CAR T-cell immunotherapy: A safe and potent living drug technique for cancer treatment (Review)

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Received April 4, 2023; Accepted August 18, 2023

DOI: 10.3892/wasj.2023.200

Abstract. For several decades, surgery, chemotherapy and radiation therapy have been the fundamental components of cancer treatment. Although these represent key therapeutic strategies, novel forms of medical treatment recently triggered an improvement in the methods with which cancer sufferers are being treated, namely with chimeric antigen receptor (CAR) T-cells. CAR T-cell immunotherapy is all set for serving the new prospect in cancer treatment. It utilizes the underlying immune potential to enhance the T-cell antigen recognition property and minimize the cytotoxicity level by engineering. CAR T-cell immunotherapy exerts minimal side-effects compared to the other available methods, such as hematological and solid cancer treatment. The Food and Drug Administration approved these 'biologically active living drugs' and highlighted the impact of this immunotherapy. Therefore, scientists are working to produce highly efficient CAR T-cells with minimal side-effects. The present review discusses the role of various generations, including the next generation of the CAR T-cells, their significance and the effects on cancer patients.

Contents

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Key words: chimeric antigen receptor T-cell antigen, living drugs, cancer treatment, generation, cytokine and immunotherapy

1. Introduction

One of the major recent advancements which have provided hope to patients with leukemia/lymphoma cancer involves the use of cells from the patient's own body or donor for the treatment of persisting cancer. T-cells are separated from the patient's body via leukapheresis. The chimeric antigen receptor (CAR) cells are engineered hybrid T-cell receptors (TCRs) which are a combination of antibody and TCRs (1). CAR has a dual function: One is to bind with the tumor antigen and the other is T-cell activation functions (2). This artificial receptor is comprised of a single-chain variable fragment (scFv) of antibodies, linkers, a transmembrane domain and an intracellular signaling domain. Normally, T-cells have variable α-chain and β-chain, and a miniscule quantity of T-cells have γ- or δ-chains and the antibody consists of two heavy chains and two light chains with one constant and one variable domains in each chain. The scFv is a heterodimer of variable heavy (VH) and variable light (VL) domains (3). Thus, the scFv antibodies which are used in this construct are produced by the hybridoma technique, having specificity against a particular cancer antigen (4).

To genetically modify T-cells, mRNA is isolated from hybridoma cells, then reverse transcribed into complementary DNA (cDNA), which then serves as a template for amplification. This cDNA is amplified by PCR and inserted into the TCR gene of the isolated T-cell (5). This construction is performed by delivering the stable CAR expression gene, either by viral or non-viral mode of gene transfer systems. The most commonly used expression vectors are lentiviral vectors, σ-retroviral vector and the transposon system (6). This genetically engineered TCR gene expresses the CAR, having defined specificity. The replacement of the both the variable α and β domains of the TCR with VH and VL immunoglobulin homologs results in the CαVH + CβVL or CαVL + CβVH immunoglobulin composition. In the 1980s, scientists designed CAR by the addition of genes coding for artificial T-cell receptor-like proteins. An effective signal for T-cell activation

is transmitted by the chimeric receptors, being expressed on the T-cells. The substitution of variable TCR region with the antibody homologs have been proven effective by endowing antibody type specificity (7). In 1993, Eshhar *et al* (8) constructed the T-cell receptor genes by combining the antibodies variable domain and T-cell receptor's constant domain. These chimeric T-cells are customized according to the target epitope binding in a particular cancer. They effectively bind to the specific epitope in a human leucocyte antigen (HLA) in an independent manner, which benefits patients with a decreased expression of the HLA gene. Upon specific interaction of the antigen and the CAR T-cell receptor, the signaling cascade is turned on, resulting in tumor elimination. This therapy can combat a number of issues associated with chemotherapy and RNAi technology, or even the issues associated with the treatment of immunocompromised patients. The overall goal of developing this therapy is to elucidate an effective immune response, which is generated by cytokine production (9). Although there are both pros and cons associated with this therapy, continuous efforts are being made to neutralize and balance these to make it more suitable for use in treatment.

