunogenicity, targeting of lesions, tumorigenicity, heterogeneity,

✉ Yang Hui
huiyang79@126.com

✉ Chongran Sun
sun.chongran@zju.edu.cn

¹ Department of Biochemistry and Molecular Biology, Harbin Medical University, 157 BaoJian Road Nangang Dist, Harbin 150086, People’s Republic of China

² Department of Neurology, The Second Affiliated Hospital of Harbin Medical University, Harbin, People’s Republic of China

³ Basic Medical Institute of Heilongjiang Medical Science Academy, Harbin, China

⁴ Department of Neurosurgery, The Second Affiliated Hospital of Zhejiang University School of Medicine, 866 Yuhangtang Road, Hangzhou City, Xihu District 310058, People’s Republic of China

inability to monitor cell activity, and inability to control *in vivo* differentiation [9]. In addition, other challenges are how to choose the transplant route and how to guarantee the persistence of transplanted cells *in vivo*. To solve these problems, the main strategies are to modify the cell transplantation matrix, add cytokines and trophic factors to ensure cell viability, and use biomaterials, nanomaterials and 3D printing to enhance the stability of the transplant conformation [10, 11]. Magnetic field guidance, chemokine induction and other methods can improve stem cell homing and can also solve the dilemma of low treatment efficiency. Cytokines and chemokines used in pretreatment can also be expressed by stem cells themselves through gene editing [12].

To further find solutions to current problems, we summarized the current core technologies and derived strategies in the field of immune cell therapy, as well as the core advances in the field of synthetic biology in the field of cell therapy, including current breakthroughs in stem cell research with gene editing. The main strategies addressed in this paper are a) cell bridging strategies, b) "off-the-shelf" cell strategies, c) important gene target modification strategies, and d) gene control system strategies. We hope to use this review to summarize the latest developments in this field to develop innovative ideas and research methods in the field of stem cell therapy.

Cell Bridging Strategies

Chimeric Antigen Receptor (CAR)

At present, 5 CAR-T products have been launched in the United States, and the number of products under development in the world has exceeded 400 [13]. CAR-T cells have made great strides in the treatment of B-cell malignancies, and there are also numerous studies on other malignant hematological tumors and solid tumors. Currently, CAR has undergone four iterations of its structure to further improve its efficacy. The main challenge that CAR-T cells currently face is cytokine release syndrome (CRS) caused by excessive activation of T cells *in vivo* and off-target effects caused by incorrect killing of normal cells. Clinical trials have shown that CAR-T therapy can also damage the central nervous system, resulting in CAR-T encephalopathy syndrome (CRES). These clinical side effects often prevent patients with poor physical condition from continuing to receive CAR-T therapy. Due to changes in the cell distribution and a decrease in the number of CAR-T cells in the body, some patients experience tumor recurrence after treatment [14]. The research field of CAR-T therapy for solid tumors is in a difficult position, mainly due to the capsule of solid tumors and the unique immunosuppressive microenvironment in tumor tissues. Finding new targets, such as tumor

blood vessels and tumor chemokines, may bring good news, but it is also crucial to further upgrade the CAR structure itself. To solve these problems, researchers have developed a new generation of CAR-T-modifying methods based on gene editing techniques. The upgraded CAR-T cells showed increased functionality, longer survival and better targeting (Fig. 1). TandemCAR and trivalentCAR target multiple tumor-associated antigens to further improve the accuracy of identification but sometimes face the problem that the vector is too large to be transfected [15, 16]. The Bites-CAR is a combination of bispecific antibodies and CAR-T cells. The secreted bispecific antibodies can activate CAR-T cells and activate endogenous T cells to achieve a stronger tumor killing effect. However, this strategy lacks a termination system and may be plagued by adverse effects during treatment [17]. AT-CAR and SUPRA CAR are somewhat similar in structure. They use the biotin-avidin system and leucine zippers to separate the single-chain variable fragment (scFv) from the receptor part, which helps to control the activity of T cells in the body. When the patient is cured or cannot tolerate the treatment, it can be terminated by stopping the injection of the scFv. However, *in vitro* synthesis of scFv increases the cost of treatment and brings potential immunogenicity [18, 19]. In addition to chimeric antigen receptors, there are other approaches to cell bridging in stem cells or immune cells that can be combined into a variety of new engineering cell strategies (Fig. 2).

T-cell Antigen Coupler

Because the adverse effects of CAR-T cells in clinical therapy are related to the function of CAR-T cells, studies of T-cell receptor (TCR)-based engineered TCR-T cells have recently shown fewer side effects than CAR-T cells [20]. In addition to directly engineering TCRs, recruiting TCRs to work with artificial receptors can also be a feasible solution. Helsen et al. created a TCR-dependent receptor called the T-cell antigen coupler (TAC). As a membrane receptor, TAC does not need to identify major histocompatibility complex (MHC) molecules for activation [21]. The TAC consists of one antigen-binding domain, one TCR recruitment domain and one CD4/CD8 α coreceptor domain. The antigen binding field is a scFv targeting tumor-associated antigens. The TCR recruiting domain may specifically link CD3 ϵ and connect the entire structure to the TCR. Compared with CAR-T cells, TAC-T cells showed comparable cytotoxicity and tumor removal in both *in vitro* and *in vivo* models, and there were no adverse reactions similar to those seen in second-generation CAR-T cells. Moreover, TAC-T cells have a stronger effect on solid tumor infiltration than CAR-T cells, suggesting that TAC-T cells have low toxicity and can be an effective strategy for the treatment of solid tumors. This strategy demonstrates that certain forms of cellular bridging

Fig. 1 Upgrades of various CAR constructs. Classic CAR structure: Extracellular to intracellular is scFv, hinge, TM, CD3 ζ and costimulatory domain. TanCAR: IL-13 binds to SCFV and can bind to IL-13 receptors that are specifically expressed on tumor cells. TriCAR: Three CARs targeting different tumor-associated antigens (TAA) were expressed on the same T cell. Bites-CAR: Expressing bispecific antibodies (BiTEs). One side can bind to TAA, the other side can bind to CD3 and activate T cells. AT-CAR: Exogenous injection of biotinylated antibodies. The extracellular domain of CAR is the avidin that matches the biotin. SUPRA CAR: an exogenous injection of antibody with A zip was given First. scFv binds to T cells with the tyrosine zipper

