Adoptive therapy with CAR redirected T cells for hematological malignancies

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The survival of patients with hematological malignancies has been significantly improved due to the development of new therapeutic agents. However, relapse remains a major matter for concern. Recently, T cells engineered with chimeric antigen receptor (CAR) were reported to show unprecedented responses in a range of hematological malignancies. The persistence of the CAR-T cell can last for years and tends toward long-term antitumor memory by which relapses can be effectively prevented. The primary side effects that appear in most clinical trials are cytokine release syndrome and neurotoxicity. However, these symptoms can be treated and reversed. In this review, we describe CAR structure and function and summarize recent advances in CAR-T cell therapy in hematological malignancies.

cancer, hematological malignancies, immunotherapy, chimeric antigen receptor, T-cell therapy

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BASIC CONCEPTS CONCERNING CAR

Chimeric antigen receptor (CAR) is an artificial receptor consisting of a single-chain variable fragment (scFv), a hinge chain and a transmembrane and an intracellular signal region. In 1989, for the first time, Gross et al. generated and expressed chimeric T-cell receptor (TcR) genes composed of the TcR constant (C) domains fused to the antibody's variable (V) domains (Gross et al., 1989). Compared with autologous T cells modified with tumor-specific T cell receptors (TCRs), CAR-T cells can overcome major histocompatibility complex (MHC) restriction and thus exhibit better anti-tumor activity.

The scFv is the most important part of CAR because it determines the target. The ideal target antigen for CAR should be protein epitopes, carbohydrates and glycolipids presented on the tumor cell surface. Moreover, other antigens such as lineage-specific antigens, highly expressed in

tumor tissues can also be considered in special cases.

Fully activated T cells require both a TCR signal and co-stimulatory signals, such as CD28, CD40L, and CD137, which are usually absent from tumor cells. Because of this, such co-stimulatory signals as CD28, OX40, CD40L, CD137 and CD27, were introduced into the intracellular CAR domain. This construction greatly improved T cell survival and cytokine secretion (Carpenito et al., 2009; Curran et al., 2015; Hombach et al., 2012).

PROGRESS OF CAR-T THERAPY IN HEMATOLOGICAL MALIGNANCY

Early breakthroughs in CAR-T

Although genetically modified T cell therapy has been present for many years, the efficiency of early genetically modified T cells was not satisfactory. Recently, the chimeric antigen receptor modified T cell has garnered much attention because a major breakthrough has been achieved. In

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2011, three cases of chronic lymphoblastic leukemia (CLL), which were all refractory, and relapsed chronic B cell lymphocytic leukemias were treated with CD19 targeted CAR-T as reported by June and his workers (Kalos et al., 2011; Porter et al., 2011). Two of the three patients were p53-deficient, which indicated a poor prognosis (Dohner et al., 1995). After treatment, two patients achieved complete remission (CR), and another achieved partial remission (PR). One of the CR patients received only a very low dose of CAR-T ($1.46 \times 10^5 \text{ kg}^{-1}$ CAR-T cells), but the treatment still showed excellent therapeutic efficiency at such a low dosage; 23 d after infusion, adenopathy was no longer present. A fluorescence *in situ* hybridization (FISH) test showed that P53 loss was negative, flow cytometry analysis showed that no CLL or B cells were detected, and 31 d after infusion, CT showed remission of the adenopathy. At the time the report was published, the remission had lasted for 10 months (Porter et al., 2011).