2. Reasons for the use of CAR T-cell immunotherapy

Recently, immunotherapy has been viewed as a useful and alluring therapeutic strategy in a variety of malignancies, including colorectal cancer. Chimeric antigen receptor (CAR) T-cell and CAR-natural killer cell therapy are two immunotherapy techniques that have had notable success, mostly in the treatment of hematological malignancies (10). A schematic representation of CAR T-cell immunotherapy is presented in Fig. 1.

CAR T-cell immunotherapy has become a life-saving approach, highly compatible with biomolecules of the human body, as drugs from this immunotherapy began to gain Food and Drug Administration (FDA) approval since 2017 (11). Currently, CAR T-cell immunotherapy has become a more acceptable approach due to the following reasons:

i) Major histocompatibility complex (MHC) independence. The T-cell response is only produced when the antigen is processed and presented by the protein known as MHC or MHC molecules. Following the recognition of the antigen, a triad complex, comprising of antigenic peptide, MHC and T-cells forms, which activates the signaling cascade to eliminate the tumor; however, defects exist in the machinery, which downregulates the signaling and hence, allow for tumor escape (12). To overcome this issue, immunotherapy needs to be MHC-independent, and this therapy follows the same route of independence that benefits the MHC downregulation.

ii) $CD4^+$ and $CD8^+$ T-cell redirection to CAR TCR. The T-cell subsets, $CD4^+$ and $CD8^+$, can be redirected for target cell recognition by detouring both the classes of MHC molecule restriction. $CD4^+$ CAR and $CD8^+$ CAR T-cells are potent cytotoxic cells against defined opted targets, although $CD8^+$ CAR T-cells are considered more potent (13).

iii) Live drugs. CAR T-cell immunotherapy is known as a 'living drug as the specific T-cells used in this therapy are first obtained by leukapheresis then modified into a robust receptor and subsequently, infused into the patient with leukemia/lymphoma. In the case that the infusion is performed in the same patient from whom the T-cells were obtained, this is termed autologous infusion, whereas in the case that the cells are administered to a different patient, this is termed allogenic infusion. In the whole process of CAR T-cell generation, the T-cells do not lose their potency. This CAR TCR recognizes and kills the cancer cells possessing a specific antigen on their surface. Their persistence is high due to their capacity for proliferation, signal initiation and adequate killing of cancer cells until the antigen is present on its surface (14). CAR T-cells have a high proliferative capacity, which enables maximum interaction and accessibility with the tumor antigen for more efficient tumor clearance.

iv) Specificity. The immune response is initiated, only upon the specific interaction of the CAR and the target cell, which results in the production of IFN-γ, IL-6 and IL-15 (15). This specificity is acquired due to the presence of the single-chain variable fragment (scFv) region of the antibody. The basic structure of CAR has a single ζ-chain, which produces cytokines, thus rendering these CAR T-cells more specific towards their target, and various generations of CAR T-cells are developed. A summary of CAR T-cell immunotherapy drugs of various generations and trial phases is presented Table I.

3. The CAR T-cell generations

The CAR T-cell is classified into different generations depending on the type and number of co-stimulatory domains attached to the construct. These domains are altered to elevate the level of cytokine production for optimal tumor cell killing; cytokines are small protein or peptide or glycoprotein messengers released on the specific interaction between cell to cell or cell to a receptor. They have a broad anti-tumor spectrum, as it recruits immune effector cells at the tumor site, enhances tumor cell recognition, recruits natural killer cells, inhibits p53 tumor-suppressor function and enhances T cell function (16). The response generated by the CAR TCR is solely dependent upon the activation domain. The generational classification is illustrated in Fig. 2A.

First-generation CARs. The first-generation CARs have an intracellular signaling CD3ζ domain or FcRγ coupled with scFv domain. Experimentally, it has been determined that ζ-chain signaling is insufficient for CAR T-cell persistence and a lasting response (8).