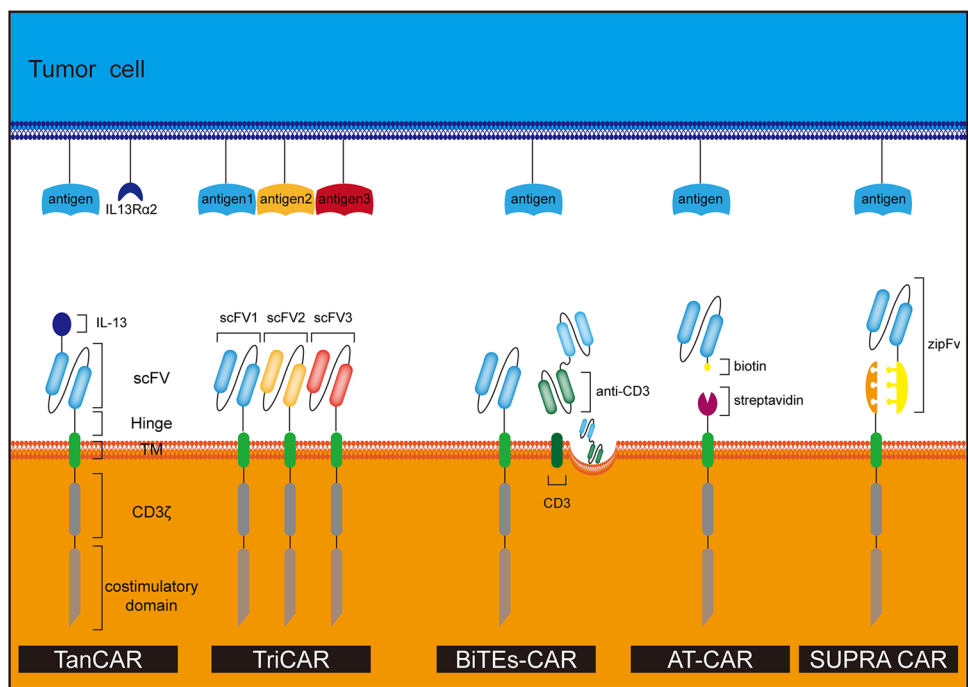
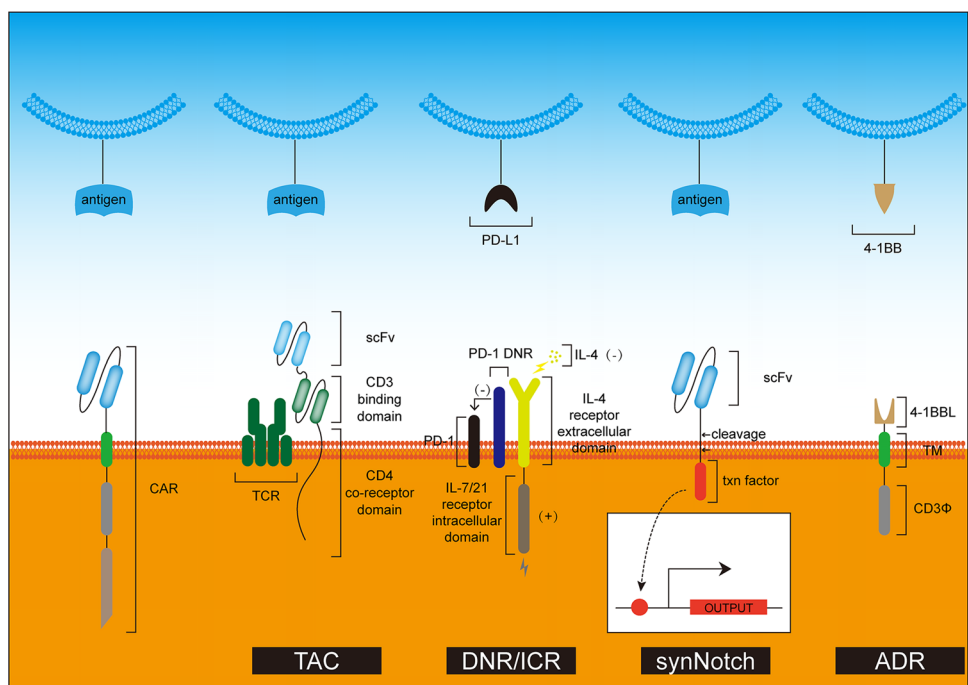


Fig. 2 Engineering receptor strategies. TAC: There are three parts scFv, CD3 binding domain and CD4 co-receptor domain. CD3 binding domain can bind TAC to TCR. DNR/ICR: PD-1 DNR can combine competitively with PD-L1. ICR has IL-4 receptor extracellular domain and IL-7/21 receptor intracellular domain. ICR can bind IL-4 and activate T cell. SynNotch receptor: When SynNotch receptor binds to ligand, it forms transcriptional activation structures to activate downstream gene expression. ADR: There are three parts 4-1BBL, transmembrane domain and CD3 Φ . ADR can bind 4-1BB of T cell to prevent immune rejection



designed to rely on natural receptors are not only safer but may also function more optimally.

Dominant Negative Receptor/Inverted Cytokine Receptor

The programmed death-1/programmed cell death-ligand 1 (PD-1/PD-L1) pathway is critical for T-cell development and function, and the expression of PD-L1 on the tumor

cell surface is also associated with immune escape. Studies have shown that blocking PD-1/PD-L1 with monoclonal antibodies can significantly remove this immune escape and improve the antitumor activity of CAR-T cells [22]. In addition to directly blocking this pathway using a PD-1 monoclonal antibody, the expression of PD-1 dominant negative receptor (DNR) on the surface of T cells is also a way to improve the function of CAR-T cells [23]. When T cells meet tumor cells, PD-1 DNR on T cells can competitively

bind to PD-L1 on the surface of tumor cells, thereby reducing the inhibitory effect of the PD-1/PD-L1 pathway on T cells and initiating T-cell killing of tumor cells.