Well-designed clinical trials

After June's report of a CAR-T breakthrough in hematological malignancy, reports of modified T cell-related clinical research in hematological malignancy increased, most of which applied to CD19-targeted CAR-T. In 2012, the national cancer research center reported eight cases of CD19-targeted CAR-T in the treatment of B cell malignancies, for which the co-stimulation signal was CD28. All of the patients received chemotherapy before the CAR-T infusion and received an IL-2 infusion after the CAR-T infusion. Six patients achieved remission (Kochenderfer et al., 2012). One year later, Brentjens et al. published the results of their clinical trial at the Memorial Sloan-Kettering Cancer Center on the treatment of five acute lymphocytic leukemia (ALL) patients using CD19-targeted CAR-T with a CD28 co-stimulation signal. Chemotherapy was conducted before infusion and amazingly, the ALL patients achieved rapid depletion of leukemic cells and MRD (minimal residual disease negative)-complete remission; the start of the remission ranged from 8 to 59 d after the CAR-T infusion (Brentjens et al., 2013). In contrast to previous studies, Baylor College of Medicine enrolled and treated eight leukemia patients using CD19-targeted CAR-T with a CD28 co-stimulation signal. This was done without chemotherapy and before infusion. Two of the patients had already achieved CR before the CAR-T infusion, and among the other six patients, five showed a response to the CAR-T treatment (Cruz et al., 2013). using the same type of CAR-T, June et al. repeated their success in two children with ALL. About one month after infusion, both patients achieved morphological remission, and a clonal immunoglobulin heavy chain (IGH) rearrangement test showed that remaining ALL cells were cleared. Unfortunately however, 23 d after infusion, an IGH test found a malignant transformation in one child, and her leukemia relapsed two months after infusion. The relapsed cells were CD45dim⁺, CD34⁺

and CD19⁻, so it was inferred that the malignant cells had escaped via a CD19-loss mutation. Fortunately, the other child experienced continued remission (Grupp et al., 2013).

After 2014, studies with more cases began to be reported, and studies on the survival rate as well as a relapse analysis were also conducted. These studies mainly included researches at the University of Pennsylvania, the Memorial Sloan-Kettering Cancer Center, the Baylor College of Medicine and the National Cancer Institute (Table 1). Davila et al. reported fourteen complete remissions among sixteen patients with a CR rate of 88% (Davila et al., 2014). June et al. still used CD137 (4-1BB) as a co-stimulation signal and reported twenty seven out of thirty treated patients had achieved complete remission, resulting in a CR rate of 90%. The continued event-free survival rate after six months was 67%, and the total survival rate was 78%. At the sixth month after infusion, the rate of the existence of CAR-T cells was 68%. In 27 patients, morphologically complete remission had been achieved at one month after infusion, and in twenty two patients, flow cytometry analysis detected no malignant residues. Two patients with leukemic cells detected in the spinal fluid had their central nervous system (CNS) malignant cells eradicated after infusion and maintained complete remission beyond six months. The disease relapsed at six weeks to eight and a half months after infusion in seven CR patients. Among these seven, three patients were found to have lost their CAR-T cells two weeks to three months after infusion, and all of the remaining cells were CD19 positive. Four relapsed patients still maintained CAR-T cells. Along with normal B cell recovery from the second to third month, three patients relapsed. Three patients relapsed because of the loss of CD19 in the ALL cells. Among 27 CR patients, 19 achieved durable remission, and 15 patients did not accept any other treatment (Maude et al., 2014b). In 2015, Rosenberg and his workers reported CAR-T with CD28 treatment of B cell malignancies (Kochenderfer et al., 2015; Lee et al., 2015). Four complete remissions were achieved in seven diffuse large B lymphoma patients, and three of them achieved durable remission (beyond 9–22 months). All six chronic B cell malignant patients achieved complete or partial remission, and 3 of 4 CLL patients maintained long term remission. Among 20 ALL patients, eight had allogeneic hemopoietic stem cell transplants before the CAR-T infusion, and 14 patients showed a response to the infusion; the reaction rate was 70% in which twelve patients achieved MRD-negative complete remission. The 10 months event-free survival at a median follow-up of ten months in those patients was 51.6% at 9.7 months and beyond.

Non-CD19 targeted CAR-T in hematological malignancies

Above, we discussed clinical trials conducted with CD19 targeted CAR-T cells. Moreover, clinical trials of non-

