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Review Paper

The Gene Manipulation and Cellular Immunotherapy Combination in the Treatment of Cancer

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Context: The immune system is directly linked to the tumors, from tumor formation to the tumor's development and metastasis. So, the interest of scientists over the protective immunological mechanisms has increased and shown gifted strategy in cancer treatment.

Evidence acquisition: Genetic engineering and cellular immunotherapy are two different advanced molecular mechanisms to modify the immune responses and genome. Gene manipulation is the bioengineering technology that allows vectors to transfer new genetic information into the target cells. Cellular immunotherapy is an excellent strategy that connects the body's immune system to fight cancer.

Results & Conclusions: This review described that combination of genetic engineering and cellular immunotherapy has brought the novel antitumor repressive molecules stopping the tumor tissue immune tolerance and significantly expanding cancer therapy's effectiveness. Usually, cell immunotherapy and genetic engineering are considered two independent processes, and, in this review, we believe them in combinations. Here, we review these two novel approaches, and they are both combinations in terms of technological advances and clinical experience.

Keywords: Chimeric Antigen Receptor T cell; Engineered T cell receptor; Natural Killer cell; Tumor-Infiltrating Lymphocyte

1. Background

Cancer progression is directly linked to the function of the immune system, which is responsible for antitumor response and reduces tumor eradication. So, there is an extreme interest in tumor therapy based on immunotherapy. Tumor immunotherapy can trigger the immune system stimulatory agonists or the immune-checkpoint blockade (1). Genetic engineering can modify the gene expression profile of the tumor for improving the antitumor potential of immune system cells. By way of illustrations, tumor cell transduction with some new genes like "suicide genes" is a mostly inspected approach of gene editing strategies (2). Tumor cells can change their surface markers repeatedly to escape from an immune system like expressing Tumor-Associated Antigens (TAA) or decrease the Major Histocompatibility Complex (MHC) class I expression. During the adaptive response, special TAA is presented by the MHC class I and II molecules from Antigen-Presenting Cells (APCs) to the exact receptors of CD8+ (cytotoxic) T cells and CD4+ helper T cells.

Regulatory T (Treg) cells can inhibit extreme immune responses to self/non-self-antigens to keep immune homeostasis. Treg cells have a role in tumor progression

because they prevent antitumor immunity. There are numerous suppressive mechanisms for Treg, including stopping co-stimulatory signals by dendritic cells over cytotoxic T-lymphocyte antigen-4, Interleukin (IL)-2 consumption, and inhibitory cytokine secretion, tryptophan, and adenosine metabolism, and direct killing of effector T cells (3).

Tumors mostly escape from immune response, so scientists nowadays focus on improving the immune system to target several stages and different immune cells, acting as a network. Such mechanisms can be (i) Tumor-Infiltrating Lymphocyte (TIL) therapy, (ii) Engineered T Cell Receptor (TCR) therapy, Chimeric Antigen Receptor (CAR) T cell therapy, and Natural Killer (NK) cell therapy. Here we aim to consider these methods in which cell therapy is merged to gene therapy.

2. Cancer Immunotherapy

It has been about 150 years since the first time immunotherapy has been considered to treat cancers. Recently, research has concluded that immunotherapy can prolong the survival of patients with refractory cancers and cause less harm than conventional therapies. This strategy has become the first-line treatment for many cancers in stage IV of Colorectal Cancers (CRCs) (4).

Currently, there are three key immunotherapeutic tactics for tumor battling: Immune checkpoint blockade, Adoptive cell therapy, and cancer vaccines.

Immune checkpoint blockade uses particular antibodies to restrain negative regulatory checkpoints and unleash T cell responses. The most potent checkpoint molecules are Cytotoxic T-Lymphocyte-Associated protein 4 (CTLA-4) and Programmed cell Death protein 1 (PD1). Additionally, T Cell Immunoreceptor With Ig And ITIM domains (TIGIT) is expressed on activated T cells, regulatory T cells (T reg), and Natural Killer (NK) cells. Blockade of TIGIT results in T cell antitumor immunity and prevents NK. cells exhaustion which is promising.

CTLA-4 is expressed on activated T cells, and T reg cells in lymphoid tissue bind to B7-1 and B7-2 ligands on Antigen-Presenting Cells (APC), inhibiting T cells' activation and proliferation other words, T cell activation (5). It is structural and biochemical akin to CD28, and they compete to bind the same ligand, but CTLA-4 has higher affinity and avidity.

Expression of PD1 molecules is on T cells and T reg cells in peripheral tissues. The ligands of these molecules are PDL1 and PDL2 on APCs or cancer cells and promote T cell exhaustion, a condition that the T cells response becomes chronic. Level of T cells expression of PD1 and tumor expression of PDL1 could be prognostic markers of multiple malignancies (6). Loss of CTLA-4 or PD1 function can induce autoimmune disease.

Inhibiting PD1 or PDL1 is efficient in treating different kinds of cancers. Pembrolizumab and Nivolumab are two kinds of potent anti-pd1 antibodies. In March 2021, the FDA approved Pembrolizumab combined with platinum and fluoropyrimidine-based chemotherapy for metastatic or advanced esophageal or gastroesophageal carcinoma (7). The Nivolumab is another PD1 blocking antibody that has been approved for Renal Cell Carcinoma (RCC) (8) and unresectable malignant pleural mesothelioma (9). The combined usage of Nivolumab and Ipilimumab is suggested for the first-line treatment in several cancers like Metastatic or Recurrent Non-Small Cell Lung Cancer (10).

As we mentioned earlier, CTLA-4 and PD1 are two important molecules of T cells tolerance to self-tissues. Consumption of CTLA-4 blocking antibodies can create dermatitis, enterocolitis, uveitis, hepatitis, nephritis, and hypophysitis. A mixture of anti-CTLA4 and PD1 may generate a variety of autoimmune disorders. The most common symptoms are skin rashes, diarrhea, and abdominal pain. A meta-analysis study revealed that patients treated with PD1 or PDL1 inhibitors suffered less from toxicities than those who underwent chemotherapy. However, most of these side effects can be treated by consuming drugs based on guidelines like glucocorticoids (**Fig. 1**) (11).

Adoptive Cell Therapy (ACT) is the infusion of allogeneic T cells into a cancer patient. This treatment has been the most beneficial therapy for metastatic melanoma, and 51% of patients achieved objective clinical responses.

