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CAR-T Cell Therapy: Limitations and Improvements

Pranit K. Rao

Lincoln-Sudbury Regional High School, 390 Lincoln Rd, Sudbury, MA 01776; pranit.rao@gmail.com

ABSTRACT: The immune system consists of the body's defense against viruses, diseases, and many other versatile materials. Immunotherapy, which uses a combination of the body's immune system defense mechanisms, is vital to developing treatments for various cancers—specifically, Chimeric Antigen Receptor (CAR) -T cell therapy. Our understanding of CAR-T cell therapy is improving, but specific challenges in the treatment remain present, such as the tumor microenvironment (TME) and T-cell exhaustion, just to name a few. As a result, many researchers are developing creative approaches by using improvements to CAR-T cell therapy, which can increase the efficacy and results of cancer treatment. Topics researched include immune checkpoints such as PD-1 and CTLA-4, which are used in some clinical and preclinical trials. Cytokine integration with CAR-T cell therapy, including IL-2 and IL-12, has been tested. Another model includes BiTEs secreted from CAR-T cells to recruit other endogenous immune cells to kill the tumor including many other treatments not discussed. These studies have shown promising results, but much effort must be made to overcome obstacles. Overall, these findings and many more in the future possess viable treatments for many forms and types of cancer.

KEYWORDS: Immunology, Cancer Immunotherapy, CAR-T Cell Therapy, Cytokine, Checkpoint Inhibitor.

Introduction

Vertebrates have evolved into a complex but fascinating system that defends against a variety of pathogens. The immune system holds the answers to many diseases such as many cancers. The intricate system has two major lines of defense: innate and adaptive immunity. Around the turn of the 20th century, crucial discoveries, such as Elias Metchnikoff's phagocytosis, established immunology.[1] Furthermore, the discovery of neutralizing antibodies by Emil Behring and Paul Ehrlich aided immunology research as well, at a smaller scale.[1] These studies laid the foundation for immunology today and allowed for other discoveries to help reveal the concepts of the interactions between innate and adaptive immunity.[1] The immune system's two lines of defense are distinct. Innate immunity is the first defense against pathogens, such as viruses, bacteria, fungi, and parasites.[2] The innate immune system works independently with no prior recollection.[2] In other words, it combats external substances similarly, regardless of their composition or identity.[2] On the other hand, adaptive immunity is antigen-dependent, meaning it takes longer to implement due to its higher specificity of antigens. It also possesses the ability to remember instances of when compromise occurs, allowing it to form an immune memory.[2]

The application of immunotherapy is broad. However, cancer presents as a huge avenue as it affects one in five individuals worldwide at some point in their lifetime.[3] Cancer immunotherapy uses the body's immune system to recognize and destroy tumors. Furthermore, when compared to more traditional treatments such as radiation, surgery, and chemotherapy, it tends to be less invasive and more natural.[4] When successful, it can train the immune system to destroy a cancer cell and reduce the relapse rate.[5] There are many types of immunotherapies, including immune checkpoint inhibitors, chimeric antigen receptor (CAR) T-cell therapy, vaccines, cytokines, and many more.[4] Some immunotherapies include genetically engineered cells as treatments such as CAR-T cell therapy. As the years go on, immunotherapy research will increase, and more treatments involving CAR-T cell therapy will be developed.

Specifically, T-cells are a type of cell in the immune system that can be genetically engineered to specialize in killing cancer cells in the form of CAR-T cells.[7] At their core, CAR-T cells are engineered cells with synthetic receptors given to a patient that introduce chimeric antigen receptor (CAR) into the T cell genome leading to the production of CAR protein and its expression on the T-cell surface to recognize certain antigens and destroy them.[7] CARs have an antigen binding domain, a hinge region, a transmembrane domain, and one or many intracellular signaling domains.[7,8] The antigen binding domain is the part of a CAR that allows it to recognize and bind with specific antigens.[7,8] The hinge region of a CAR connects the binding regions to the transmembrane domain.[7,8] Issues have been observed in the transmembrane domain because of its frequent changes due to the requirements of the spacers and the signaling domains' changing requirements.[7] One of the transmembrane domain's most important roles is to anchor the CAR to the cell membrane, but other evidence indicates that it impacts the functioning of the CAR-T cell.[7,8]

Chimeric antigen receptor (CAR) -T cell therapy is a groundbreaking cancer treatment with a rich history. In 1987, the first findings about the possibility of CAR-T therapy were reported by Dr. Yoshikazu Kurosawa and his team.[6] Just two years later, Israeli immunologist Dr. Zelig Eshhar and his team found similar evidence that proved the possibility of manipulating the T-cells to recognize antigens; they also showed T-cell activation and killing of cancer cells by the chimeric receptor.[6] These findings stimulated research, particularly surrounding

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interleukin-2, among many others, adding to the studies previously conducted.[6] Fast forward to August 30th, 2017, the Food and Drug Administration approved the first T-cell therapy called tisagenlecleucel (Kymriah).[6] This therapy allows for the treatment of Acute Lymphoblastic Leukemia, specifically B-cell Acute Lymphoblastic Leukemia, for children and young adults.[6] Following the first approval, the FDA approved several other therapies, including three other CD19-specific treatments: Yescarta, Tecartus, and Breyanzi which are all second-generation CAR-T cells.[6] Figure 1 shows the interaction between the CAR-T cell and CD19.