CD19 CAR-T have also been explored (Table 2). Early in 2012, a “third generation” of CD20-specific CAR-T with CD28 and 4-1BB costimulatory elements was generated to treat three patients with lymphoma after cyclophosphamide lymphodepletion. All three patients showed remission after infusion, and two remained progression-free for more than 12 months (Till et al., 2012). In 2013, Ritchie et al. reported a trial of anti-LeY CAR-T coupled with CD28 in acute myeloid leukemia (AML). Following fludarabine-containing preconditioning, five patients were enrolled, and four patients received a cell infusion. Three of these four patients had evidence of a biological response to the CAR-T cell infusion, and one patient remained in remission for over 23 months (Ritchie et al., 2013). In 2014, Wang et al. designed CD20-specific CAR-T cells coupled with CD137 and enrolled seven patients with lymphoma. Five patients achieved tumor regression, and one patient with no bulky tumor burden attained ongoing complete remission for 14 months (Wang et al., 2014). Wang et al. reported another trial using anti-CD33 CAR-T and treated a 41-year-old male patient with AML. The patient showed a marked decrease of blasts in the bone marrow two weeks after therapy, but the disease had progressed at nine weeks after cell infusion (Wang et al., 2015). Compared with CD19-targeted CAR-T, published data on non-CD19-targeted CAR-T clinical trials did not

show the same excellent therapeutic efficiency, but did show that non-CD19 targeted CAR-T can make a difference in other hematological malignancies such as lymphoma and AML. Therefore, exploring better applications of CAR-T in non-B-cell-malignancies is an attractive and challenging field of study.

From the discussion above, we can see that second generation CAR-T with a co-stimulation signal, especially CD19-targeted CAR-T, can act efficiently in the treatment of hematological malignancy. In many studies, the response rates to CAR-T are above 50%. We believe that CAR-T is a major breakthrough in cell therapy as well as in the treatment of malignancies. Compared with the current therapies for hematological malignancy, CAR-T has more specific tumor-cytotoxicity and does not require a bone marrow match. However, monitoring and controlling side effects are still a disadvantage of clinical CAR-T applications.

Side effects in treatment of hematological malignancies

In general, CAR-T therapy is relatively safe for the treatment of hematological malignancy. However, CAR-T did indeed exhibit some extended side effects, mostly syndromes (Table 3). The primary side effect is cytokine release syndrome (CRS) also known as a cytokine storm, a syndrome caused by excessive cytokine release that is

Table 1 Summary of the main clinical trials conducted in CD19⁺ hematological malignancies^{a)}

Institution	UPenn	NCI	MSKCC	BCM
Registered code	NCT01626495 NCT01029366	NCT01593696 NCT00924326 NCT01087294	NCT00466531 NCT01044069	NCT00840853 NCT00586391
CAR endodomain	CD137+CD3 ζ	CD28+CD3 ζ	CD28+CD3 ζ	CD3 ζ vs. CD28+CD3 ζ
CD19 ⁺ Disease	ALL CLL	ALL CLL NHL	ALL CLL	ALL CLL NHL
Patients enrolled	44	52	24	14
Preconditioning	Variable	Flu/Cy or appendix vs. none*	Variable	None
Gene vector	Lentivirus	Retrovirus	Retrovirus	Retrovirus
Infused CAR ⁺ T cell number	1.4 $\times 10^7$ –1.1 $\times 10^9$	4.0 $\times 10^5$ –3.0 $\times 10^7$ kg ⁻¹	1.8 $\times 10^8$ –3.2 $\times 10^9$	2.0 $\times 10^7$ –2.0 $\times 10^8$ m ^{-2§}
Patients responded	35	33	15	6

a) CAR, chimeric antigen receptor. UPenn, University of Pennsylvania. NCI, National Cancer Institute. MSKCC, Memorial Sloan-Kettering Cancer Center. BCM, Baylor College of Medicine. CLL, chronic lymphocytic leukemia. NHL, non-Hodgkin lymphoma. ALL, acute lymphoblastic leukemia. Flu, Fludarabine. Cy, cyclophosphamide. *, In the trial registered as NCT01087294, all 10 enrolled patients had undergone allogeneic hematopoietic stem cell transplantation (alloHSCT) and did not take any depleting chemotherapies before receiving CAR-T cells infusion. §, Number of total cells infused.