In Tumor-Infiltrating Lymphocytes (TILs), lymphocytes are extracted from a cancer biopsy and are expanded with Interleukin-2 (IL-2) and infused into the patient. lymphodepleting chemotherapy in patients with melanoma before TILs appeared to be efficient, while EBV-associated nasopharyngeal carcinoma did not enhance the TILs therapy (12). Also, TILs are prognostic biomarkers in cancers such as ovarian cancers, Esophagus Cancers, and breast cancers (13-16).

Isolated T cells are genetically engineered, and Chimeric Antigen Receptors (CAR) are added to identify and target cancer cells. Then these cells are infused into the

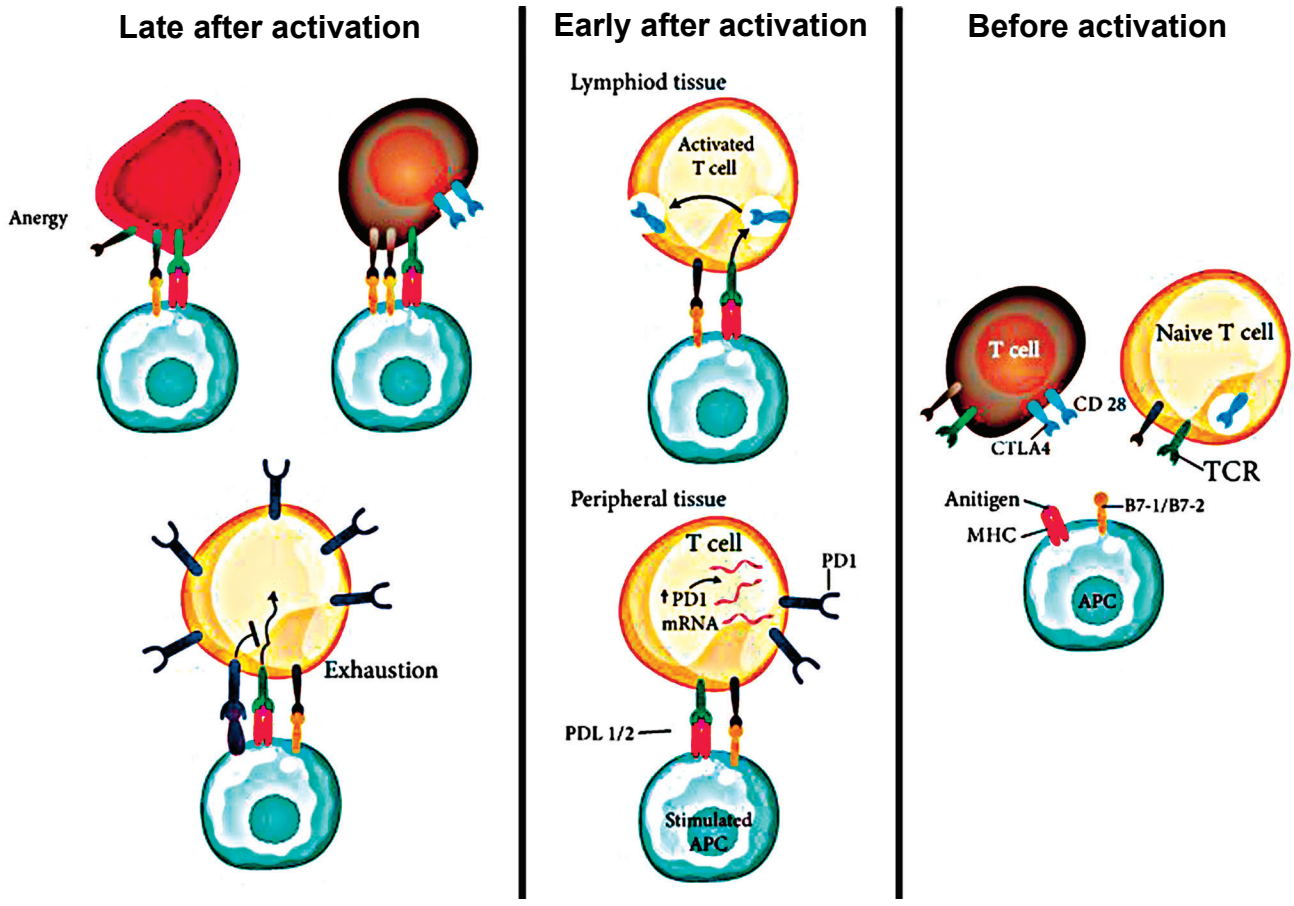

Figure 1. Function of PD1 and CTLA-4. Cytotoxic T Lymphocyte Antigen 4 (CTLA4) is carried in intracellular vesicles of naïve T cells, then CTLA-4 expresses on Treg and activated T cells in lymphoid tissues. T cells become activated when T-Cell Receptors (TCRs) bind to their specific antigen presented by an Antigen-Presenting Cell (APC) in addition to CD28 binding to B7-1 and B7-2. CTLA-4 competes with CD28 to bind the same ligands: B7-1 and B7-2. Joining CTLA-4 to B7-1/B7-2 ligands results in anger of T cells. Early after activation of T cells, Programmed cell Death 1 (PD1) is expressed at the mRNA level. Later, PD1 is expressed on the surface of activated T cells to bind APCs and cancer cells in peripheral tissues, and finally causes exhaustion of T cells.

patient's body. This strategy is called chimeric antigen receptors T cell therapy or CAR T cell therapy. These synthetic CAR T-cells have four generations. The first only has the CD3ζ domain, the second and third have co-stimulatory molecules (CD28 and 4-1BB), and the fourth generation can secretion cytokines like IL-2 (17). Generation by generation, CAR t cells become more effective.

The FDA approval of CAR-T cell therapy for CD19 landmark led to treat relapsed and refractory-cell Acute Lymphoblastic Leukemia (B-ALL) and other B cell malignancies. CD22 CAR-T therapy has successful remission in patients with relapsed B-ALL. CD19/CD22 CAR-T therapy could be effective in relapsed B-ALL, as well (18). Glypican 3 (GPC3) in Hepato Cellular Carcinoma (HCC) has shown promise as a target for CAR T cell therapy (18). CAR-T cell therapy seems hopeful to treat advanced prostate cancers and NSCLC (19, 20). However, more clinical investigations are needed.

CAR T cell therapy may affect other cells rather than cancer cells and cause toxicities. The most common symptoms are Cytokine Release Syndrome (CRS) and neurotoxicity, mostly at mild levels. Some mild symptoms are solved with antihistamines, antipyretics, and hydration, followed by corticosteroid therapy if needed, but severe and life-threatening symptoms just are treated with tocilizumab. Patients after CAR T cell

therapy might be at the risk of infection (21) and other rare symptoms such as encephalopathy and Tumor Lysis Syndrome (TLS).