Second-generation CARs. The lack of an extra co-stimulatory domain results in T-cell apoptosis; thus, second-generation CARs are required. To increase the level of cytokine production, co-stimulatory signaling segments, such as CD28 and 4-1BB (or CD137) are used for the construction of second-generation CAR T-cells. The CAR TCR acknowledges the antigenic peptide MHC complex, and the signal is then transduced from the co-stimulatory domain, which renders the generation of cytokine IL-2 to activate the T-cells (17). Second-generation CAR drugs, such as tisagenlecleucel (Kymriah) and axicabtagene (Yescarta) ciloleucel have been approved by US FDA respectively (18,19).

Table I. CAR-T Cell Immunotherapy drugs of various generations in trial phases.

Generations of CAR T-cells	Targeted antigen	Clinical trial ID	Type of cancer	Phase	(Refs.)
First	FR-α	NCT00019136	Ovarian cancer	I	(50)
	CAIX	DDHK97-29	Renal carcinoma	II	(51,52)
	L1-CAM	NCT00006480	Neuroblastoma	I	(53)
	IL13Rα2	NCT00730613	Glioblastoma	I	(23)
		NCT01082926	Glioblastoma	I	(54)
	GD2	NCT00085930	Neuroblastoma	I (Active, not recruiting)	(55)
Second	MSLN	NCT01355965	Malignant pleural mesothelioma	I	(56,57)
	HER2	NCT01109095	Glioblastoma multiforme	I	(58)
	CEA	NCT01373047	Liver metastases	I	(59,60)
		NCT02416466	Liver metastases	I	(61)
	FAP	NCT01722149	Malignant pleural mesothelioma	I	(62)
	MUC16ecto	NCT02498912	Solid tumors	I	(63)
Third	EGFRvIII	NCT01454596	Glioma, glioblastoma, brain tumor	I/II	(64)
	GD2	NCT01822652	Neuroblastoma	I (Active, not recruiting)	(65)
		NCT01953900	Sarcomas	I (Active, not recruiting)	(66)
		NCT02107963	Osteosarcoma, neuroblastoma, melanoma	I	(67)
Fourth	FR-α	NCT03185468	Urothelial cancer	I/II (recruiting)	(68)
	GD2	NCT02765243	Neuroblastoma	II (suspended)	(69)
	PSMA	NCT03185468	Bladder cancer	I(recruiting)	(70)