Table 2 Summary of clinical trials conducted with non-CD19-targeted CAR-T in hematological malignancies

Identifier	Target	Disease	Status	Institute	Location
NCT02259556	CD30	Lymphoma	recruiting	Chinese PLA General Hospital	China
NCT01864902	CD33	Myeloid Leukemia	recruiting		
NCT01886976	CD138	Multiple Myeloma	recruiting		
NCT02274584	CD30	Lymphoma	recruiting	Peking University	
NCT01716364	LeY	Multiple Myeloma; AML; Myelodysplastic Syndrome	unknown	Peter MacCallum Cancer Centre	Australia
NCT02315612	CD22	Follicular Lymphoma; ALL; NHL; Large Cell Lymphoma	recruiting	National Cancer Institute	America
NCT01316146	CD30	Non-Hodgkin's Lymphoma; Hodgkin's Lymphoma	Active, not recruiting	Baylor College of Medicine	
NCT02159495	CD123	AML	recruiting	City of Hope Medical Center	
NCT02546167	BCMA	Multiple Myeloma	recruiting	Abramson Cancer Center of the University of Pennsylvania	

Table 3 Case summary of the main toxicities reported in clinic trials that are currently conducted^{a)}

Institution	NCI	MSKCC	Total
Patients enrolled	52	24	76
Fever	24	19	43
Hypotension	13	8	21
Hypoxia/ dyspnea	7	2	9
Kidney toxicity	4	1	5
Cardiac disorders	5	2	7
Respiratory disorders	6	1	7
Fatigue/rigors/ chills	3	7	10
Dysphasia/ obtundation	9	6	15
Headache	6	0	6

a) Cytopenia is frequently discovered after depleting chemotherapy and is not listed here. All of the adverse events listed here are Grade 3 or Grade 4. In trials at UPenn, all of the patients with ALL developed CRS in which eight patients had severe CRS. They did not report details of adverse events; thus, those studies at UPenn are not listed here. Patients treated at BCM showed few adverse events and are also not listed here.

usually observed in CAR-T-related therapy. In immunization therapy, immune system activation can become uncontrolled, which leads to a clinical reaction such as a systemic inflammatory response. Other reported side effects include tumor lysis syndrome, myalgia, neurotoxicity, B cell deficiency, capillary leak syndrome, respiratory distress syndrome, macrophage activation syndrome, diarrhea and coagulation disorders.

DIAGNOSIS AND MANAGEMENT OF CRS

Mild CRS does not require special treatment, but severe life-threatening CRS requires powerful intervention. Hence, the diagnosis and management of severe CRS are the key focuses of monitoring after infusion. Cytokines that were reported as significantly elevated after infusion are IFN- γ , IL-6, TNF- α , IL-2, GM-CSF, IL-10, IL-8, IL-5, and fractalkine (Davila et al., 2014; Grupp et al., 2013; Kalos et al., 2011; Kochenderfer et al., 2012). Three of these cytokines are thought to be highly related to CRS: IL-6, IFN- γ and TNF- α . IL-6 is an anti-inflammatory cytokine that has multiple effects and is thought to be a central regulatory factor in CRS (Lee et al., 2014). TNF is a potent factor that induces low blood pressure (Schiller et al., 1991). IFN- γ can induce fever, fatigue and myalgia (Foon et al., 1985; Vadhan-Raj et al., 1986). Potentially life-threatening complications of CRS include respiratory distress syndrome, cardiac dysfunction, neurologic toxicity, renal and/or hepatic failure and disseminated intravascular coagulation. Cardiac failure should be paid special attention (Maude et al., 2014a). Severe CRS can transform into macrophage activation syndrome (Grupp et al., 2013).

Knowing that excessive cytokine release is the cause of CRS, it is quite expected that we can predict CRS by monitoring cytokine levels. Considering that different individuals have different cytokine base values, it is better to compare increases in multiple cytokines instead of setting a threshold cytokine level. Brentjens et al. identified seven cytokines associated with CRS: IFN- γ , IL-5, IL-6, IL-10, Flt-3L,

GM-CSF and fractalkine. They thought that CRS should be considered severe if two of the seven cytokines' levels were elevated over 75-fold or one cytokine level was elevated over 250-fold. However, current clinical laboratory tests do not include all of these cytokines. Moreover, cytokine levels may be inconsistent with clinical signs because of the various individual sensitivities. Thus, they considered C reaction protein (CRP) as an ideal indicator. In their trials, if $>20 \text{ mg dL}^{-1}$ was set as the threshold for CRS, the sensitivity was 86%, and the accuracy rate was 100% (Davila et al., 2014). However, as an inflammatory factor, CRP can be affected by many factors and fluctuates frequently. The greatest interference is from infection, and it is difficult to distinguish infection from CRS using only CRP. In another study, only 50% of patients with $\text{CRP} > 20 \text{ mg dL}^{-1}$ showed severe CRS, and the authors suggested that the effectiveness of CRP in predicting CRS was associated with the CAR design (Maude et al., 2014b).

