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• REVIEW •

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Establishing guidelines for CAR-T cells: challenges and considerations

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T cells, genetically modified by chimeric antigen receptors (CAR-T), are endowed with specificity to a desired antigen and are cytotoxic to cells expressing the targeted antigen. CAR-T-based cancer immunotherapy is a promising therapy for curing hematological malignancy, such as acute lymphoid leukemia, and is promising for extending their efficacy to defeat solid tumors. To date, dozens of different CAR-T cells have been evaluated in clinical trials to treat tumors; this necessitates the establishment of guidelines for the production and application of CAR-T cells. However, it is challenging to standardize CAR-T cancer therapy because it involves a combination of gene therapy and cell therapy. In this review, we compare the existing guidelines for CAR-T cells and discuss the challenges and considerations for establishing guidance for CAR-T-based cancer immunotherapy.

chimeric antigen receptor, CAR-T cells, guideline, cancer immunotherapy

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INTRODUCTION

Immunotherapy has long been a research focus for cancer treatment. The adoptive transfer of immune cells is efficacious for inhibiting the growth and invasion of cancer cells (Rosenberg et al., 2008). Cytotoxicity elicited by effector cells contributes to tumor-killing efficacy. By producing lymphokine-activated killers, cytokine-induced killers, and selecting and expanding tumor-infiltrating lymphocytes, researchers have developed and significantly advanced the application of cancer immunotherapy, including substantially enhancing the quality of effector cells, for sophisticated clinical utilization. Since the end of 1990s (Gilham et al., 2012; Gross et al., 1989; Kuwana et al., 1987), T lymphocytes have been genetically equipped with chimeric antigen receptors (CAR), by which the specificity of T cells can be modulated to a desired antigen. Distinct from unmodified effector T cells, CAR-modified T (CAR-T) cells exert tumor-killing responses in a human leukocyte antigen-independent manner. Another effector cells, Natural Killer cells (NK) are redirected by CARs (Li et al., 2015). With the development of the first generation CAR construct through the third generation, various CAR-T cells have been generated to treat different tumors. Currently, more than 50 clinical trials involving CAR-T cells to treat cancers are under phase I/II studies. Notably, CAR-T cells with CD19 specificity showed high efficacy for the treatment of refractory/relapsed hematological malignancies (Brentjens et al., 2013; Grupp et al., 2013; IKalos et al., 2011; Kochenderfer et al., 2010; Porter et al., 2011). CAR-T-based cancer

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immunotherapy, along with monoclonal antibodies of immune checkpoints, led a scientific breakthrough in 2013 (Couzin-Frankel, 2013).

NECESSITY OF GUIDELINES FOR CAR-T CELLS

It is widely accepted that medical drugs should be regulated by specific administration under set guidelines. Guidelines for medical drugs should be developed by regulation departments as well as experienced researchers and physicians. Well-established guidelines warrant the quality of the product and guarantee the preliminary requirement for safe and efficacious applications. Importantly, guidelines support the advancement of related techniques and accelerate the application of regulated products. For CAR-T cells, guidance from administration under set guidelines is essential. Different academic institutions generated CAR-T cells with their own modifications, leading to variations in the phenotype, biological potency, and other characteristics. Therefore, it is important to establish guidelines for mandating preliminary requests for CAR-T cells and the production process. Indeed, it is challenging to establish well-accepted guidelines for the entire CAR-T industry because clinical data of CAR-T cells with distinct phenotypes or production methods often show equal efficacy. The guidelines, therefore, are regulations describing the minimal and fundamental criteria for production, in-process releasing tests, and certificate analysis (Gee, 2015).

EXISTING GUIDELINES SUITABLE FOR CAR-T CELLS

Unlike traditional "off-the-shelf" drugs, CAR-T cells have unique features. CAR-T cells are somatic cells that are under the regulation guidelines for cell therapy. Their genetic modification categorizes them gene therapy products. To date, more than 70 clinical trials have been registered, with 46 trials in the United States and 16 trials in China (data according to www.clinicaltrials.gov). These CAR-T cells must be regulated by domestic guidelines.