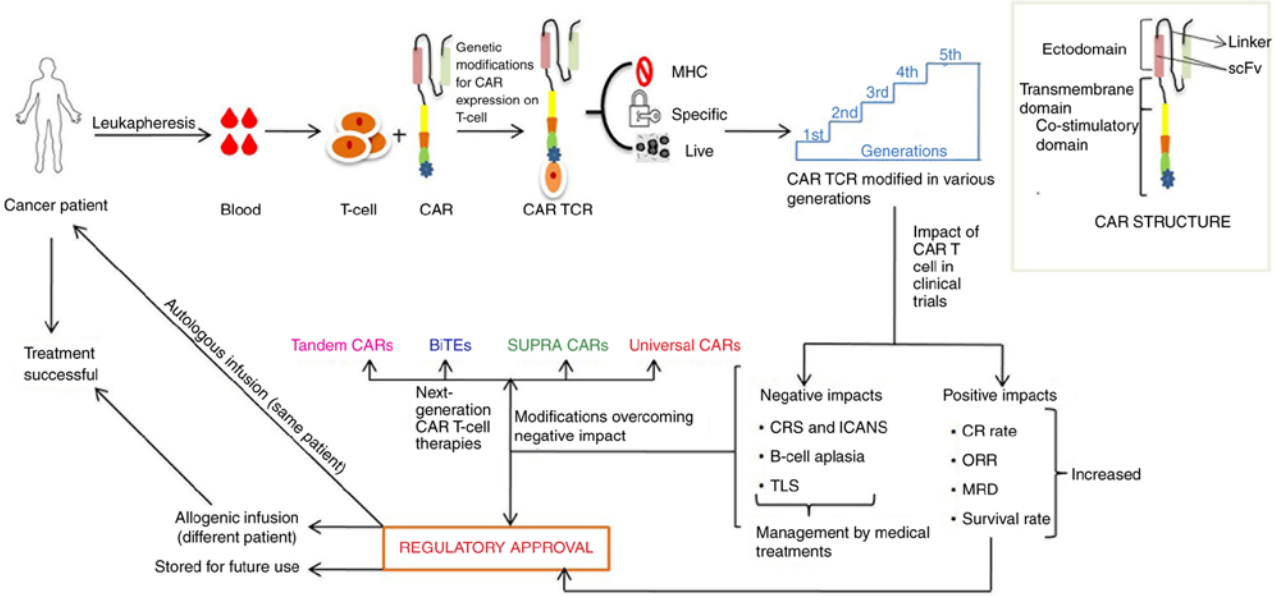

Figure 1. Schematic representation of CAR T-cell immunotherapy development steps. CAR, chimeric antigen receptor; TCR, T-cell receptor; MHC, major histocompatibility complex; CRS, cytokine release syndrome; ICANS, immune effector cell-associated neurotoxicity syndrome; TLS, tumor lysis syndrome; CR, complete response; ORR, objective response rate; MRD, minimal residual disease; scFv, single-chain variable fragment; BiTEs, bi-specific T-cell engagers.

Third-generation CARs. The early exhaustion of second-generation CARs has led to the development of third-generation CARs, which are constructed by uniting several co-stimulatory domains. CD3ζ-CD28-41BB or CD3ζ-CD28-OX40 is used in combination to increase cytokine production and exhaustion (20); to enhance the