Currently, no laboratory test can precisely predict and diagnose CRS or reliably judge the severity of CRS depending on a patient's clinical signs. An article about CRS contains a standard for CRS grading and principles for its management. Grade 1 CRS mainly presents as a fever and other mild constitutional symptoms; grade 2 CRS presents as low blood pressure, which can respond to a single pressor drug or fluid infusion, anoxia responsive to $<40\%$ oxygen and $\leq$ grade 2 organ toxicity; grade 3 CRS presents as low blood pressure that requires multiple or high doses of pressor drugs, anoxia unresponsive to $<40\%$ oxygen and grade 3 organ toxicity; grade 4 CRS presents as grade 4 organ toxicity that requires mechanical ventilation. Additionally, grade 3 and 4 CRS require the use of Tocilizumab or steroids for symptomatic treatment (Maude et al., 2014a).

Steroids always play an important role in immune related diseases, and this applies to CRS management. A current report indicates that CRS can be quickly resolved after the use of steroids (Brentjens et al., 2013; Maude et al., 2014b). However, steroids can greatly damage CAR-T cells and even eliminate them. Patients managed with steroids always

relapse, and the relapse will still respond to CAR-T cells (Brentjens et al., 2013; Davila et al., 2014). The IL-6 receptor inhibitor Tocilizumab seems to be an excellent drug to resolve CRS without greatly affecting CAR-T cells. Experience with the clinical use of Tocilizumab has demonstrated good CRS control, and CRS remission usually occurs within 24 h (Davila et al., 2014; Grupp et al., 2013; Maude et al., 2014b). Other recommended drugs are anti-TNF- α mAbs (infliximab), soluble TNF- α receptor (etanercept) and IL-1R based inhibitors (anakinra) (Maude et al., 2014a).

Factors affecting side effects of CAR-T and its solution

Many factors may affect the side effects of CAR-T, and many factors still require more study. However, the plasma inflammatory cytokine level is definitely important. We surmise that the principal factors may be patient tumor load, infused cell dosage, chemotherapy before infusion, the patient's sensitivity to cytokines and the type of co-stimulatory signal.

Most studies support the view that under a heavy tumor load, the risk of CRS is higher (Brentjens et al., 2013; Kochenderfer et al., 2012; Lee et al., 2015; Maude et al., 2014b). Kochenderfer et al. suggested that the ratio of target cells should be decreased to reduce the risk of serious side effects induced by excessive cytokines, that is, treat patients with a lower tumor load or administer B cell-depleting pharmaceuticals such as Rituximab before cell infusion. A split-dosage and low infusion cell dosage are also recommended (Kochenderfer et al., 2012). "Kochenderfer's clinical results also showed that in ALL patients who responded to treatment, those with heavy tumor loads were prone to present grade 3 to 4 CRS. In six patients in whom grade 3 and higher CRS occurred, four patients showed the infiltration of over 50% malignant bone marrow cells. In those who did not respond to the CAR-T cells, none presented serious CRS, even with the infiltration of 95% malignant bone marrow cells. Brentjens et al. reported that cytokine elevation was directly associated with the tumor load and was also associated with fever after infusion. Two patients with the heaviest tumor load had the highest cytokine elevation. Four cytokines were the most elevated: sIL-2R α , IFN- γ , IL-6 and IFN-inducible protein 10 (IP10). The two patients with the heaviest tumor load experienced continued fever and low blood pressure as well as transient changes in their mental state. Depleting chemotherapy before infusion was also suggested to reduce the tumor load (Brentjens et al., 2013). In June's clinical trial, all of the patients treated showed CRS, and 27% of the patients (8/30) had serious side effects, which were thought to be associated with the tumor load as well (Maude et al., 2014b). However, a low tumor load did not equal absolute safety. Two patients showed 3 grade CRS with the infiltration of 5% and 7.7% bone marrow malignant cells. A patient's sensitivity to cytokines must also be considered (Lee et al., 2015).