On the other hand, cytokine regulation is also an essential component of T-cell activation, so the combination of inverted cytokine receptor (ICR) and CAR is also an effective method to improve the effect of T lymphocytes [24]. In the process of killing tumors, the immune attack of CAR-T cells is often weakened by the immunosuppressive signals generated by the tumor. These signals include the inhibitory cytokines IL-4, IL-10, and transforming growth factor beta (TGF- β), among others, which can be produced by cellular or stromal components of the tumor microenvironment [25]. Cho et al. used the intracellular structure of the IL-7/IL-21 receptor to replace the natural intracellular structure of the IL-4 receptor and turn it into a newly modified receptor, which prevents CAR-T cells from activating the signal transducer and activator of transcription 6 (STAT6) pathway after being stimulated by IL-4, inhibiting the differentiation of T cells to the Th2 phenotype. This allows the signal to be converted into STAT3 and STAT5, promoting the th1- and th17-like polarization of CAR-T cells, thereby retaining CAR-T-cell tumor-targeted toxicity. This strategy has demonstrated killing activity against IL-4 + solid tumors *in vitro* and *in vivo*.

The two engineered receptors share a common ability to bypass T-cell inhibitory signals and even turn negative signals into positive signals during both the internal environment and cellular communication. Regarding immune checkpoints, newly discovered immune checkpoints are emerging one after another. In addition to PD-1, other immune checkpoints, such as T-cell immunoglobulin and mucin-containing molecule 3 (Tim-3), lymphocyte activation gene 3 (LAG-3) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), are also being used to create new DNR targets. The modification of cytokine receptors is more complicated, the signal transduction matrix of many receptors has not been elucidated, and the modification of G protein-coupled receptors and receptor tyrosine kinases is relatively difficult. The idea of changing the factor inhibition signal can be used as a reference, but the specific implementation method needs further study. However, the safety of modified cytokine receptors still needs to be strictly controlled to prevent adverse reactions caused by excessive cell activation.

synNotch Receptor

The SynNotch receptor is an engineered receptor that retains the intrinsic transmembrane domain of the Notch receptor and replaces the intracellular and extracellular domains with custom domains that enable it to recognize specific ligands while simultaneously activating specific

genes. When the SynNotch receptor binds to its ligand, the transmembrane domain undergoes two hydrolyses, thereby releasing the intracellular domain, allowing it to form an active transcriptional regulatory protein, which then enters the nucleus and regulates the expression of its target genes. This transcriptional regulation can be either activating or repressive. Because SynNotch receptors can target specific antigens and control gene expression, the combined application of SynNotch and CAR can improve the accuracy of tumor recognition by engineered cells, reduce off-target occurrences, and even improve therapeutic effects. Cho et al. proposed a strategy to express SynNotch and CAR on the surface of T cells, and the two receptors target different antigens. While improving the targeting of T cells to tumor cells, SynNotch can also be used to regulate the expression of cytokines [26]. Luo et al. performed another study using SynNotch-NK cells targeting Glypican-3 (GPC3) to kill tumor cells along with CAR-T cells targeting GPC3. Expression of the SynNotch receptor allows NK cells to acquire targeted killing capability, and its downstream effector can control the expression of IL-12. It can improve the activity of CAR-T cells and achieve a better clearing effect on tumor cells [27]. This strategy is an ideal combination that ingeniously brings NK cells into the tumor treatment process while simultaneously regulating CAR-T cells at the cytokine level. SynNotch receptors can also be used to regulate CAR expression; for example, the CAR "switch" and SynNotch receptors can link universal tumor antigen control to downstream CAR expression. After injection of SynNotch-T cells *in vivo*, SynNotch receptors can make T cells recognize tumor cells and enrich them while simultaneously activating gene expression of the CAR. After that, the CAR receptor recognizes different antigens of the same tumor and then initiates cell killing. This strategy can improve the specificity of engineered T cells for tumors and reduce the systemic toxicity of T cells and accidental injury to normal tissue cells [28, 29]. The SynNotch receptor can be widely used in engineered cells due to its single signal transduction system. For CAR-T cells, the most promising strategy is to use SynNotch to control the expression of CAR, which can greatly reduce the systemic toxicity of CAR-T cells and is very promising for achieving good results in clinical research. For stem cells, the use of SynNotch receptors may achieve gene-specific expression or directed differentiation at specific locations at specific times, while the use of the specificity of scFv in the extracellular region may improve the efficiency of homing and enrichment of stem cells *in vivo*. However, due to the self-activation problem of classical SynNotch, designing a more stable SynNotch system can better enhance the control of genes and the safety of treatment.

Allogeneic Immune Defense Receptor

The allogeneic immune defense receptor (ADR) is a customized receptor that mediates the clearance of autoreactive T cells and can effectively avoid allogeneic T-cell-mediated rejection [30]. A study by Mo et al. reported the structure of ADR, which consists of an extracellular domain (4-1BB ligand), a transmembrane domain, and an intracellular domain (CD3 Φ). Activated lymphocytes can temporarily regulate the expression of tumor necrosis factor receptor superfamily member 9 (TNFRSF9/4-1BB), and the ADR receptor can bind to 4-1BB of activated lymphocytes to eliminate the attack of lymphocytes on ADR-T. Allogeneic T lymphocytes expressing both ADR and CAR can prevent host rejection without affecting normal CAR function. These cells can survive well in mouse models and produce sustained tumor eradication in mouse models of hematopoietic and solid cancers. We can expect this strategy to be a successful method for delivering universal CAR-T products.

Additional artificial receptors, such as Tango, modular extracellular sensor architecture (Mesa) and generalized extracellular molecule sensor (GEMS), are also available for cell engineering [31–33]. The fundamental purpose of artificial cell bridging is to improve the efficiency of targeted migration of cells and the degree of activation of cells when they perform their functions and even participate in the manufacture of universal cell products. To a certain degree, stem cells also have similar problems of poor targeting and low migration efficiency and may be able to achieve breakthroughs in the application of engineered receptors. Combining research in the fields of synthetic biology and cell therapy, the artificial cell receptor strategy has broad application prospects, and this therapeutic approach is expected to fundamentally change the diagnosis, treatment and prevention of diseases.