Cancer vaccines are to manipulate the immune system to identify and eliminate cancer cells. Cancer vaccines can be therapeutic or prophylactic.The Sipuleucel-T vaccine and PSA-TRICOM vaccine prolonged overall survival in advanced and metastatic prostate cancer (22). Personalized recombinant cancer vaccines have been noticed recently, but finding the right neoantigen to have the potent defense against tumors is complex and needs more investigation (23).

Cytokines are small proteins that mediate cell-to-cell communication for response to inflammation and immune attack. Nowadays, the therapeutic use of a class of cytokines called interleukins has successfully translated several interleukins with demonstrated therapeutic potential from the lab to the patient. The potential antitumor activities of some pro-inflammatory cytokines in animal studies suggest the recombinant interferon-alpha and interleukin-2 for cancer treatment.

Cancer vaccines manipulate the immune system to identify and eliminate cancer cells and strengthen the immune response. Tumor antigens are two groups: mutational antigens and Tumor-Associated Antigens (TAAs). Tumor antigens are overexpressed in cancer cells and are significant in tumor onset, development, and metastasis. Cancer vaccines can provide long-lasting immunity against TAs. Cancer vaccines can be therapeutic or prophylactic. Prophylactic vaccines such as Hepatitis B vaccination can effectively prevent liver cancers. Autologous tumor cell vaccines, allogeneic tumor cell vaccines, Dendritic Cell (DC) vaccines, protein/peptide-based cancer vaccines, and genetic vaccines such as DNA, RNA, and Viral-based vaccines are different kinds of therapeutic vaccines. DNA vaccines, which are plasmids delivering genes of TAs, have shown favorable responses towards adaptive and innate immune systems.

Therapeutic vaccines, for instance, the Sipuleucel-T vaccine and the PSA-TRICOM vaccine Prolonged OS in advanced and metastatic prostate cancer, respectively (24). Vaccines against stage III/IV NSCLC have been suggested adjuvant to chemotherapy and radiation. The Nelipepimut-S vaccine has passed clinical trial phase III against HER2-positive breast cancer. Personalized recombinant cancer vaccines have been noticed recently, but finding the right neoantigen to have the potent defense against tumors is complex and needs more investigation.

3. Cancer Gene Immunotherapy

Gene therapy is the genetic manipulation correcting altered (mutated) genes or site-specific alterations. Most recent clinical trials have treatment potential for diseases caused by recessive gene disorders like Cystic Fibrosis (CF), hemophilia, muscular dystrophy, and Sickle Cell Anemia (SCA), rheumatoid arthritis, Gaucher disease, and cancer (25). In the gene therapy process, mostly recombinant DNA harboring the target or healthy gene is inserted into a molecular carrier called a "vector." Vectors can be viral and non-viral, including plasmid, Retroviral, Lentiviral, Adenoviral, and Aden associated. Several protocols improve the gene therapy process, but it has still been challenged, and its efficacy is under debate. Vectors should be particular, usually are needed in large quantities, and high purity can release one or more target genes in the exact sizes and should not be known by the immune system. When the vector is injected into the patient, it should express the gene for the patient's entire life with no risk of allergic reactions or inflammatory processes. The target cell type of gene therapy can be germline gene therapy in most monogenic disorders and somatic cells gene therapy that inserts a human gene into a living person's somatic cells but not eggs and sperm that transfer to the next generation.

Hematopoietic Stem Cells (HSCs) are the stem cells that create other blood are perfect targets for gene transfer because of their self-renewal power. The combination of gene therapy and stem cells needs particular vectors that can induce Pluripotent Stem cells (iPS).

More than inserting gene fragments through vectors, several new strategies exist to change the genome sequence using sequence-specific endonucleases. The central part of the genome-editing procedure is creating a Double-Stranded Break (DSB) at the target place within the genome. These genome editing technologies include Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the RNA-guided CRISPR-Cas's nuclease system (**Fig. 2**). The ZFNs and TALENs usually practice a policy of roping endonuclease catalytic domains to modular DNA-binding proteins for making designed DNA Double-Stranded Breaks (DSBs) at exact genomic loci. Whereas, Cas9 is a nuclease directed by short RNAs over Watson-

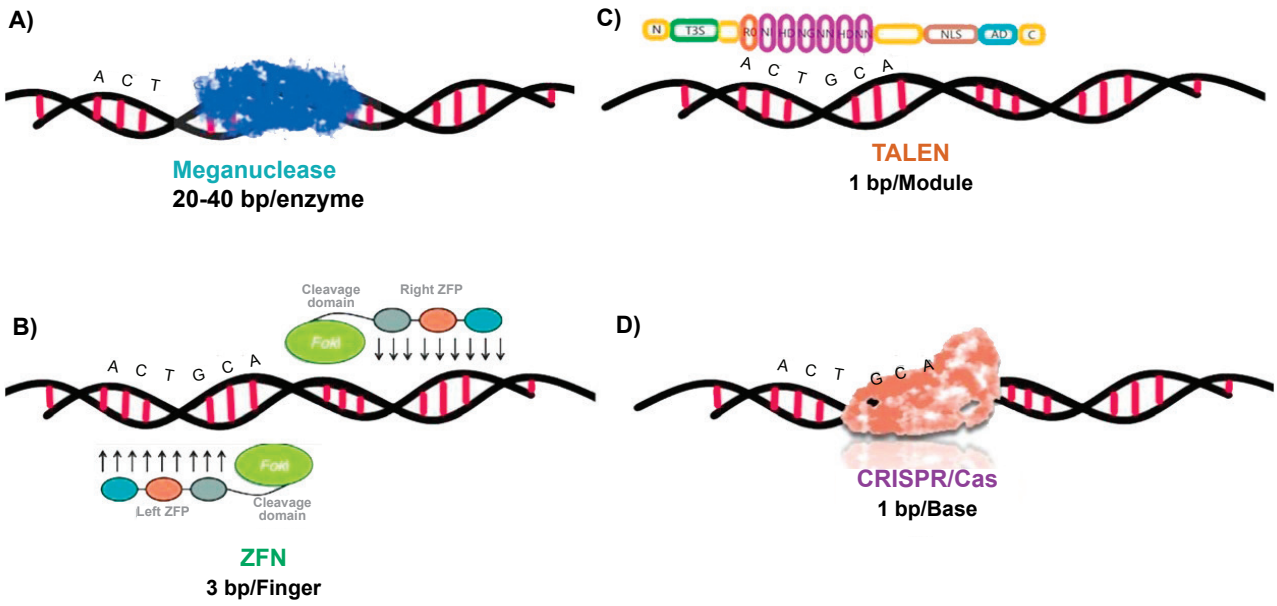

Figure 2. Four main mechanisms of genetic engineering tools, **A)** Meganuclease as the "molecular DNA scissors" that can replace, eliminate or change sequences in a very targeted method, **B)** Zinc finger domains which are planned to aim at specific DNA sequences and this allows zinc-finger nucleases to mark unique sequences within complex genomes. **C)** Transcription Activator-Like Effector Nucleases (TALEN) are restriction enzymes like ZFN, and CRISPR–Cas that employ non-homologous end-joining and homology-directed repair mechanisms **D)**.