The Food and Drug Administration (FDA) in United States has classified CAR-T cells as 351 biological products and regulates them through "Considerations for the design of early-phase clinical trials of cellular and gene therapy products" and "Guidance for industry: preclinical assessment of investigational cellular and gene therapy products", dated November 2013, direct by the Office of Cellular, Tissue, and Gene Therapies in the Center for Biologics Evaluation and Research. Additionally, CAR-T cells should meet the criteria for chemistry, manufacturing, control (CMC) information following the released guidance "Guidance for FDA reviewers and sponsors: content and review of chemistry, manufacturing, and control (CMC) information for human somatic cell therapy investigational new drug applications (INDs)"; dated April 2008 and "Guidance for FDA reviewers and sponsors: content and review of chemistry, manufacturing, and control (CMC) information for human gene therapy investigational new drug applications (INDs); dated April 2008". These regulations direct the manufacturing, in-process control, release testing, and quality control of the CAR-T cells.

In Europe, the medical administration is regulated by the European medicines agency (EMA). The issued guideline "Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells", finalized in 2012, is suitable for CAR-T cells. This guideline defines the scientific principles and provides guidance for the development and evaluation of medicinal products containing genetically modified cells intended for use in humans and presented for marketing authorization. The focus of this guideline is on the quality, safety, and efficacy requirements of genetically modified cells developed as medicinal products. In addition, the manufacture of CAR-T cells should be regulated by the basic guidelines for medical products, including the regulation on advanced therapy medicinal products No 1394/2007 and guidance on quality, preclinical and clinical aspects of gene transfer medicinal products (CPMP/BWP/3088/99). Distinct from FDA's guidelines, EMA issued specific documents to regulate genetically modified cells as medical products.

The China Food and Drug Administration issued a panel of guidelines for gene and cell therapy. In 2003, a guideline named the "Guideline for research and quality control technology of human gene therapy products" was issued. This document defines the basic requirements for medical products under consideration for clinical trials. In this guideline, medical products are specified as genetically modified cells and vectors (viral and non-viral) carrying functional genes. CAR-T cells products should be regulated by this guideline. As a cellular therapy products, CAR-T cells should also be regulated by the "Guidelines for research and quality control technology of human somatic cellular therapy products", issued in 2003. For clinical application, CAR-T cell products are regulated by the universal guideline for drugs applying for clinical trials, the "Quality management standard for drugs applying for clinical trials", issued by the China Food and Drug Administration in August 2003. In fact, CAR-T cells have some unique characteristics that are beyond the regulation of current guidelines, but are necessary for their safety and biological potency, such as the transduction efficiency. With the release of guidelines unique for stem cells for clinical trials in 2015, administrative documents specific for CAR-T cells are expected to be issued in China.

Nations allowing clinical trials of CAR-T cells issue their own domestic guidelines. To merge their regulatory experiences, interexchange between administrations of different nations often occurs, such as between the FDA and EMA. It is clear that CAR-modified T cells should be regulated by

different guidelines within one nation, and there are specific administrative documents suitable for CAR-T cells. A good guideline should guarantee the requirement for safety and enable maximum efficacy.

KEY ASPECTS OF CAR-T CELL PRODUCTS MUST BE REGULATED

The production of CAR-T cells involves vector production, modification of T lymphocytes, and expansion of CAR-T cells. Standard operation protocols should be established and good manufacturing practice facilities are required. During the entire production process, detailed in-process control parameters and release testing standards are defined to regulate the production of CAR-T cells (Figure 1). Before modifying T cells, vectors carrying CAR genes have to be produced. The uniformity and homogeneity are determined, and the T cells are separated. The entire process is monitored in two parts, a vector-producing part and a CAR-T cell-producing part. The first part is typically regulated by the guidelines issued for gene therapy products, while the second part is always regulated by the guidelines issued for cellular therapy and gene therapy. Although there are numerous minor differences between the guidelines regulating CAR-T cells in different nations, the main aspects to be regulated are similar.