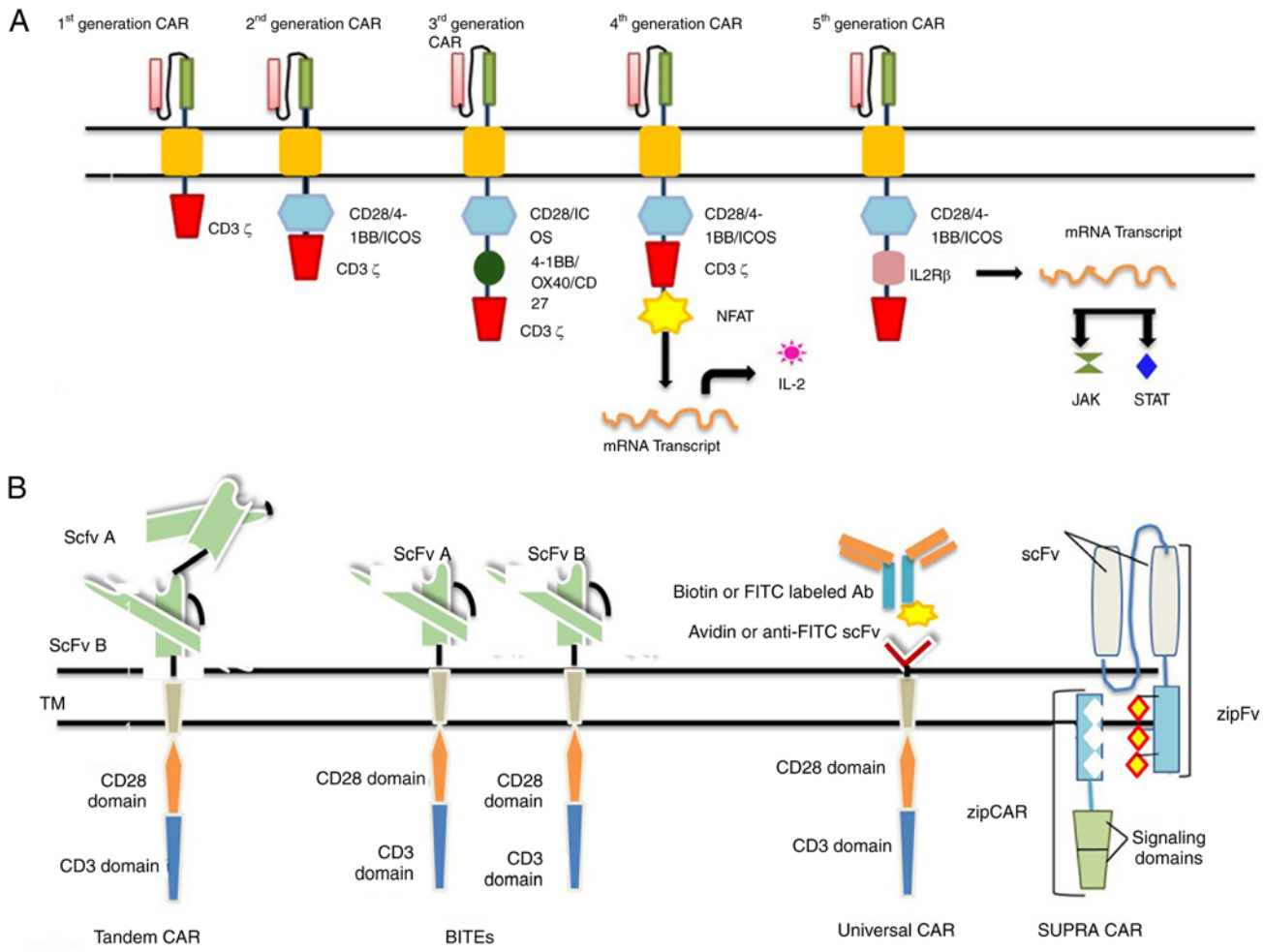

Figure 2. (A) CAR T-cell generations. (B) Next-generation CARs. CAR, chimeric antigen receptor; NFAT, nuclear factor of activated T-cells; STAT, signal transducer and activator of transcription; scFv, single-chain variable fragment; BiTEs, bi-specific T-cell engagers; CAR, chimeric antigen receptor; NFAT, nuclear factor of activated T-cells; STAT, signal transducer and activator of transcription; scFv, single-chain variable fragment; BiTEs, bi-specific T-cell engagers.

antitumor properties, fourth-generation CARs have been designed.

Fourth-generation CARs. The fourth-generation CAR T-cells are termed T-cells redirected for universal cytokine-mediated killing (TRUCKs). These CAR T-cells are modified by the integration of the desired gene construct in the promoter region, which alters the expression of the TCR gene. This desired gene codes for cytokines that will be delivered to the tumor site. When the CAR binds to the antigenic epitope on tumor cells, it initiates the CD3ζ chain downstream signaling that causes the phosphorylation of nuclear factor of activated T-cells (NFAT), a T cell transcription factor; thus, NFAT is activated, which in turn activates the TCR that attaches to the NFAT-response element-IL-2 promoter. This results in the production of the transgenic proteins (i.e., cytokines) which accumulate at the target site to eliminate cancer cells with minimal toxicity to normal cells (21).

Fifth-generation CARs. The need for fifth-generation CAR T-cells emerged due to insufficient tumor elimination and the partial activation of antitumor T-cell functions. For optimal tumor elimination, proper cytokine engagement is a crucial step for improved T-cell activation and proliferation. This generation differs, it has a novel CAR design with a supplementary IL-2Rβ activation domain between the CD28 and CD3ζ domains of the CAR. Furtermore, by the addition of a tyrosine motif by site-directed mutagenesis at the C-terminus of the CD3ζ domain, the signal transducer and activator of transcription (STAT) transcription factor is recruited as the antigen binds to the CAR T-cell. The signal is then transduced for the recruitment of STAT, which promotes CAR T-cell proliferation and promotes the activation of programmed death ligand in tumor cells. Ultimately, this will result in tumor eradication to a maximum extent (22). The drugs used in this immunotherapy belonging to all the aforementioned five generations are markedly efficient in blood cancers and pave the way towards their use in the treatment of solid cancers as well. Drugs for the treatment of solid cancers are currently in clinical trials, as presented in Table I.