Gene-Edited “Off-The-Shelf” Cell Strategies

Autologous cell therapy is currently the main method of adoptive cell therapy. During the process of treatment, the immune system may reject certain types of stem cells or immune cells and reduce their efficacy [34, 35]. In addition, the appearance of graft versus host disease (GVHD) will also affect the effect of cell therapy. In clinical use, some drugs, cytokines or monoclonal antibodies can reduce the immune response and mediate tolerance to prevent graft-versus-host disease [36]. If a batch of cells can be prepared in advance and directly made into a cellular product, the current dilemma brought by autologous cells to cell therapy can be changed. With the gradual maturity of gene editing technology, the production of allogeneic “off-the-shelf” CAR-T products has gradually become a reality. Compared

with autologous cells, it has outstanding advantages such as low cost, personalization, and customization [37].

Gene editing for the elimination of TCR or human leukocyte antigen (HLA) has been shown to be an effective method for reducing the immunogenicity of CAR-T cells [38, 39]. One reason for choosing this strategy is that donor T lymphocytes can induce GVHD through TCR recognition of host “nonindividual” antigens [40]. Wiebking et al. used CRISPR/Cas9 to integrate the CD19-targeting CAR gene into the *TRAC* locus so that the TCR gene could be knocked out and the CAR could be expressed simultaneously. TCR was silenced in 90% of $\alpha\beta$ T cells, and CAR expression was also observed in 75% of $\alpha\beta$ T cells. The results showed that CAR-T cells exhibited normal toxicity to CD19+ tumor cells in vitro and good tumor clearance in vivo and did not cause graft-versus-host disease [41]. On the other hand, transplant rejection occurs when the host immune system recognizes that the HLA molecules of the transplanted allogeneic cells do not match their own. One of the ways to avoid host rejection is to remove HLA-I molecules from donor T cells. Lee et al. used CRISPR/Cas9 to knock out *B2M*, which targets α chains of HLA-II (*DPA*, *DQA*, and *DRA*), in CAR-T cells, and the results showed that HLA-I/II-negative T lymphocytes maintained the phenotype and function of normal T lymphocytes in vitro. This strategy also provides an efficient means for the universal generation of CAR-T cells [42]. Furthermore, the mixed responses of lymphocytes in vitro suggest that HLA-II plays a dominant role in immune rejection, suggesting that HLA-II may become a hot topic for future “off-the-shelf” cell studies. iPSCs are classic “off-the-shelf” cell types and are an ideal source of derived cell products [43]. Wang, B. et al. presented a novel strategy for iPSC-derived T cells, which is a new strategy for producing universal CAR-T cells. In the same way, to regulate the activation of immune cells, *B2M* is knocked out to inhibit CD8+ T cells, *CITTA* is neutralized to inhibit CD4+ T cells, and HLA-E is knocked out to inhibit NK cells. In an effort to further inhibit NK-cell activity, researchers also eliminated *PVR*, which encodes the ligand of the NK-cell-activating receptor DNAX accessory molecule 1 (DNAM-1). The edited iPSCs were later differentiated into T cells (iPS-T cells) to kill tumor cells. The researchers found that CAR-iPS-T cells had good anticancer effects without stimulating immune cells. In addition, inserting other genes also allows the production of universal cells. In studying human herpesvirus 8, researchers found that the virus can protect itself from the host's immune system through *K3/K5*. Studies have demonstrated that the *K3* gene encodes a ubiquitous E3 membrane ligase, which can silence the most important components of the major histocompatibility complex class I (MHC I). *K5* codes a similar E3 ligase with greater specificity. *K3/K5* can regulate the expression of MHC molecules on the surface of the virus and prevent monitoring of T and

NK cells. Based on this self-protective mechanism of the virus, Wang et al. used retroviral transduction of the *K3/K5* gene into CAR-T cells to regulate the expression of MHC-Ia and MHC-II cells and reduce the immunogenicity of allogeneic T cells. This method only changed the HLA phenotype of T cells and had no effect on cytotoxicity, cell growth or cytokine secretion [44].

In stem cell research, similar strategies exist that use gene editing to circumvent immune rejection. Han et al. used CRISPR/Cas9 to remove the expression of highly polymorphic HLA-A/-B/-C and HLA Class II in human pluripotent stem cells. This strategy knocked out *CIITA* to eliminate HLA II molecules and could insert PD-L1, HLA-G, and CD47 into the *AAVS1* site, preventing immune surveillance by T cells, NK cells, and macrophages. PD-L1 is the ligand of the immune checkpoint PD-1, HLA-G is an NK-cell inhibitor ligand expressed at the maternal–fetal interface during pregnancy, and CD47 is a "do not eat me" signal that may help cells escape phagocytosis by macrophages. Cotransfection of these three genes became a versatile iPSC strategy with low immunogenicity [45]. Deuse et al. performed a similar study using CRISPR/Cas9 to target *B2M* and *CIITA* and overexpressed CD47, successfully producing iPSCs with low immunogenicity while preserving pluripotency and differentiation capacity [46]. Stem cells have characteristics that are less immunogenic than other cells, but as a universal cell product in treatment, allogeneic cell transplantation will always bring abnormalities to the treatment. Another advantage is that the development of "off-the-shelf" stem cell products may address the low in vivo activity and short half-life. The specific mechanisms and strategies are still under study.