Crick base pairing of the double-stranded molecule of target DNA is more specific to the wide variety of cell types (26).

The usual genome editing procedure depends on the DSB and includes two main pathways; non-homologous end construction and homology-directed repair. In the non-homologous end-joining method, several enzymes can directly attach to the DNA. In contrast, in homology-directed repair, the homologous DNA sequence is required as a template for repairing misplaced DNA sequences at the cleavage site. The ZFNs represent the native restriction enzyme FokI enzyme which attaches to the N-terminal of DNA and is cut upstream or downstream at shifted cleavage. Initial forms of chimeric FokI-zinc finger were in places that imitated the native FokI site. The detection that nuclease domains of two FokI monomers dimerized to cut DNA required a modification in the strategy of chimeric zinc finger nucleases DNA-recognition domains. The TALEs have a unique repeating structure made of 34 amino acid repeats as a unique DNA-binding domain. The repeats are highly conserved and mainly vary in two adjacent amino acids (positions 12 and 13) termed Repeat-Variable residue (RVD). Combining the FokI nuclease domain to TALEs (making TALE nucleases or TALENs) created a genome-editing element with superior DNA targeting and greater specificity than ZFN.

The pioneer gene editing cell therapy was HIV co-receptor gene CCR5 (transmembrane chemokine receptor) by ZFNs in autologous CD4+ T-cells of HIV patients. The first TALEN-edited cell therapies in clinical trials solve some of the ZFN's difficulties. For example, several gene editing plans for autologous T-cell immunotherapy have been designed to make anti-CD19 CAR-Ts across relapsed/refractory aggressive B-cell non-Hodgkin lymphoma. Cellectis SA has an advanced allogeneic method named Universal Chimeric Antigen Receptor (CAR) T-cells targeting CD19 (UCART19) (27). More than transposons, ZFN, and TALEN, the CRISPR gene editing structure goes into a clinical trial to change the immune cell's genome. The first report was in a patient with advanced non-small-cell lung cancer that CRISPR–Cas9 recruited to knock out the immune checkpoint inhibitor PD1 before reinfusion into the patient.

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) indicated the DNA sequences

typically present in prokaryotic cells initially derived from bacteriophages. The CRISPR-Cas is a microbial adaptive immune system that recruited RNA-guided nucleases to break external genetic elements (28). The CRISPR/Cas9 includes three main components, small crRNAs (CRISPR RNAs), trans-activating CRISPR RNA (tracrRNA), and the CRISPR-associated protein 9 (Cascade 9). The general immunity points of entire CRISPR-Cas systems are the Cas proteins. Now according to the collection of *cas* genes and their complexes, six types (I-VI) of CRISPR-Cas systems have been recognized and clustered to classes 1 and 2. Class 1, counting types I, III, and IV are determined by multi-Cas proteins, contrary to class 2, including types II, V, and VI containing one Cas protein (29). Cascade 9 similarly needs a small sequence downstream of the hybrid section called PAM (Protospacer Adjacent Motif) that acts as a directing element.

CRISPR/Cas9 knowledge is appropriate for oncogenes' silencing or repairing tumor suppressors and apoptotic and immune-stimulatory purposes. TP53 mutation happens commonly in most human tumors. A mutant TP53 and Albers *et al*. in a xenograft model presented inhibition of tumor suppressor Transformation related Protein 53 (TrP53) and expression of oncogene H-Ras by the CRISPR/Cas9.

Human Estrogen Receptor 2 (HER2) gene is another famous oncogene overexpressed in tumors like breast cancer. It is shown that co-expression of Cas9 and three sgRNAs directing HER2 exons 5, 10, and 12 meaningfully increase cell death and tumorigenicity in Her2-positive breast cancer cells. Other examples are Epidermal Growth Factor Receptor (EGFR), Nuclear Factor Erythroid 2-Related Factor (NRF2), Granulin (GRN), and targeted E6 and E7 HPV. Many studies have to work on genome-wide knockout libraries (GeCKO) and the assembly of sgRNAs in contradiction of target cancer genes.

4. Cancer Gene- and Cell- Immunotherapy

The tumor surrounding environment is called the Tumor Microenvironment (TME), has a role in reducing an immune attack expected to detect adaptive cellular immunity. TME contains tumor cells, tumor stromal cells with stromal fibroblasts, endothelial cells, and immune cells like microglia, macrophages, and lymphocytes more than the non-cellular components of the extracellular matrix such as collagen fibronectin, hyaluronan, laminin, among others. Cancer cells can prevent the immune system or trigger immune tolerance over the secretion of extrinsic factors that impact the Tumor Microenvironment (TME). CRISPR/Cas-mediated genetic manipulation has attempted to solve some challenges of immune system misfunctioning during the tumor formation process. Numerous components have a role in tumor cells. The M2 macrophages interaction like macrophage Signal Regulatory Protein a (SIRPa), Kindlin2, Osteopontin (OPN), Lysosome Associated Membrane Protein Type 2A (LAMP2a), IL-8, Tumor-Secreted Protein S (Pros1) that are the candidate of changing by CRISPR/Cas for boosting the patient's immunity against tumor (30).

5. Immune Cell Gene Therapy

There is a novel method of gene manipulation to stimulate patients' immune systems to discover and kill tumor cells called Adoptive Cell Transfer therapy (ACT). There are several kinds of ACT. There are several groups, including (i) Tumor-Infiltrating Lymphocytes (TIL) that is T-cells are grown from the tumor itself, (ii) Endogenous T-cell therapy which is tumor-specific T-cells are grown from the blood, (iii) Chimeric Antigen Receptor (CAR T) includes chimeric antibody/T-cell receptor gene is put into peripheral T-cells, (iv) TCR transduced T-cells which are genetic edited t-cell receptor to recognize tumor is put into peripheral T-cells, and (v) CAR-NK cell therapies that are gene manipulated Natural Killer cells to express Chimeric Antigen Receptor(CAR).