4. Positive impact of CAR T-cell immunotherapy on patients

CAR T-cell immunotherapy, as a modern-day innovation, is customized according to the selected target present on the cancer cell. The selected targets are proteins, which are widely

expressed on the malignant B-cell surface, benefiting the specific CAR and target interaction. The target antigen should be cancer-specific; particularly, CD19, CD22, CD33, CD38, CD5, CD7, IL3A, SDCI, MS4A1, NCAM1 and ULBPI are used as prominent targets. The main antigens targeted in solid cancers are Her2, CEA, GD2, PSMA and CAIX (23).Thus, the impacts of these targeted drugs are listed as follows:

High complete remission (CR) rate. CR indicates the disappearance of all the signs of cancer in response to the therapy. The efficiency of this therapy is summarized in Table II, demonstrating increased CR rates in approved drugs. The conventional chemotherapy had only a 20-40% CR rate or symptom reduction; however, this immunotherapy treatment results in a 68-93% CR rate, which is improved compared with other available immunotherapies (24,25).

High objective response rate (ORR). ORR indicates the percentage of patients with a reduced tumor size within a specific period. The ORR following CAR T-immunotherapy was found in the range of 58-87%, which indicates a sufficient amount of tumor size reduction in patients (26).

Elevated minimal residual disease (MRD). MRD indicates the number of cancer cells remaining either during therapy or following therapy. An elevated MRD-negative CR was obtained in the range of 75-93% (27,28).

Higher survival rate. As previously demonstrated, 75 to 90% of patients with a specific type of leukemia such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, Hodgkin's lymphoma and B-cell non-Hodgkin's lymphoma recovered following this immunotherapy; this thus indicates a high survival rate of patients treated with CAR T-cell immunotherapy (29,30).

Easily permeable in the blood-brain barrier process. Drugs usually lack the ability to cross the blood-brain barrier or to penetrate in the tumor tissue to access the antigen in solid tumors; however, CAR constructs have the potential to activate existing T-cells inside the body that can even cross the blood-brain barrier.

Availability. This therapy has been approved to treat two groups of patients, one is adults and the other is children, as well as young adults <25 year of age. There are various ongoing studies evaluating the effectiveness of CAR T-cell therapy in pregnant women (31). CAR T-cell therapy can be administered to patients with any stage of cancer, although if the tumor burden is very high it is used in combination with chemotherapy or radiation priming (32). Independence from HLA recognition makes it easier for any patient with a down-regulated or very low HLA gene expression to opt for this type of treatment.

5. Post-therapy challenges and their management

The success of the CAR T-cell therapy is notable; however, in some patients, there are associated side-effects that can be managed with various medical treatments, and are either

Table II. Characteristics of approved drugs for CAR T-cell immunotherapy.

Sr. no.	CAR-T approved drugs	Type of lymphoma	Scfv	Costimulatory domain	Other names	Trial study	Cells extracted (defined)	ORR (%)	CR (%)	Clinical trial ID	(Refs.)
1	Axicabtagene ciloleucel	Adult DLBCL	FMC63	CD28	KTE-C19, Axi-cel	ZUMA-1	No	82	54	NCT03391466	(71,72)
2	Tisagenlecleucel	Adult DLBCL	FMC63	4-1BB CD137	CTL019, CART-19	JULIET	No	54	40	NCT02445248	(73)
3	Lisocabtagene maraleucel	Adult DLBCL	FMC63	4-1BB CD137	Liso-cel, JCAR017	TRANSCEND-001	CD4:CD8 (fixed)	73	53	NCT02631044	(74)
4	Idecabtagene vicleucel	RR-MM	Anti-BCMA	CD137 4-1BB and CD3-ζ	Ide-cel, bb2121	KarMMa	CD4:CD8 (variable)	73	45	NCT03361748	(75)
5	Brexucabtagene autoleucel	MCL	Anti-CD19	CD28 and CD3- ζ	Tecartus, KTE-X19	ZUMA-2	No	87	62	NCT02601313	(76)

CAR, chimeric antigen receptor; scFv, single-chain variable fragment; DLBCL, diffuse large B-cell lymphoma; RR-MM, relapsed/refractory multiple myeloma; MCL, Mantle cell lymphoma; ORR, objective response rate; CR, complete response.