The dosage of infused cells is also an important factor

related to side effects. Especially in trials in which chemotherapy is given before infusion, the dosage can greatly influence the severity and the time of appearance of side effects. After drastic chemotherapy, the risk of severe CRS is greatly increased. In a death report, patients who did not receive depleting chemotherapy received a 1.2×10^7 – 3.0×10^7 CAR-T cells kg^{-1} infusion and did not respond to the therapy. Afterwards, one patient received two days of cyclophosphamide chemotherapy and then received the same cell infusion dose as the three previous patients. The patient experienced drastic side effects that presented as sepsis-like low blood pressure and renal failure. Although the autopsy did not indicate that CAR-T cells were the direct cause of death, subsequent to that case, the infusion dosage was adjusted to 1/3 of the previous dosage and was split over two days. The new treatment regime was not as dangerous (Brentjens et al., 2011). Another case of death occurred several minutes after infusion with an order of magnitude of 10^{10} cells (Morgan et al., 2010). In later clinical trials, dosage over 10^{10} was rarely reported.

Compared with target differences, the effects of various co-stimulatory signals may play a more important role in side effects, although more studies are still necessary. The most used co-stimulatory signals are CD28 and CD137 (4-1BB). In pre-clinical studies, it was observed that CD137 caused less secretion of IL-2 and TNF- α (Milone et al., 2009), and *in vivo* IL-2 and TNF- α did not significantly increase. So, the researchers indicated that using CD137 instead of CD28 may be a strategy to reduce toxicity of cytokines (Kalos et al., 2011). However, in clinical trials using these two co-stimulatory signals, severe side effects have been reported. In CLL patients, a reaction to cytokines with CD137 occurred slightly late, usually ten days later. As for CD28, side effects occurred more quickly, within 3–5 d (Brentjens et al., 2013). The differences in side effects and the time of occurrence due to co-stimulatory signal selection still need further study.

Moreover, the different sensitivities of patients to cytokines may offer an explanation as to why some patients presented severe CRS at a low tumor load and low infusion dosage. In a trial, one patient who developed severe CRS following CD19-CAR therapy for ALL was found to carry a mutation in the perforin gene. It is believed that this is what predisposed the patient to macrophage activation syndrome (HLH) (Lee et al., 2014). This suggests that different gene expression levels may have enormous influence.

CAR-T related death

Although CAR-T is thought to be a relatively safe treatment, deaths in trials have still been reported. There have been three deaths reported in CAR-T related clinical trials (Brentjens et al., 2010; Kochenderfer et al., 2015; Morgan et al., 2010). The first death reported was a patient who received two days cyclophosphamide chemotherapy (1.5 g m^{-2}) before CAR-T infusion. After the CAR-T infu-

sion, the patient presented low blood pressure, dyspnea and renal failure. 20 h after infusion the patient had a continued fever accompanied by low blood pressure. Then, acute renal failure appeared, and the patient became anuric. In intensive care unit (ICU), pressure support was conducted, and the low blood pressure was resolved. However, the anuria continued and was accompanied by a gradual increase of serum potassium and serum phosphate. The patient died four days after infusion. However, the autopsy did not indicate cytokine release syndrome as the cause of death. Finally, the patient was thought to have died of infection after immunosuppression, instead of from the CAR-T therapy itself (Brentjens et al., 2010). In 2015, Rosenberg reported a death case in which CD19-targeted CAR-T cells with CD28 were used. The patient died of an unknown cause 16 d after infusion without any symptoms of cytokine release syndrome. Considering that the patient had underlying cardiac disease, the cause of death was thought to be a heart disorder, so the CAR-T therapy had no relationship to the cause of death (Kochenderfer et al., 2015). Another death case report was directly related to CAR-T but did not occur with a hematological malignancy. Morgan et al. designed an ERBB2 targeted CAR-T with CD28, 4-1BB and CD3 ζ signal molecules. A patient with a colon cancer metastasis in the lungs and liver received a CAR-T infusion at a dosage of 10^{10} in which 79% of the cells were CAR-T cells. The patient immediately exhibited respiratory failure 15 min after infusion, and chest radiography indicated an exudate in the lungs. The patient died 5 d after the infusion. During the progression of side effects, the cytokines were elevated; therefore, the cause of death was thought to be directly related to CAR-T. It seems that pulmonary epithelial cells also express a small amount of ERBB2 and thus are attacked by CAR-T cells (Morgan et al., 2010). Therefore, in the treatment of hematological malignancy, no cases of death were reported as directly caused by the CAR-T therapy itself. However, caution must be taken to prevent infection during treatment.