6. Tumor-Infiltrating Lymphocyte (TIL) Therapy

As mentioned, Adopting Cell Therapies (ACT) introduces a class of immunotherapy. Chimeric Antigen Receptor (CAR) T-cell therapy and tumor-infiltrating lymphocyte (TIL) therapy can be named as the types of cellular therapies adopted. In Chimeric Antigen Receptor (CAR) T-cell therapy, T cells are removed from peripheral blood cells, and they are modified to express specific receptors and injected into the patient again.

Primary clinical responses in the TIL therapy published by Rosenberg and his colleagues in the late 1980s, in which infusion of TIL was joint with lymphodepleting conditioning regimes and HD IL-2 to destroy the cells that suppress the immune system and finally improve TILS function. The patient's tumor is sampled in this treatment instead of testing the T cells from the peripheral

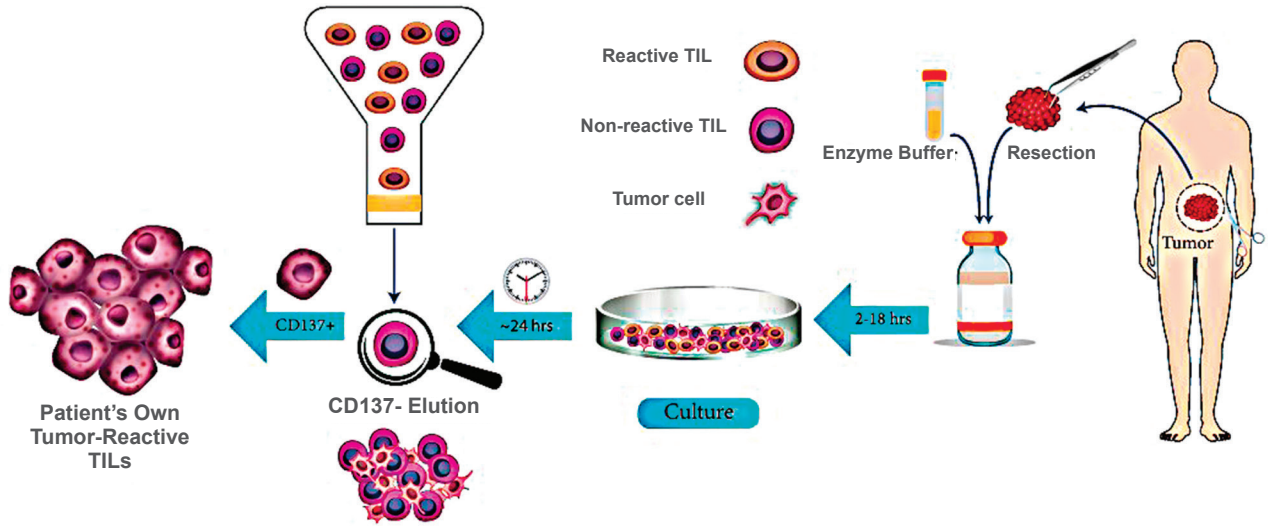

Figure 3. In CAR T-cell therapy, T cells are removed from peripheral blood cells and they are modified to express specific receptors and injected into the patient again. But in TIL therapy instead of sampling the T cells from the peripheral blood cells, the patient's tumor is sampled.

blood cells (**Fig. 3**). Then the samples are placed in a medium enriched with interleukin 2 (IL-2), an anti-CD3 antibody, and blood mononuclear feeder cells (31). The DNA in the sampled tumor cells is then sequenced to detect existing mutations. The mutated neoepitopes are then inserted into the autologous dendritic antigen-presenting cell and cultured with T cells. According to the secretion of cytokines to evaluate the ability of T cells to detect specific neoantigen, TILs are selected, propagated, and re-injected into the patient. They can join activated cytotoxic T lymphocytes (CTL) by identifying their associated ligand to kill the tumor cell and create an antitumor immune response (32). Because TIL treatment uses the patient's tumor for treatment, it can be considered a kind of personal treatment.

Success in TIL treatment depends on two factors: Antigenic characteristics are diverse compared to the autologous tumor showed by TIL and their lytic ability to eradicate tumors (33). However, there are many challenges to success in this treatment, for example, the role of the host with lymphodepleting conditioning, the role of interleukin 2, the number of cells injected, and other factors. Among the problems in this method, we can mention these cases, which are expressed below. Tumors can be non-immunogenic, depending on their types. The cytotoxic activity of TILs can be restricted by immunosuppressive agents (32). Because TIL is considered a personal treatment, a unique product should be produced for each patient, that this in itself imposes a relatively high cost and time (34, 35).

In recent years, the relationship between the presence of lymphocyte infiltrations in tumors and the clinical outcome of patients in several types of tumors, including metastatic melanoma, ovarian cancer, colon cancer, and breast cancer, has been found. This treatment has led to unprecedented responses in some patients, like treatment of cell transfer with autologous TILs to patients with melanoma which can lead to complete and lasting answers. However, in many cases, it does not lead to a solution (34). So, TIL has not yet been approved as a cancer treatment.

7. T-Cell Receptors Therapy

About 50 years ago, the concept of "Gene Therapy" was introduced, but it has just been a decade that the treatments have become successful. Vectors (as the genetic carriers) are usually nonpathogenic recombinant viruses are used to transfer the genes. The genome of a recombinant virus consists of thousands of such engineered T cells, or T cell receptors are effective therapies against several forms of cancer and a range of malignancies. Still, recent advances depend on autologous T cells, which demands too much effort and money to produce (36). Recent clinical reports are over TCR engineered-T cells to spot New York esophageal squa-

mous cell carcinoma (NY-ESO-1) (37).