Table III. Toxicity level of CAR T-cell immunotherapy in B-cell lymphoma with CD19 as the target.

Sr. no.	Drug study name	JULIET (%)	TRANSCEND (%)	ZUMA-1 (%)	(Ref.)
1	CRS (all grades)	58	37	93	(77)
2	CRS (grade ≥3)	22	1	13	
3	Neurotoxicity (all grades)	21	25	65	
4	Grade ≥3 (cytopenia ongoing on day 30)	32	N/A	28	

CRS, cytokine release syndrome.

resolved on their own or require further modification in the CAR T-cell construct. Some of these are as follows:

Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). CRS is a systemic inflammatory response caused by the interaction between CAR T-cells and the mononuclear phagocyte lineage cells. This is commonly known as the 'cytokine storm', which is the response produced due to a high level of cytokine production. These effects can be controlled by blocking the cytokine receptors (33). The second most acute toxicity associated with CAR T-cell immunotherapy is ICANS, which presents with symptoms, such as aphasia, confusion and difficulty in finding words, which may lead to depression, seizures, coma, motor weakness and cerebral edema (34). Neurotoxicity can be managed by the addition of more specificity in the receptor genes. These toxicities have been graded into groups as presented in Table III.

B-cell aplasia (off the tumor and on target). B-cell aplasia is the destruction of normal B-cells or when anti-CD19 CAR T-cells inadvertently damage normal B-lymphocytes that express CD19, which causes hypogammaglobulinemia; consequently, patients are typically at a high risk of developing infections. Despite the death of 1 patient from influenza A, the infections have been shown to be manageable with a low mortality rate. This can be controlled by intravenous immunoglobulin replacement therapy which increases the antibody levels in the body (35,36).

Tumor lysis syndrome (TLS) and anaphylaxis. As a result of this immunotherapy, tumor cells are broken down, which release constituents into the bloodstream that leads to the development of hypocalcemia, hyperkalemia, hyperphosphatemia and hyperuricemia (37). TLS can be managed carefully by maintaining fluid uptake, balancing the electrolytes, etc. Anaphylaxis is a severe allergic reaction caused by the domains of the murine antibody origin (38); efforts are being made to improvise the construct into the humanized source.

6. Next-generation CAR T-cell therapies

Tandem CARs (Tan-CARs). Tan-CARs have a unique structure, comprising two domains joined by a linker in a tandem orientation to the intracellular signaling domain; both are expressed together as one single unit of CAR on the cell surface. This synchronized targeting of both antigens enhances the therapeutic potential of this immunotherapy by elucidating an effective immune response. This increases their avidity and therapeutic potential (39). Grada *et al* (40) constructed and studied the trivalent CAR T-cells that co-targeted multiple antigens one after the other, as a result of which glioblastoma tumor was cleared with 100% efficiency.

Bi-specific T-cell engagers. To overcome the cancer relapse due to tumor escape, bispecific or dual CARs are synthesized. Bi-specific T-cell engagers (BiTEs) are recombinant bispecific proteins that have two linked scFvs of monoclonal antibodies. The only drug to gain FDA approval on March 29, 2018 was Blinatumomab (BLINCYTO®; Amgen, Inc.) for the treatment of acute lymphoblastic leukemia, which belongs to the BiTEs generation of CARs (41). One end of scFv targets CD19 and the other targets CD3. This drug drives a synergistic cascade of effector molecules by activating $CD4^+$ and $CD8^+$ T-effector memory cells; as a result, the optimal killing of tumor cells is achieved (42).