Important Gene Target Modification Strategies

The core idea and basic approach of genome editing to modify cells is to insert new genes or eliminate genes to improve cell function, which has a positive effect on the cells for treatment. For CAR-T cells, the insertion/removal of genes can facilitate or eliminate clinical adverse events. As a proinflammatory factor, the primary function of granulocyte macrophage colony stimulating factor (GM-CSF) is to increase the proliferation and activation of macrophages and blood monocytes. Combined with the gene knockout method, GM-CSF can be specifically suppressed in CAR-T cells, and this strategy can reduce the expression of GM-CSF before the emergence of CRS compared with antibodies that neutralize anti-GM-CSF. Sachdeva et al. found that the use of transcription activator-like effector nuclease (TALEN) can achieve knockout of GM-CSF, and it did not affect the normal proliferation and activation of CAR-T cells. The

downregulation of GM-CSF expression significantly abolished the secretion of CRS biomarkers, including monocyte chemoattractant protein 1 (MCP-1), IL-6, and IL-8. This suggests that the treatment that uses GM-CSF KO CAR-T cells does not cause patients to develop CRS [47]. This also enhances the safety of the treatment, as knocking out the gene directly prevents the toxic side effects caused by antibody intervention. In the latest study, homologous recombination can be performed at the same time as gene knockout, and the DNA fragment encoding the CAR can be directly inserted at the mutation site, which can avoid the second gene editing of the cell. It can lower manufacturing costs to some extent, shorten the production cycle of products, and improve the safety of gene editing.

Immune escape of tumors is considered one of the key factors limiting the efficacy of CAR-T cells, so regulating the immune control points of donor cells can also be used to improve the efficacy of cell therapy. Zhang et al. used CRISPR–Cas9 to successfully generate LAG-3 KO CAR-T cells. LAG-3 is a negative regulator of T-cell activity, and LAG-3 KO CAR-T cells showed no significant changes in activity or immunophenotype during in vitro culture. Compared to classical CAR-T cells, LAG-3 KO CAR-T cells showed strong antigen-specific antitumor activity in a mouse xenotransplantation model [48]. PD-1, which is a T-cell immune control point, is another recent hot immune checkpoint. The main optional strategies include monoclonal antibody blockade and drug inhibition. Direct knockout of PD-1 can also enhance the efficacy of CAR-T cells to an extent. A study by Rupp et al. demonstrated that CRISPR/CAS9-mediated PD-1 knockout targeting the *Pdcd1* locus combined with lentiviral transduction of CAR is also an efficient strategy to enhance T-cell function [49]. CAR-T cells with PD-1 knockout showed a more significant killing effect on tumor cells in vitro. However, the effect of inhibition of this pathway has not been studied, and systemic inhibition of PD-1 may improve toxicity in vivo. In addition, inactivation of *Pdcd1* may inadvertently lead to premature failure of T lymphocytes [50]. In the future, further studies on the PD1/PD-L1 pathway may lead to the emergence of new modified approaches, which will facilitate the study of a new generation of CAR-T cells related to PD-1.

The off-target effect of CAR-T cells is also a serious adverse reaction that can be fatal. Certain tumor-associated antigens have limitations because they are also expressed in normal cells. For example, research shows that treating acute myeloid leukemia (AML) with anti-CD33 CAR-T cells can eliminate all leukemia cells but will inhibit the growth of normal bone marrow cells. The CAR-T cells remaining in the patient's body can also clear the transplanted hematopoietic stem cells (HSCs) in the body, causing the failure of stem cell transplantation. Kim et al. used CD33 KO hematopoietic stem cells to rebuild an antigen-negative

hematopoietic system. Autologous CD33 KO HSCs succeeded in avoiding attack from CAR-T cells and became an ideal therapeutic combination [51].

Similarly, many more genes can be modified in stem cells. IL-10 is an anti-inflammatory agent that mediates the anti-inflammatory activity of the body by activating STAT3 [52]. In addition, IL-10 inhibits the production of proinflammatory cytokines by reducing the damage to oxidative tissues caused by hemoglobin due to the positive feedback loop of CD163, heme oxygenase-1 (HO-1), and IL-10. Peruzzaro et al. demonstrated that the application of lentiviral overexpression of IL-10 in mesenchymal stem cells enhanced its anti-inflammatory effect in vivo. MSCs overexpressing IL-10 showed strong anti-inflammatory effects and functional recovery following intracerebral transplantation in traumatic brain injury (TBI) rats [53]. Brain-derived neurotrophic factor (BDNF) is an important neurotrophic factor that can help build new neural connections and protect healthy neurons. Studies have demonstrated that BDNF-overexpressing mesenchymal stem cells can effectively alleviate striatal atrophy in Huntington's disease transgenic mice [54]. Glial cell line-derived neurotrophic factor (GDNF) is expressed in a variety of nerve cells and nerve-related cells and has the effect of target-derived neurotrophic factor, which can promote the survival of neurons, affect the development and differentiation of neurons, and have a nutritional effect on neurons. A study by Shahrezaie et al. showed that overexpression of GDNF in MSCs can enhance their neuroprotective function [55]. The findings indicated that GDNF-overexpressing bone mesenchymal stem cells (BMSCs) showed a better therapeutic effect on spinal cord injury than

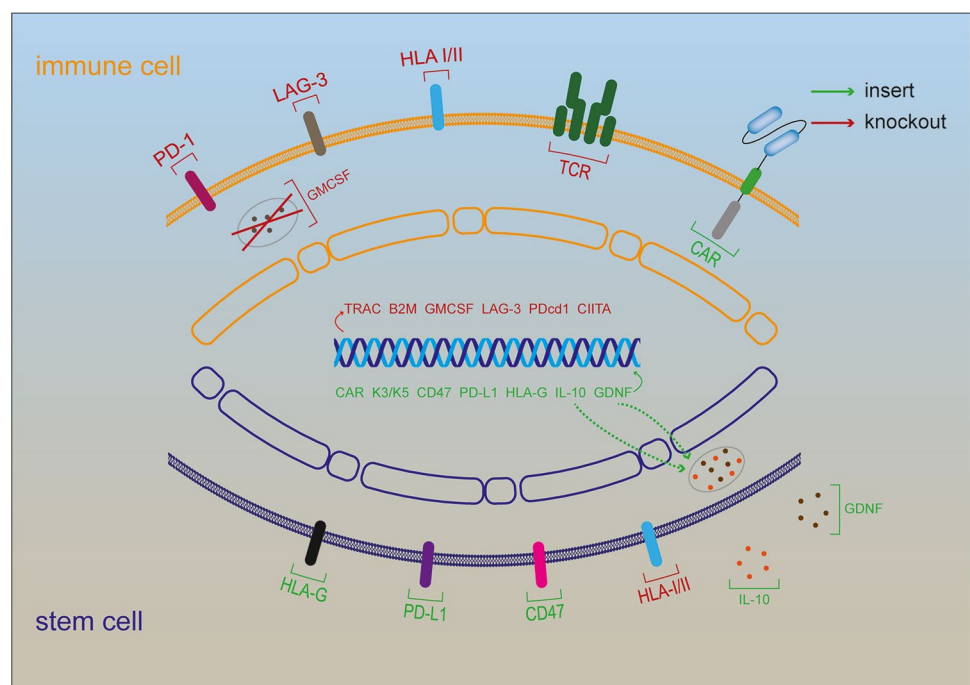
unmodified BMSCs. These studies suggest possible strategies for treating mesenchymal stem cells in neurological diseases. Other studies have shown that overexpression of lymphoid enhancer-binding factor 1 (LEF1) can promote the proliferation and antiapoptotic effect of HU-MSCs [56].