T cells can specifically recognize and eliminate their target cells. The T Cell Receptor mediates physiological antigen recognition (TCR), an alpha-beta heterodimer comprising rearranged V, D, and J genes. T cells are extracted from the tumor tissue or the patient's blood. Then, TCRs α and β chains are detached from single T-cell clones and injected into a viral vector such as retrovirus or lentivirus. T cells obtained from patients can be modified with a viral vector to encode the wanted TCR α-β sequences. After TCR-expressing cassettes production, they were cloned in retrovirus vectors. This process took place within 24 hours by unbiased PCR amplification of TCR α and β chain variable regions constructed with TCR constant regions (38). Peripheral T cells were extracted from healthy people and used for tumor-derived TCR libraries expression. These libraries were screened for tumor antigen-specific TCR and TCR-expressing transgenes recovered from isolated T cells (tetramer-positive). This method can provide tumor-specific TCR-expressing viral vectors to produce personalized antitumor T-cell products. (38). 5T4 is a transmembrane tumor antigen that is mainly expressed on 90% of primary renal cell carcinomas. This antigen is also expressed in many human carcinomas, but it has no place in most somatic adult tissues. 5T4 offers us a compelling and unknown pathway for (TCR)-engineered T-cell therapy and encourages us to clinically test 5T4-targeted antibodies and vaccine therapies. In 2019 XU Y. and his colleagues indicated the preclinical development of isolating CD8+ T-cell clones with high avidity for an HLA-A2-restricted 5T4 epitope (residue 17–25; 5T4p17). This action was done by cytotoxicity and cytokine release assays. All seven TCRs showed high expression on CD8+ T-cells with transduction efficiencies in a range of 59 to 89%. TCR-transduced CD8+ T-cells proved redirected cytotoxicity and cytokine release in response to 5T4p17 on target cells. They killed 5T4+/HLA-A2+ colorectal-, kidney-, and breast tumor cell lines beside primary RCC tumor cells *in vitro*.

8. Chimeric Antigen Receptor (CAR) T Cell Therapy

Chimeric Antigen Receptor T cells (CAR T cells) are genetically engineered T cells that can recognize tumor antigens and create an engineered T-cell receptor. CAR T cell therapy as adoptive immunotherapy has recently established excessive consideration and took extraordinary revolutions (39). For security, CAR-T cells are planned to be exact to an antigen just expressed on a tumor and not expressed on non-tumor cells (**Fig. 4**).

At first, CAR-T cell research was mainly zoomed-in blood cancers like acute lymphoblastic leukemia (ALL) and Diffused Large B-cell Lymphoma (DLBCL) (40).

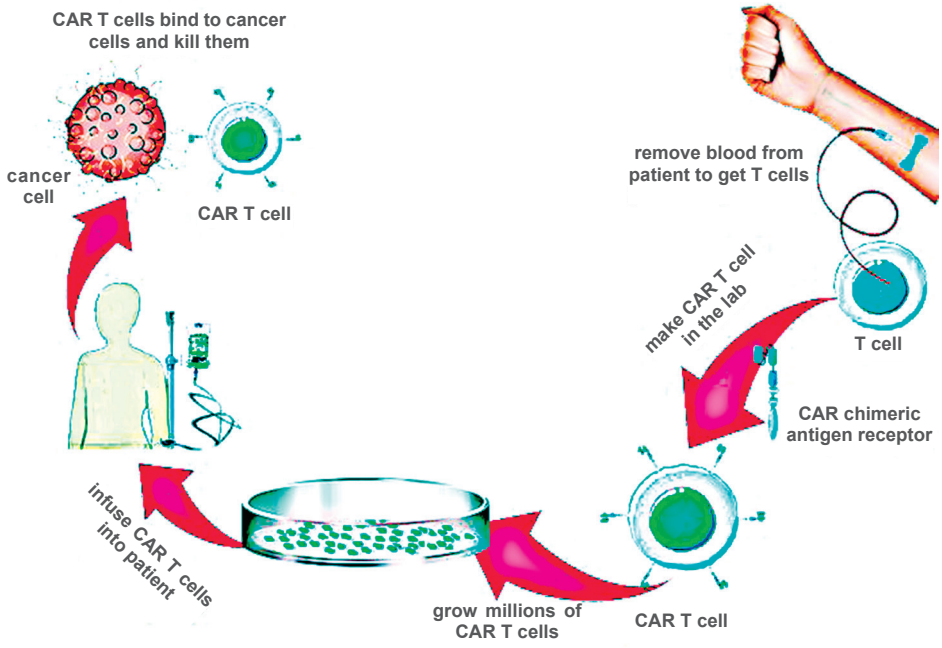

Figure 4. Schematic presentations of CAR T cell process from cell recruiting to cell returning to the patient.

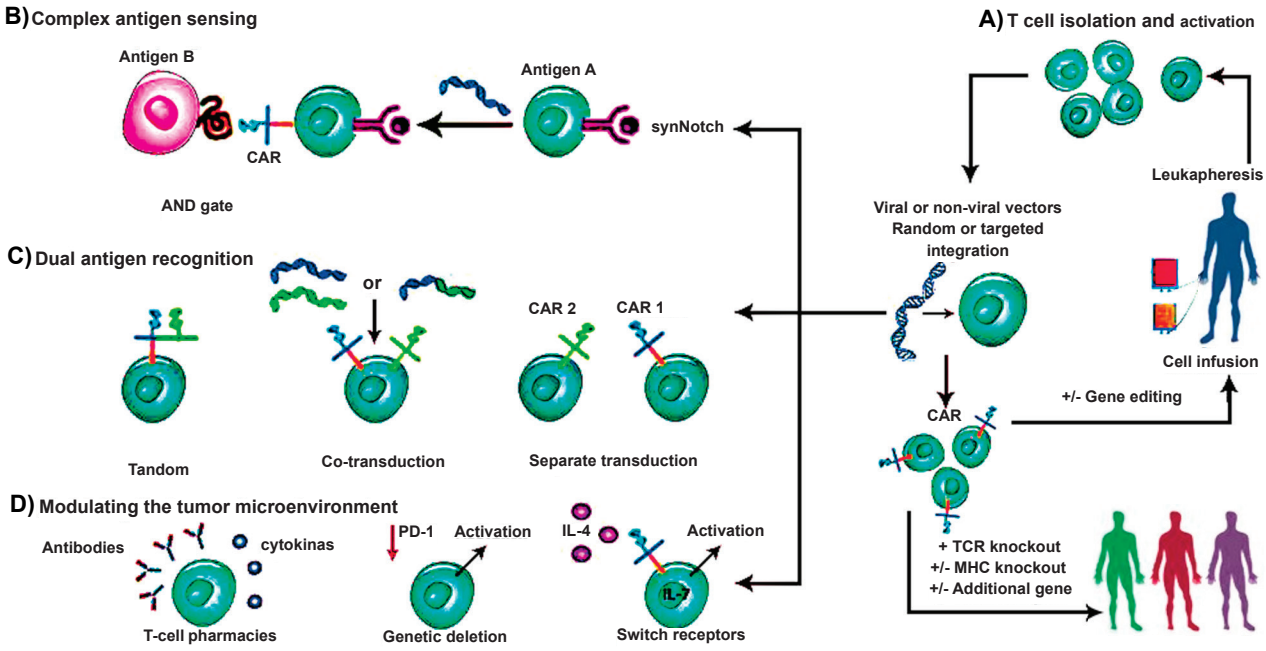

Figure 5. Novel CAR formats for reducing the Car T cell toxicity and increase its efficacy. **A)** the process of cell isolation and activation, **B)** Complex antigen targeting has the potential to expand the efficacy of CAR T cells immunotherapy, **C)**Tandem CAR T cells can identify either tumor antigen A or B and may prevent antigen escape, **D)** To increase CAR T cell effectiveness and reduce the immunosuppressive tumor microenvironment, CAR T cells engineered in the way that secrete cytokines, such as IL-7, PD-1 and reduce the immunosuppressive tumor microenvironment.