Universal CARs. To increase the antigen specificity, scalability and widen the antigen recognition spectrum, improved CARs are designed. The universal CARs are constructed using a 'third party', which could be biotin or anti-fluorescein isothiocyanate (FITC) scFv region (43). The extracellular region is composed of avidin, joined with an intracellular T-cell region. The biotin attached to the CARs is recognized by the biotin-binding immune receptors, which switches on the signaling cascade; hence, the tumor is eliminated. CD19-uCAR-T cells (NCT03229876) are under clinical trials for the treatment of acute lymphoblastic leukemia and non-Hodgkin's lymphoma (44).

SUPRA CARs. A system having two constituents was invented to improve the controllability and flexibility of CAR T-cells. One of the two is the universal receptor termed zip-CAR, and the other scFv adaptor segment which targets the tumor is termed zipFv. zipFv is a combination of the leucine zipper and scFv domain of the antibody and zip-CAR is another leucine zipper, that is attached to the CAR expressed on T-cells (45). The targeted antigen binds to the scFv domain, and the zipFv binds to the zipCAR that activates the T-cells and interferons are produced in response to it. To alter the level of T-cell response, binding affinity can be altered, these alterations can be used either to upregulate or downregulate the T-cell

activation; even a zipFv with no antigen specificity can terminate the toxicity. The SUPRA CAR model allows for multiple targeting without any genetic manipulations (46). These CAR systems can be used to elevate tumor recognition precision. The next-generation CAR T-cells are illustrated in Fig. 2B.

7. Challenges and limitations associated with CAR T-cell therapy

The therapeutic application of CAR T-cell therapy for solid tumors has advanced significantly. However, there are a number of obstacles to CAR T-cell therapy in solid tumors that are related to the tumor microenvironment, including the absence of tumor-specific antigen, a poor CAR T-cell trafficking efficiency, migration into tumor locations and the presence of an immunosuppressive tumor microenvironment (47).

Tumor resistance to single-antigen targeting CAR constructions is one of the most difficult limitations of CAR T-cell treatment. The malignant cells of a sizable fraction of patients treated with these CAR T-cells exhibit either a partial or complete loss of target antigen expression, despite the fact that single-antigen targeting CAR T-cells initially have the potential to produce high response rates; this is known as antigen escape (48). Another limitation is the selective pressure of the CAR T-cells that can cause tumor cells to downregulate antigens. On-target off-tumor effects can still occur even with proper antigen targeting, leading to related toxicity (49).

8. Conclusions and future perspectives

Genetic engineering has played a vital role in the diagnosis and treatment of various ailments, one of the major achievements being CAR T-cell immunotherapy. This immunotherapy is becoming the most effective and prominent treatment for various types of cancer, with an improved survival rate along with fewer post-therapy side-effects in comparison to other therapies available for cancer treatments. These are the new generation treatment options with better persistence, proliferation and tumor elimination. These CAR T-cells are on the way to being stored in reservoirs serving as off-the-shelf therapeutic cells. The race to design the most efficient CARs with the least possible side-effects, has come a long way from the development of new CAR T-cells from the laboratory to the therapeutic approach. Continuous improvements have been made to cover all types of cancers, including solid cancers. CAR T-cell immunotherapy drugs developed for solid cancers are currently undergoing clinical trials for approval; once approved these drugs will open a new door for cancer treatment. Modifications are required to construct humanized CAR T-cells which can cover and treat almost all types of cancer with a maximum survival rate and minimal post-therapeutic challenges.

Acknowledgements

The authors would like to express their sincere gratitude to Professor Javed Musarrat, the Hon'ble Vice-Chancellor of Integral University Lucknow, India, for his motivational support and for providing the necessary facility. The authors would also like to give special thanks to Dr Saif Khan (Department of Basic Dental and Medical Sciences, College of Dentistry, University of Ha'il, Ha'il 2440, Saudi Arabia) for supporting the conception of the present review.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

All the authors contributed equally to the preparation and design of the manuscript. AA was involved in the conception and design of the study. MH was involved in the articulation the contents, and in drafting and editing the manuscript. SAK was involved in data mining for data to be included in the present review. FK was involved in editing the manuscript. NA was involved in editing the technical part of the manuscript. SK was involved in the organization of the data to be included in the review. SJ was involved in the design the figures and tables. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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