Important gene-targeted modification strategies can be used for research in the field of immune cells and stem cells, as well as for the manufacture of universal cells (Fig. 3). At present, in this regard, the main criterion for selecting the type of gene to be modified is to control or reduce the adverse effects of CAR-T cells. For stem cells, the method of gene overexpression or deletion is the mainstream method of gene modification research. It is worth noting that the editing of a specific gene should not only consider its enhanced function but also consider its negative effects when editing genes that control multiple functions and pathways in cells. Furthermore, inserting genes should also avoid destroying the normal function of the original genome, and attention should be paid to safety issues when using viruses as gene delivery vehicles. The implementation of this strategy requires a thorough evaluation to ensure the safety of gene-edited cells as a cellular product for clinical treatment.

Gene Control System Strategies

The gene control system refers to the situation in which the transfected gene is silenced under natural conditions; when the cell takes in a signal molecule added by us, the expression of the transfected gene will be started. The addition of gene control systems can better regulate gene expression and

Fig. 3 Gene insertion/knockout and "off-the-shelf" cell strategies. Immune cell: Knockout of PD-1, LAG-3, HLA-I/II, and TCR reduced immunogenicity. Knockout of GMCSF can reduce the occurrence of CRS. TRAC encodes TCR, PDcd1 encodes PD-1. B2M and CIITA are involved in encoding HLA-I/II. The expression of MHC-IA and MHC-II can be down-regulated by K3/K5 insertion. Gene knockout can simultaneously integrate CAR. Stem cell: Knockout of HLA-I/II and expression of HLA-G, PD-L1 and CD17 can reduce immune rejection. The expression of IL-10 can increase the anti-inflammatory activity of the cells. The expression of GDNF can increase the neuroprotective function of cells



improve the efficiency and safety of cell therapy, but traditional gene control systems, such as tetracycline-mediated *TetR*, have some shortcomings that limit its application. For example, long-term tetracycline induction can lead to cell resistance, and tetracycline itself can cause some adverse reactions [57–59]. Some new studies have developed a new generation of gene regulation systems using vanillic acid (VA), benzoate or phloretin and have performed better than the tetracycline control system [60–62]. However, these molecules are not present in normal food, so the treatment must provide these additional molecules, which leads to the result that the efficacy of the treatment depends on the patient's compliance. If these molecules cannot be absorbed through the digestive system, they can only be ingested by injection, which can cause additional harm and pain to the patient. These molecules can also become potential allergens, leading to uncontrollable adverse effects during treatment. Yin et al. conducted a cell model study to control the expression of specific genes by drinking green tea, and the signaling molecule of this method is actually a secondary metabolite of tea polyphenols, namely, protocatechuic acid [63]. Research has made progress in three areas: combined application with the CRISPR/Cas system, the design of biological computers, and the biological treatment of diabetes. The protocatechuic acid (PCA) control system was designed based on a streptomycin-derived transcriptional repressor, *PcaV*. In the natural state, downstream genes are silent. When PCA enters the cell as an exogenous factor, it will bind to the protein responsible for gene silencing, causing it to lose its inhibitory function, thereby activating the expression of downstream genes. The team also studied genetic control systems based on other signal factors, such as the sodium ferula system and the far-red light system [64, 65].

In another study, Bai et al. designed the Coolsens gene switch using menthol as an inductor based on the transient receptor potential cation channel, subfamily M, member 8 (TRPM8) activation mechanism, which may stimulate gene expression downstream by external administration of menthol [66]. Coolsens cells were encapsulated in brown algal microcapsules, injected subcutaneously into mice and demonstrated a normal response to Menthol signals. These studies utilize synthetic biology methods to control gene expression through exogenous small molecules or stimulatory signals such as light waves to generate a complete gene control system in cells and provide an ideal way to control cell behavior and gene expression at specific times.

In the field of CAR-T research, there are also studies to control T lymphocytes using a similar "on/off" approach to achieve toxicity and fate control. The first solution was to incorporate an apoptosis induction system into CAR-T cells; examples include ICas9 and HSV-TK. However, these methods can only permanently halt the function of T cells. Recent studies hope to freely control the "on" and "off" of

T-cell killing activity by some methods, so new methods, such as drug-induced assembly of CARs and sCARs, have been developed [67]. Giordano-Attianese et al. added a chemically disruptive heterodimer (CDH) structure to classical CAR, allowing the CAR molecule to be inactivated by small molecular drugs to form a customized CAR called Stop-CAR [68]. This strategy allows us to suppress T-cell activation once treatment is complete and adverse events occur. Furthermore, metabolic engineering can also control the activity of T cells; for example, uridine is a nutrient needed to activate T lymphocytes, and T lymphocytes can only obtain uridine from autogenesis. Wiebking et al. used CRISPR/Cas9 to knock out genes encoding UMPs in the T-cell genome, which prevented T-cell synthesis of uridine, leaving T cells dependent on exogenous uridine for development and activation [69]. After the transplanted T cells were cut off from the uridine supply, their growth and function stopped, and the restoration of the uridine supply allowed the blocked T cells to function again. This strategy is a very valuable T-cell "on/off" strategy.