Materials

All materials for CAR-T cells production (including the reagents used for vector production) should be clinical grade if available. The materials and reagents should be high-quality, including testing of sterility, and free of endotoxins and adventitious contaminations. Detailed information regarding the materials should be recorded, including the supplier, lot number, and expiration date. If the vector used to transduce T cells is provided by a manufacturer, detailed information should be provided along with the vectors. Culture media should be serum-free if possible. If the serum is indispensable, documents validating the necessity of the serum should be provided. Similarly, it is strong-

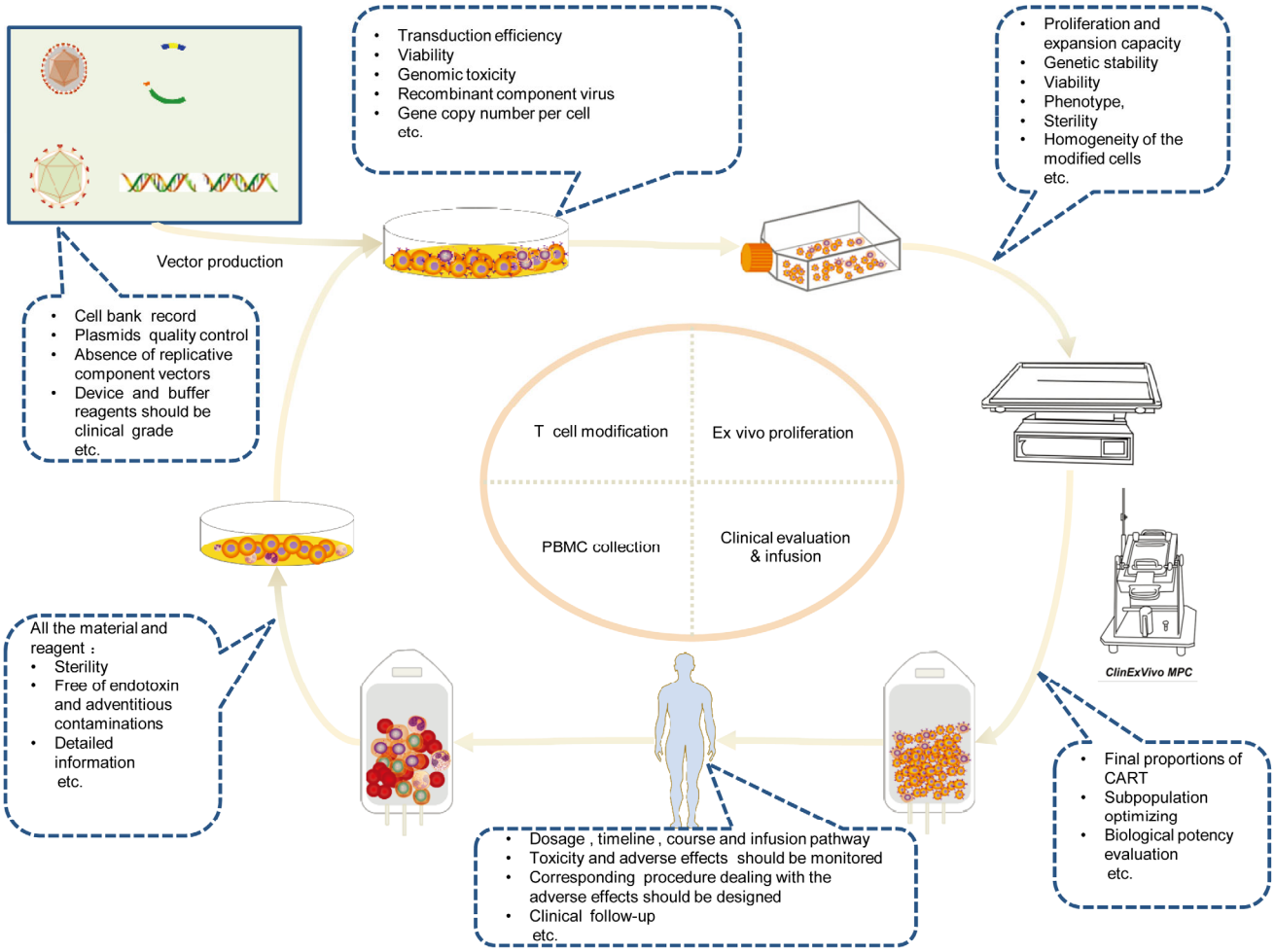

Figure 1 Schematic illustration of the CART manufacturing process and the quality control of each step regulated by current guidelines. Peripheral blood mononuclear cells are selected from whole blood and activated. Activated T cells are transduced with CAR retroviral/lentiviral vectors or transposons/mRNA. Modified cells are expanded using an appropriate bioreactor, such as a WAVE bioreactor. Magnetic Dynabeads are removed from the cells with MPC and then the cells are evaluated for infusion. Each step regulated by the guidelines is boxed with a dotted line.

ly recommended that other additives such as cytokines, antibiotics, and others, be removed.

Vector production

Viral vectors, including lentivirus and retrovirus, are the most commonly used vectors for transducing T cells into CAR-T. Electroporation of mRNA, DNA, and the transposon/transposase system also showed efficient transduction of T cells. When used with viral vectors, the production, collection, and purification steps should be monitored. Banking systems should be established where the master cell bank and working cell bank are requisites. Quality control of the plasmids used to generate single infectious pseudo-virus must be validated and plasmid constructs should be verified before the production step. Appropriate purification technology should be established to meet the releasing criteria under the guideline of gene therapy products. Finally, quality control of the vectors should be validated, including testing for sterility, endotoxins, identity, purity, absence of replicative component vectors, genome integration manner, and infectious potency.