FACTORS AFFECTING THE THERAPEUTIC EFFECT OF CAR-T

Lymphocyte-depleting chemotherapy before infusion

The role of depleting chemotherapy before CAR-T cell infusion has been gradually recognized for the following reasons: (i) to reduce the tumor load and thus decrease the risk of severe side effects and (ii) to deplete some lymphocytes and secure enough proliferation space for the infused CAR-T cells (Cui et al., 2009; Kohn et al., 2011). A study revealed that lymphatic clearance is closely related to therapeutic effects (Wrzesinski et al., 2010). In a trial that aimed to compare the therapeutic efficiency between the first and second generation CAR-T, all six NHL patients did not receive chemotherapy before infusion. No patients

showed obvious reaction to either first or second generation CAR-T therapy (Savoldo et al., 2011). We previously discussed the death case reported by Brentjens et al. Patients who did not receive depleting chemotherapy before infusion did not show an objective reaction to CAR-T, but a fourth patient had drastic side effects at the same dose of CAR-T cells because the patient received two days of cyclophosphamide chemotherapy before infusion. After the death, the other patients continued to receive pre-infusion chemotherapy but at a lower dose. Then, the patients began to achieve remission (Brentjens et al., 2011). This suggests an association between side effects and therapeutic effects. Brentjens showed no relationship between CRS and anti-tumor efficiency (Brentjens et al., 2013). However in Rosenberg's trial, patients who experienced the best therapeutic efficiency all presented CRS (Kochenderfer et al., 2013). Maude et al. also suggested that some degree of CRS might be necessary for the anti-tumor effect (Maude et al., 2014b). The relation between CAR-T therapy and the anti-tumor effect still requires further study. When selecting a chemotherapy strategy, a balance must be struck: mild chemotherapy limits proliferation of CAR-T cells, while, on the other hand, drastic chemotherapy may drive CAR-T cells to proliferate too quickly and increase the risk of severe CRS.

The design of CAR influences the efficacy of treatment

The long-term existence of CAR-T cells (>4 weeks) and a high interferon- γ (peak value >200 pg mL⁻¹) are associated with better clinical outcomes (Xu et al., 2013). In patients who achieved continued remission, CAR-T cells and their proliferation can always be stably detected. (Maude et al., 2014b). The longest time of CAR-T endurance reported was over four years (Porter et al., 2015), and the persistence of CD19-targeted CAR-T is always accompanied by a loss of B cells. Because many relapsed patients have B cell recovery, it is an important consideration to extend the time of CAR-T cell persistence to prevent relapse. The addition of a co-stimulatory signal caused the second generation CAR-T cell breakthrough as compared with the first generation CAR-T cells. Second generation cells with a CD28 co-stimulatory signal stably proliferated two weeks after infusion, but CAR-T cells without a co-stimulatory signal did not proliferate and disappeared after six weeks. (Savoldo et al., 2011). At present, CD28 and CD137 (4-1BB) are the two signals used most commonly and it is found that they exhibit good therapeutic effects. Different studies yield diverse opinions about these two signals. In Brentjens's pre-clinical research, the anti-tumor effect of CD137 co-stimulatory was not as good as CD28 (Brentjens et al., 2007; Zhong et al., 2010). However, in other studies, CD137 was thought to have better persistence and tumor-cytotoxicity. To verify the respective advantages of these two signals, more clinical research is still necessary.