The strategy of gene switching is a promising strategy for cellular therapy. For CAR-T cells, when adverse reactions occur, we should quickly suspend the activity of T lymphocytes in the patient or control their toxicity and then restore T-cell function after the patient can adapt and allow continued treatment. In addition, we can use the gene control system to make T cells express a detectable signal molecule when they play a killing function and then judge the toxicity of CAR-T in vivo by the strength of the signal. This is helpful for the development of individualized treatment plans according to the patient's condition.

For stem cells, gene control systems can control the expression of specific genes at specific times and even create a fluorescent signal system to enable the tracking of stem cells in vivo. The challenge of this strategy is the efficiency of tracing to ensure that the strength of the exogenous signal is sufficient to activate the switch system of the engineered cells, and the exogenous signal should not cause toxic side effects to the body. In the future, the generation of a stable and safe gene control system will undoubtedly lead to huge progress in the development of cell therapy.

Discussion

In the era of genetic engineering, the field of cell therapy has achieved the latest progress, that is, to control the characteristics, fate and function of cells from the gene level and to obtain customized cells through genome modification, which are also the inevitable development direction of the next generation of cell therapy (Fig. 4).

Gene editing strategies in the field of cell therapy may be generic across different cell types. For example, regulatory

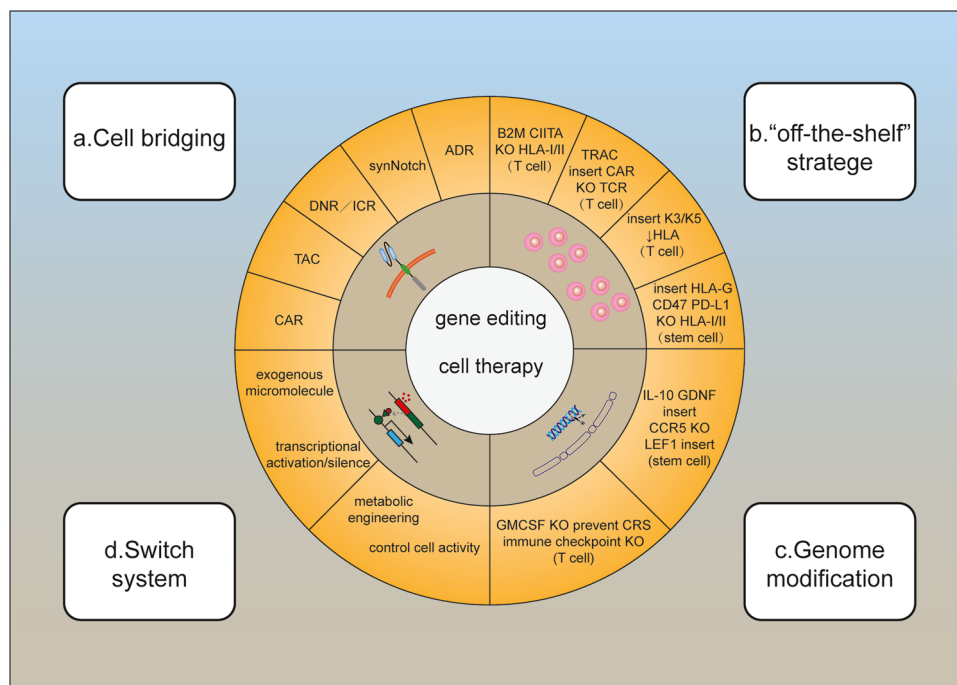


Fig. 4 Gene-edited strategies for cell therapy. **a**, Engineering receptor strategies, include CAR, TAC, DNR/ICR, synNotch receptor and ADR. **b**, “Off-the-shelf” cells strategies. knockout of HLA-I/II and TCR could reduce immunogenicity. Expression of CD47, PD-L1 and HLA-G could also reduce immunogenicity. **c**, Gene insert/knockout strategies. Knockout of GMCSF could prevent CRS. Expression of IL-10/GDNF could increase the effect of stem cell therapy. Overex-

pression of LEF1 can promote the proliferation and anti-apoptotic effect of MSCs. Knockout of CCR5 can enable human stem cells to acquire HIV-1 resistance. **d**, Switch system strategies. Gene control systems that rely on exogenous signals can control the expression of specific genes. Knockout of UMPs temporarily inhibit T-cell activity in vivo

T cells (Treg) are a subset of T cells that control autoimmune responses, and recent research advances have greatly promoted the development of Treg therapy. Rosado-Sanchez I et al. reviewed the remarkable therapeutic effect of CAR-Tregs and further optimization methods [70]. This is a major advance in the treatment of autoimmune diseases with cell products, and it also suggests that existing strategies have potential applications in other cell subsets.

In the future, cell bridging strategies, universal cell strategies, and gene switch strategies are promising reference strategies for genetically modified stem cells. Cell bridging strategies are widely used in the field of immune cells, and whether these strategies can be used to engineer stem cells has become an interesting topic. There are many application directions of gene switch systems in stem cell research, such as using stem cells as carriers to achieve directional drug delivery or artificially regulating the expression of transcription factors, controlling cells to maintain stemness or differentiation, etc. These approaches can address the problems of immune rejection during treatment and poor transplant survival and allow us to control the migration and activity of stem cells in the body. There are also many considerations when applying these technical strategies. First, the engineered receptor required for cell bridging is an

artificial gene whose transfection has the potential to disrupt the cell's own signal transduction. Receptor self-activation is a common adverse situation of artificial receptors, which may bring uncontrollable adverse reactions to cells, which is often directly related to the structure and function design of receptor proteins. In research and application, we need more monitoring methods to ensure the safety of stem cell therapy. In addition, in the selection of gene editing methods, incorrect insertion mutations should be avoided as much as possible. Building a gene control system in stem cells has not been shown to be compatible and may be hampered in the course of future research. Therefore, the study of a set of gene control switches suitable for stem cells and the selection of corresponding gene delivery methods have become important topics of synthetic biology in the field of stem cell gene editing. After cell transplantation, the long-term survival of cells in vivo has also become a major challenge for long-term cell therapy, which requires us to master the correct and efficient stem cell encapsulation technology and transplantation approach.