When used with electroporation, the quality control of the mRNA or DNA plasmids is mandated. The device should be clinically applicable, and the buffer reagents should be clinical grade if possible.

Modification of T cells

Viral and non-viral modifications cause genomic toxicity (Verma and Somia, 1997). Viral vectors always integrate transgenes into T cells, and the absence of insertional mutagenesis and recombinant virus components should validated (Hacein-Bey-Abina et al., 2003), although previous studies have demonstrated non-preferred integration sites by lentiviral vectors (Aiuti et al., 2013; Biffi et al., 2013). After the emergence of the recombinant virus component, the entire batch should be discarded. In order to minimize genomic toxicity, a minimal multiplicity of infection is suggested, provided that the transduction efficiency is not affected by the use of viral vectors. Viability, phenotype, sterility, and homogeneity of the modified cells must be determined. Transduction efficiency should be determined by flow cytometry or equivalent methods. The gene copy number per cell should be determined. In addition, the *ex vivo* proliferation and expansion capacity and genetic stability of the modified cells are recommended to be determined.

Clinical considerations

Clinical considerations are the final step of the CAR-T cell application. Well-established regulations facilitate the clinical management and benefit maximization of CAR-T based therapy. Approval from national or local ethics committees is a preliminary requisite. Inclusion and exclusion criteria must be established before the start of the trial. Any modifications to the criteria should be approved by the committee and recorded appropriately. Detailed information regarding the patients should be recorded over the long-term, such as 20–30 years. Dosage, timeline, course, and infusion pathway must be recorded. Efficacy should be evaluated appropriately. Toxicity and adverse effects must be monitored during the entire course or longer, and the corresponding procedures for dealing with adverse effects should be well-designed. Clinical follow-up is required for clinical trials of CAR-T cell therapy to monitor the efficacy and early or delayed risks.