Then several clinical trials proceeding to engineer CARs directing many other blood cancer antigens like CD30 (TNFRSF8), CD33 (sialic acid-binding Ig-like lectin3 or p67), CD123 (interleukin-3 receptor), CD135 (Fetal liver kinase-2 or Flk2) (41).

CAR T usually is more complicated because discrimination of the beat antigens is challenging. The target antigen must be over-expressed in most cancer cells and absent in normal cells (42). Moreover, the efficient entering of CAR-T cells into the middle of tumor masses is challenging because the hostile tumor microenvironment like blood vessels, signaling molecules, and the Extracellular Matrix (ECM) can suppress T cell activity (**Fig. 5**).

Interestingly, CAR T are engineered to make Armored CAR T cells that constitutively or conditionally secrete cytokines, such as IL-12, IL-15, and IL-18. It increases CAR T cell effectiveness and reduces the immunosuppressive tumor microenvironment. Also, dominant-negative receptors can deactivate immunosuppressive ligands. In contrast, chimeric switch receptors might be considered to alter repressive signals into T cell stimulatory indications. The failure of tumor-specific T cells well traffic and attack to the tumor can be addressed by the concomitant expression of chemokine receptors or enzymes, which reduce materials of the extracellular matrix (43).

T-cells may similarly keep self-antigens safe from autoimmune reactions like the mechanism of happening in organ transplantation or rheumatic diseases like lupus (44). In several CAR-T cell therapies more than killing cancer cells directly, some chemotherapy strategies like Fludarabine (FA) and Cyclophosphamide (CTX) are considering simultaneously to induce synergistic immunobiological effects. Chemotherapy combination with Cyclophosphamide (CTX), 5-Fluorouracil (FU), Folinic Acid (FA), and Docetaxel (DTX) are suggested as the pretreatment in cancer immunotherapy. By way of illustrations, pretreatment with CTX 300 mg/m2/day for three consecutive days and FA 30 mg/m2/day for three straight days is recruited when FRα (Folate Receptor α) CAR-T cells is a candidate for the treatment of end-stage ovarian (45).

Cytokine Release Syndrome (CRS) can be another negative consequence of CAR-T cell therapy. Medication of tocilizumab (Actemra) made life-threatening CRS in patients two years of age and older (46). It is shown that a systemic inflammatory reaction triggered by a fast increase in cytokines such as IL-1 and IL-6 can be seen in a mouse model. CRS typically happens within two days after CAR-T cell injection, and the worst reaction is 1–2 weeks after injection (47).

Several CAR-T cell toxicities are stated;

- On-target on-tumor type (excessive cytokine release or tumor cell necrosis)
- On-target off-tumor toxicity after a direct attack on normal tissues with the same targeted antigen
- Off-target toxicity follows by attacks an antigen except for the target

More than toxicities, the risk of immunosuppression, immunogenicity, genotoxicity (integrating viral vectors as an oncogenic to the normal DNA) is possible. So, even in a small number of CAR T lymphocytes injection observation might have been worth hesitating side effects. The development of "off-switches" in the system of suicide genes, such as inducible-caspase-9 (iCASP9), Herpes Simplex Virus Thymidine Kinase (HSV-TK), or truncated surface receptors make the selective excision of altered T cells possible (48). Also, an inhibitory CAR (iCAR) practice can support T cell activation in the existence of target antigens and can be applied to keep healthy tissue safe from CAR T cell-mediated deletion.

9. CAR-Natural Killer Cell Therapies

Recently, researchers have received much attention from Chimeric Antigen Receptor-T cells (CAR) and have shown significant improvements in cancer immunotherapy strategies, especially for hematologic malignancies. Moreover, two products based on CD19-targeted CAR-T cells under the names of Kymriah and Yescarta were approved by the FDA to treat hematologic cancers (49).

Furthermore, NK Cells are highly potent, long-life lasting, and besides its cytotoxic and antitumor properties, it is safe and cost-effective. NK cells do not show significant toxicity in trials. Since they do not have any side effects, they require shorter post-treatment hospitalization than CAR-T cell therapy, which means less overall treatment costs (50, 51).

As mentioned, NK cells, unlike T cells, do not have antigen receptors to detect cancer cells on their surface, and what facilitates distinguishing between healthy cells and foreign, infected, or malignant transferred cells by them is the estimation of the received signals in the absence of an inhibitory signal that leads to the release of cytotoxic granules at the target site (52).

In other words, NK cells sources are the NK cell line (NK-92), cord blood NK cell, Peripheral Blood NK cells, and induced pluripotent stem cells (iPSC). NK-92 cell line is the primary source for NK. cells in most research trials. Although NK-92 cells are very phenotypically similar to blood $CD56^{brights}$, they exhibit a high cytotoxic function. The activator receptors of these NK cells are NKP30, NKP46, NKG2D, and their inhibitory receptors are ILT-2, NKG2A, and KIR2DL4.

The unrestricted ability of NK cells for proliferation and resisting freeze/thaw cycle repetitions makes it an acceptable source for off-the-shelf CAR-NK cell manufacturing. It is not ideally perfect since, due to its inherent tumorigenicity, it does not express CD16 and NKP44, and it loses its expansion and proliferative potential before infusion (53).

Another source of NK cell is umbilical cord blood. However, NK cells are derived from peripheral blood NK cells have a more immature phenotype, lower cytotoxicity, and reduced expression of adhesion molecules including CD2, CD11a, CD18, CD62L, CD16, and KIRs, Perforin, and granzyme B and a high expression of NKG2A inhibitor. More importantly, they have a limited number of umbilical cord blood and obtain a sufficient number of NK cells for infusion; many cell divisions are required (54).

Human PBMCs are another resource from which sufficient NK cells, 90% of $CD56^{bright}$ and CD16 +, can be derived, stimulated, and expanded. PB can be autologous and allogeneic. The donor of this PB can be KIR-ligand, and HLA mismatched without the possibility of GVHD, and this increases the possible choices for being a donor.