Furthermore, the advantages and disadvantages of autologous cell therapy and allogeneic cell therapy are still under discussion, and there may be better strategies to change the current status of autologous or allogeneic cell therapy. The

future breakthrough of autologous cell therapy lies in the shortening of the product production cycle and the optimization of product quality. Optimizing cell extraction and gene editing procedures is an important aspect to ensure that cells are kept in optimal condition after extraction and expanded to a sufficient number of cells for treatment in the shortest time possible. The requirement for gene editing tools is to introduce mutations quickly and accurately and to ensure stable modification of cells in the shortest time. This includes further optimization of existing gene editing tools and the development of new gene editing tools. Because autologous cells have sufficiently low immunogenicity, the selection of modified genes is more inclined to enhance the therapeutic function of cells, including enhancing the functions of the cells themselves and adding new functions (CAR allows T cells to recognize tumor-associated antigens). Allogeneic cells are more suitable for the mass production of cell products, and the primary focus of genetic modification is to reduce immunogenicity. The transformed cell products can allow all patients to receive treatment immediately when needed, avoiding the rapid deterioration of the patient's condition during the transformed cell stage, and are also suitable for some patients from whom it is not possible to extract healthy autologous immune cells or stem cells due to cachexia. This requires a high degree of perfection of the cell product production system, and therefore the long-term maintenance of allogeneic natural cells *in vitro* is also meaningful. This can save time during cell extraction and waiting for cell proliferation and can also avoid the trauma of cell extraction to patients. In response to this problem, iPSCs have great potential and research value. At present, the research of universal allogeneic cells is still a more favorable research direction for the cellular product industry because our current control over the production cycle and cost of autologous cell products has not reached the range that patients can afford.

In addition to the caveats associated with the strategies reviewed here, the field of gene-edited stem cells presents other challenges. First, the clinical application and research on the mechanism of action of stem cells are still unclear, which makes it difficult to control the therapeutic effect of stem cells after entering the human body as an advanced therapeutic medicinal product, and the tolerance of the human body to foreign cell transplantation has not been well resolved. More stringent criteria should also be set for *in vitro* passage times of genetically modified stem cells to monitor the balance between genetic modification stability and cell pluripotency. In addition, stem cells, as bioactive medicinal products, should have an intuitive tracking system to reflect their distribution and activity *in vivo* after transplantation. Second, as a therapeutic product, genetically engineered stem cells are faced with the selection of autologous or allogeneic cells. As a product of genetic editing, its

ethics issues must also be strictly controlled. While genetic engineering can increase the curative effect of cells, ideally, the engineered cells should be universal and must be shown to be compatible and safe *in vivo*. These challenges will be the main research directions in this field in the future.

Conclusions

The ultimate goal of the cell engineering strategy is to industrialize and form real cell products that can be marketed. We believe that some gene editing strategies can enhance the effect of stem cell therapy, including existing stem cell genetic modification methods and their derivatives and immune cell genetic modification methods that can be borrowed. These strategies can be roughly divided into two broad categories. One category comprises more classical methods, that is, gene knockout or overexpression, or even transfer of new genes. The purpose is to change the phenotype of cells and make them closer to therapeutic cell products. The frontier of this part is the research of gene therapy and allogeneic cell therapy. The main challenge is to screen the most suitable target genes for editing and to ensure the safety and efficacy of gene editing. The second part is the "engineered" cell transformation method, including cell bridging and gene control systems. The purpose is to modify the cells in a precise and personalized way so that the *in vivo* cell products can "obey" our instructions and improve the therapeutic effect and efficiency. The challenges in this field are great because multiple and complex gene transfections may be involved, which need to be optimized in terms of cost, safety and production efficiency. In summary, more in-depth thinking is needed in the industry, including translational research, clinical research, optimization of production methods and many other links. This article only reviews the technical strategy level, hoping to play a certain role in the progress of the entire cell therapy system. We look forward to the hope that the vigorous development of cell therapy will bring to the biopharmaceutical industry and the good news to more patients.

Abbreviations *CIK*: Cytokine-induced killer; *TIL*: Tumor Infiltrating Lymphocytes; *IPSC*: Induced pluripotent stem cell; *ESC*: Embryonic stem cell; *CAR*: Chimeric antigen receptor; *CRS*: Cytokine release syndrome; *CRES*: CAR-T encephalopathy syndrome; *scFv*: Single-chain variable fragment; *TCR*: T cell receptor; *PD-1/PD-L1*: Programmed death-1/programmed cell death-Ligand 1; *TAC*: T cell antigen complex; *MHC*: Major histocompatibility complex; *DNR*: Dominant negative receptor; *ICR*: Inverted cytokine receptor; *TGF- β* : Transforming growth factor beta; *STAT*: Signal transducer and activator of transcription; *Tim-3*: T cell immunoglobulin and mucin-containing molecule 3; *LAG-3*: Lymphocyte Activation Gene 3; *CTLA-4*: Cytotoxic T lymphocyte-associated antigen 4; *GPC3*: Glypican-3; *ADR*: Allogeneic immune defense receptor; *TNFRSF9/4-1BB*: Tumor necrosis factor receptor superfamily member 9; *Mesa*: Modular extracellular sensor architecture; *GEMS*: Generalized extracellular molecule sensor;

GVHD: Graft versus host disease; **HLA:** Human leukocyte antigen; **DNAM-1:** DNAX accessory molecule 1; **GM-CSF:** Granulocyte macrophage colony stimulating factor; **TALLEN:** Transcription activator-like effector nuclease; **AML:** Acute myeloid leukemia; **HSCs:** Hematopoietic stem cells; **HO-1:** Heme Oxygenase-1; **TBI:** Traumatic brain injuries; **GDNF:** Glial cell line-derived neurotrophic factor; **BDNF:** Brain-derived neurotrophic factor; **BMSCs:** Bone Mesenchymal Stem Cells; **LEF1:** Lymphoid enhancer-binding factor 1; **HU-MSC:** Human umbilical cord mesenchymal stem cell; **VA:** Vanillic Acid; **PCA:** Protocatechuic acid; **TRPM8:** Transient receptor potential cation channel, subfamily M, member 8; **CDH:** Chemically disruptive heterodimer; **UMP:** Uridylic acid; **Treg:** Regulatory T cell

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