Cytokine is a double-edged sword

Although excessive cytokines are the main cause of CRS, cytokines play an important role in the function of CAR-T cells. IL-2 can improve the proliferation of T cells (Caserta et al., 2010), and CAR-T cells able to secrete IL-2 and IL-15 were demonstrated to have better tumor-cytotoxicity in pre-clinical experiments (Hoyos et al., 2010; Pegram et al., 2012). IL-2 facilitated tumor-specific T cells to generate IFN- γ (Overwijk et al., 2003), and IFN- γ was highly related to CAR-T tumor-cytotoxicity. Therefore, a clinical strategy was to infuse IL-2 after infusing CAR-T cells; among eight patients thus treated, six patients achieved remission (Kochenderfer et al., 2012). Nevertheless, in many clinical trials later, clinicians did not administer IL-2 after CAR-T infusion and still good remission was achieved. A study indicated that the use of exogenous IL-2 had no relationship with CAR-T cell persistence and proliferation (Xu et al., 2013). This may be because CAR-T cells themselves can secrete cytokines. Thus, whether exogenous cytokines are necessary and which cytokines may be necessary still require further study.

A heavy tumor load may decrease the therapeutic effect

We have previously indicated that a heavy tumor load is related to a higher risk of side effects. A heavy tumor load may play a negative role on the therapeutic effect of CAR-T. A study observed that patients with a heavy tumor load were more likely to eliminate their infused CAR-T cells as compared with those with a lower tumor load. This phenomenon seems contradictory to the phenomenon that CAR-T cells proliferate better under antigen stimulation (Brentjens et al., 2011). Moreover, another study indicated that a heavy tumor load may render CAR-T treatment ineffective (Lee et al., 2015). The necessity of targeted cells for the maintenance of CAR-T cell numbers was also mentioned in another study (Kalos et al., 2011). However, why a heavy tumor load made CAR-T cells prone to elimination under antigen stimulation was not definitely explained.

FUTURE STUDIES

We have mentioned the breakthrough of second generation CAR-T in hematological malignancy. The important role of CAR-T therapy is becoming more greatly recognized by researchers. In many trials, the remission rate can be above 50% as compared with the Food and Drug Administration (FDA) approved conventional treatment for refractory ALL in which the complete remission rate is below 25%. At present, chemotherapy for leukemia treatment has serious side effects and limited tumor-cytotoxicity. Hematopoietic stem cell transplantation requires a bone marrow match and is expensive. CAR-T therapy is a targeted therapy which does not require a bone marrow match. It is highly efficient and specifically kills malignant cells. Another advantage is that CAR-T cells can persist *in vivo* and therefore preserve the

ability to prevent a recurrence. It is highly possible that, in addition to chemotherapy and hematopoietic stem cell transplantation, CAR-T can become a conventional treatment in hematological malignant disease. However, the role of CAR-T is currently uncertain. Whether it should be used as an independent therapy or as a bridge between chemotherapy and hematopoietic stem cell transplantation still needs further study.

The efficiency and side effects seem mutually antagonistic, and balancing them is a key challenge. Based on experience acquired in early CAR-T trials, it is believed that chemotherapy before infusion which can both reduce the tumor burden and deplete lymphocytes, decreasing the cell dose infused and a split-dose infusion strategy might be the most direct ways to guarantee safety without overly affecting tumor-cytotoxicity. Considering that CAR-T cells preserve their proliferation ability *in vivo*, a high cell dose does not seem necessary. A dosage of 10^5 cells kg^{-1} has even been reported as effective. On the cellular level, optimizing the structure of the co-stimulatory signal and target as well as including suicide genes might also improve security and efficiency.

Whether the great improvement of CAR-T therapy in hematological malignancies can also be replicated in solid tumors is a question worth considering. However, CAR-T does not achieve the same success in solid tumors as it does in hematological malignancies. Summarizing the results for hematological malignant disease, relevant factors might be: (i) solid tumor cells are more concentrated than hematological malignant cells, so the contact surface between the malignant cells and CAR-T cells may be limited; (ii) solid tumors contain a heavy immunosuppressive environment, and the penetration of CAR-T cells into solid tumors is limited; (iii) no specific target is present in solid tumors.

In the context of “precision medicine”, CAR-T can act as a terminal stage in precision medicine practice. At present, CAR-T cells are designed with a standard target, but different patients have various types of mutant cells. Rooted in an analysis of large data and patient sequence data, designing a particular CAR-T for each individual patient could create more precise CAR-T with high tumor-cytotoxicities. Such a strategy could make CAR-T therapy a powerful treatment in malignant disease.

Compliance and ethics The author(s) declare that they have no conflict of interest.

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