CHALLENGES TO ESTABLISHING GUIDELINES

Typically, guidelines are established as universal regulations that determine the basic quality standard for medical products. The threshold of criteria should not be so high that application of the CAR-T therapy is limited, but the quality standard should be as high as possible to minimize risks. It is therefore challenging to devise well-accepted guidelines for CAR-T cells and there are always dilemmas when designing the guidance. A balance between encouragement and restriction is required. The following aspects presented challenges, due to uncertainty and complexity of CAR-T cells, when establishing the guidelines and will be optimized with the development of CAR-T cell-based immunotherapy.

Transduction efficiency and optimal population

The percentage of CAR-positive T cells is an important parameter of CAR-T cell products. Current guidelines require determination of transduction efficiency by flow cytometry or equivalent pathways. However, whether the *in vivo* efficacy is related to transduction efficiency or the appropriate level of efficacy is controversial. Previous studies indicated that transduction less than 10% had a strong and persistent anti-tumor response (IKalos et al., 2011; Porter et al., 2011). Thus, there is no threshold for transduction efficiency in all existing guidelines, although recording this parameter is required.

It is technically easy to produce pure CAR-positive T cells by incorporating antibiotic-resistant genes into the CAR vector and selecting CAR-T cells with the corresponding antibiotics. However, incorporation of antibiotic-resistant genes is not suggested in issued guidelines; hence, few CAR-T cells under clinical trials have incorporated these genes. Since $CD8^+$ effector cells show efficacy in the immune response, it is reasonable that infused $CD8^+$ CAR-T cells have superior efficacy compared to other populations, but previous studies demonstrated that $CD4^+$ CAR-T cells are essential for the tumor-killing efficacy (Tsuji et al., 2005). Recent studies suggested that CAR-T cells bearing the phenotype of central memory effector cells are likely key for the efficacy because of their improved *in vivo* survival and persistence (Riddell et al., 2014), although others reported that naïve memory cells are more potent

(Hinrichs et al., 2010). It remains to be determined whether there is an optimal infused population of CAR-T cells. It is suggested that the regulatory T cells subset be removed because they inhibit CAR-T function (Lee et al., 2011; Yao et al., 2012). Increasing evidence has suggested that immune-checkpoints, such as programed cell death protein 1 and cytotoxic T-lymphocytes-associated protein 4, inhibit the potency of CAR-T cells (Deng et al., 2014; Peng et al., 2012). Whether these checkpoints in CAR-T cells can be used to predict the clinical response, and whether it is necessary to establish a threshold for the expression level of these immunosuppressive checkpoints on CAR-T cells in the guidelines are under consideration.

Variations

Unlike "off-the-shelf" products, CAR-T cells are personalized medical products. From collecting T cells to infusing final CAR-T cells into patients, it is difficult to establish universal standards because of patient-to-patient variation. Populations of apheresis products from different patients vary and transduction efficiencies differ even when the same multiplicity of infection is used. After CAR modification, different proliferation capacities emerged, leading to varying *ex vivo* culture times to obtain a specific number of infused cells.

Scale-up production of vectors and stable aliquot storage are strongly recommended to maintain the transduction potency. However, gradually decreasing potency is common over extended storage time, resulting in variations in transduction efficiency.

Biological potency

The issued guidelines must state that the biological potency of CAR-T cells must be tested before infusion. Lytic activity or the release of inflammatory cytokines when encountering target cells is frequently used as an indicator of the biological potency of CAR-T cells. Current guidelines do not specify the methods that should be used to determine the biological potency of CAR-T cells. There are always discrepancies between *ex vivo* and *in vivo* efficacies because of various factors, necessitating comprehensive evaluation of the *ex vivo* biological function. Both lytic function and cytokine release should be tested. Many modifications have been made to CAR-T cells, including the ability to release interleukin-12 or other cytokines and chemokine receptor overexpression (Chinnasamy et al., 2012; Chmielewski et al., 2011; Moon et al., 2011; Pegram et al., 2012). These modifications increase the complexity of release testing for CAR-T cells. The expression levels of molecules that enhance the biological potency of CAR-T cells must be determined, and universal, well-accepted methods should be established.