The fourth source of NK cells is iPSC. This source is an essential source for clinical trials due to its unlimited proliferation feature. The ability to generate a large number of NK cells from a single CAR-transduced

iPSC cell makes it an ideal source for universal, off-the-shelf NK cells. However, it should be noted that these NK cells are derived from non-hematopoietic cells, such as fibroblasts, and are immature NK cells with lower expression of CD16 and KIR and higher expression NKG2A compared to PB NK. cells, reducing their cytotoxic potential toward target cancer cells. This drawback has been addressed by increasing the expression of required surface proteins such as CD16 through genetic engineering methods (55).

Electroporation is another method that alone does not have high efficacy; however, if NK. cell activation by cytokinesis was conducted prior, it can yield an efficacy of up to 80-90%. Nonetheless, in this method, the main problem is that the DNA entered in the cell is not part of the cell genome, and this makes the expression of CAR construct on the cell surface have a lifespan of about 3-5 days and can only be used during this time. This unfavorable feature makes this method unpractical for off-the-shelf products (56).

Table 1: FDA-approved CAR-T cell therapies clinical trials and their target genes.

Clinical Trial Identifier	Phase	Trade name	Generic name	Target Gene	Disease
NCT03123939	III	Kymriah	CTL019	CD19	Acute Lymphoblastic Leukemia
NCT01522183	II	Yescarta	KET-C10	CD19 receptors	Atypical Hemolytic-Uremic Syndrome (aHUS)
NCT02348216	I, II	Yescarta	KET-C10	CD19 receptors	Refractory Aggressive Non-Hodgkin Lymphoma (ZUMA-1)
NCT00065442	III	Provenge	Sipuleucel-T	Prostatic Acid Phosphatase (PAP)	Metastatic Prostate Cancer After Failing Hormone Therapy
NCT02902042	I, II	Keytruda	Pembrolizumab	PD-1	Advanced or unresectable melanoma
NCT02658019	II	Keytruda	Pembrolizumab	PD-1	Advanced Hepatocellular Carcinoma
NCT03226249	II	Keytruda	Pembrolizumab	PD-1	Previously Untreated Classical Hodgkin Lymphoma
NCT01515189	III	Yervoy	Ipilimumab	CTLA-4	Adult patients with inoperable or metastatic melanoma
NCT01696045	II	Yervoy	Ipilimumab	CTLA-4	Children and Adolescents (12 to < 18 Years) With Previously Treated or Untreated, Unresectable Stage III or Stage IV Malignant Melanoma
NCT02393859	III	Blincyto	Blinatumomab	CD19, CD3	Pediatric Subjects with HR First Relapse B-precursor ALL
NCT04464759	I, II	Opdivo	Nivolumab	PD-1	Advanced Melanoma (LIMIT)
NCT02574078	I, II	Opdivo	Nivolumab	PD-1	Non-Small Cell Lung Cancer (NSCLC) (CheckMate370)
NCT02060188	II	Opdivo	Nivolumab	PD-1	Recurrent and Metastatic Microsatellite High (MSI-H) and Non-MSI-H Colon Cancer
NCT02031458	II	Tecentriq	Atezolizumab	PD-L1	Advanced or Metastatic Non-Small Cell Lung Cancer (BIRCH)
NCT02603432	III	Bavencio	Avelumab	PD-L1	Locally advanced or metastatic urothelial carcinoma
NCT02155647	II	Bavencio	Avelumab	PD-L1	Metastatic Merkel Cell Carcinoma
NCT03682068	III	Imfinzi	Durvalumab	PD-L1	Unresectable Locally Advanced Urothelial Cancer or Metastatic Urothelial Cancer
NCT03871153	II	Imfinzi	Durvalumab	PD-L1	Non-Small Cell Lung Cancer Stage III or Non-small-cell Lung Cancer
NCT03628053	III	Kymriah	Tisagenlecleucel	CD19	Adult Patients with Relapsed/Refractory B-cell Precursor Acute Lymphoblastic Leukemia
NCT04002401	II	Yescarta	Axicabtagene Ciloleucel	CD19	Refractory Large B-cell Lymphoma
NCT02231749	III	Opdivo & Yervoy	Nivolumab & Ipilimumab	PD-1, CTLA-4	Previously Untreated, Advanced or Metastatic Renal Cell Carcinoma
NCT02853331	III	Keytruda & Inlyta	Pembrolizumab & Axitinib	PD-1, VEGFR	Renal Cell Carcinoma

Another method is transposon. The integration of DNA transposons (DNA transduction <100 kb) and the low immunogenicity made this method an ideal approach. This method also requires a non-transducer virus such as adenovirus to facilitate cell entry. There is a concern over the DNA, integrated into the genome, that can move and relocate and have to be used shortly before any change.

The transduction methods mentioned so far involve a random integration of genes in the genome, which in turn can lead to adverse effects such as repressor gene expression and cell apoptosis, or even malignant cell formation. However, CRISPR / Cas9 is a method in which a specific gene can be integrated into a particular part of the genome with a high degree of purposefulness. This method is the ideal method of genetic engineering for NK. cell modification (57).

Therefore, this summarizes the steps for preparing materials for use in preclinical and clinical trials. Previous studies have investigated the utilization of CAR constructs in immunotherapy, though 90% of them are concerned with T cells. Few clinical studies considering NK cells are considered **(Table. 1)**. Although the results of their final phase have not yet been reported, there are many hopes that they will be effective in cancer immunotherapy and especially solid cancers.

10. Conclusions

Undoubtedly, cancer immunotherapy can be reached by several approaches that boost the antitumor immune activity. Boosted cancer immunotherapy can target several steps to improve antitumor immunity and attacking to tumor cells would reinitiate and proliferate the cycle. Changing the protein expression profile of the immune cells by genetic engineering can have critical role in cancer therapies. Vaccines, suicide gene therapy, concurrent induction of cell death, and immune response like CAR-T cells advantage. Today joining gene-editing technology to immune cell therapy could enhance the treatment efficacy. The CRISPR/Cas9 system and viral or non-viral transgene, can progress the antitumor power of immune cell. For example, Tumor-Infiltrating Lymphocyte (TIL) therapy, Engineered T Cell Receptor (TCR) therapy, Chimeric Antigen Receptor (CAR) T cell therapy, and Natural Killer (NK) cell therapy can be potentially used in clinics to achieve better therapeutic outcomes. Maybe most future approaches will rely on several immunotherapies that work together.

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