Clinical challenges

Clinical considerations are important for the efficacy of CAR-T cells, including the dosage, pre-conditioning, clinical efficacy evaluation, follow-up, toxicity classification and approach, bio-distribution, and other factors. Some of these considerations are specified in the current guidelines and some are not. Moreover, with the development and optimization of CAR-T cells, the regulations for these factors should be updated in the guidelines.

In phase I clinical trials, infused CAR-T cells are typically split into three dose (10%, 30%, and 60%) in an increasing manner and infused over three days. All CAR-T cells can be infused into the patient at once. The total number of CAR-T cells infused varies from 10^8 to 10^{10}, calculated based on the weight or body surface area. The number of infused CAR-T cells can also be determined by the CAR-positive population. However, in current guidelines, there are no suggestions regarding the dosage, design, and amount needed for infusion. It is challenging to provide detailed requirements for these factors since the optimal dosage, design, and calculation method for infused CAR-T cells are unclear.

Previous studies demonstrated that pre-conditioning substantially improves the clinical response to cell therapy. Pre-conditioning, which always involves a non-myeloablative chemotherapy regimen, creates a therapeutic "window" for CAR-T cells as it removes various suppressive factors that inhibit anti-tumor activities. In the current guidelines suitable for CAR-T cells, pre-conditioning is not required. Given the increasing amount of pre-clinical and clinical data demonstrating the enhancement of efficacy, nearly all recently registered clinical trials have adopted pre-conditioning as a standard strategy prior to CAR-T cell infusion. It is thought that future updates of the guidelines will take pre-conditioning into consideration.

It is challenging to include clinical efficacy evaluation into the guidelines for CAR-T cells. The response evaluation criteria in solid tumors (RECIST) are a set of published rules that define improvement conditions for cancer patients after receiving a specific treatment. RECIST was developed and published in 2000 and updated in 2009. Most worldwide clinicians determine efficacy using these criteria. Although the current guidelines suitable for CAR-T cells have no specific efficacy evaluation guidelines, it is strongly recommended that potency be evaluated by RECIST. For non-solid malignancy, corresponding criteria are needed. Clinical follow-up is essential for CAR-T cell-based therapy. As a gene therapy product, a long-term (15 years or longer) follow-up schedule should be established to evaluate efficacy and safety.

Toxicity is an important consideration in clinical trials, particularly in early phase trials. Current guidelines require investigators to design comprehensive and detailed evaluation criteria for toxicity, including classification of toxicity with different grades and well-designed strategies for dealing with each grade. The toxicity of CAR-T cell-based immunotherapy varies and can be classified as "on-target",

"on-target, off tumor", and "off-target" adverse effects (Wang and Wang, 2012). Cytokine release syndrome, or cytokine storm, causes adverse effects. Steroids and other immunosuppressive drugs must be prepared in cases of cytokine release syndrome and severe adverse effects. The monoclonal antibody blocking interleukin-6 receptor significantly reduced the risk of CAR-T cell-based immunotherapy (Grupp et al., 2013). Other monitoring and care should also be taken into consideration.

OUTLOOKS

CAR-T cell-based cancer immunotherapy is a promising treatment for cancer. With the rapid enrollment in current clinical trials, it is urgent to establish and optimize guidelines suitable for CAR-T cells. Given the complexity and novelty this therapy, such work is challenging. Currently, there are no guidelines specific for CAR-T cells, but the guidelines issued for gene and cell therapy products can be utilized. Using the extensive findings and experiences gained from pre-clinical investigations and clinical trials, guidelines for CAR-T cells can be updated and optimized to provide substantial clinical benefits to cancer patients.

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

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