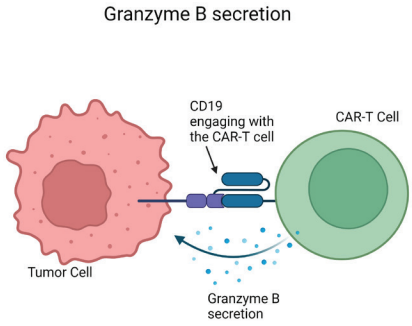

Figure 1: Made with BioRender. CD19 engages chimeric antigen receptors on the CAR-T cells producing Granzyme B and other molecules, which destroy tumor cells.

Lastly, the intracellular signaling domains are some of the most researched parts of CAR engineering. They are some of the most important parts of the CARs because they can enhance CAR-T cell therapy, in addition to being indispensable for CAR-T function similar to other parts of the molecule.[7,8] Figure 2 shows the various generations of the chimeric antigen receptor and the structure of a CAR. The first generation was developed in the 90s and had no internal costimulatory domains.[7] Costimulatory domains differ over generations and add various functions to the T-cells.[7,8] The lack of durability in the clinical trial results of the first generation indicates the importance of costimulatory domains.[7] After other studies, the importance of a costimulatory domain became evident concerning B-cell malignancies but is not limited to them.[7,8] The costimulatory domain added durability for CAR-T cell therapy, leading to the second generation of CARs containing one costimulatory domain.[7] Costimulatory domains are extremely different from each other in functional and metabolic ways, similar to how the two most common FDA-approved CAR-T cell products' costimulatory domains, CD28 and 4-1BB (CD137), are different.[8] Third-generation CARs have two costimulatory domains, but trials have yet to produce concrete results.[8] In addition, fourth-generation CARs have other associated functions on top of the two costimulatory domains.[7,8]

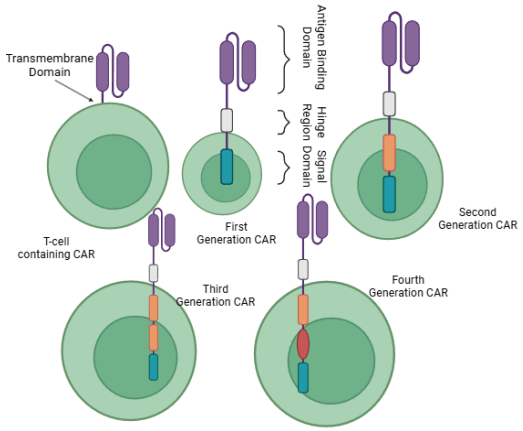

Figure 2: Made with BioRender. Structure of CAR-T cells and the various generations of CARs.

Although studies have proven CAR-T cell therapy to be a breakthrough in cancer treatment, many drawbacks and limitations remain. Some include the antigen escape, trafficking and infiltration of tumors, immunosuppressive tumor microenvironment, and toxicities.[7] Thankfully, integrating CAR-T cell therapy with other immunotherapies can be the future. This review aims to discuss a few specific drawbacks and potential improvements to CAR-T cell therapy as it currently stands through checkpoint inhibitors, cytokines, and secreted factors.

Discussion

Drawbacks of CAR-T Cell Therapy:

CAR-T cell therapy has achieved promising responses, but unfortunately, only 30-40% of patients with relapsed or refractory large B-cell lymphomas treated with the therapy reached remission in the long term, with many others experiencing short-term remissions.[9] Those numbers point to a need for improvement and opportunities to improve effectiveness in CAR-T cell therapy. Moreover, CAR-T cell therapy has many challenges but the two which will be discussed are the tumor microenvironment and T-cell exhaustion.

First, the tumor microenvironment (TME) is the complex atmosphere, including the tumor and the surrounding tissues and cells.[10] It consists of signaling molecules and immune cells, including macrophages, polymorphonuclear cells, mast cells, lymphocytes, myeloid-derived suppressor cells, and Treg cells.[23] The TME is made up of bone marrow-derived inflammatory cells, lymphocytes, blood vessels, fibroblastic cells, and extracellular matrix (collagen, proteoglycans, and many others).[10] The TME plays an intricate role in tumor growth and tumor progression—TMEs face a threat to immunotherapies such as CAR-T cell therapy.[10] For instance, they can create immunosuppressive microenvironments with analogous functions to normal tissues, making bypassing the immune system's controls easier.[10] The TME can also limit the amount of T-cells, giving it full rein over the immune system and acting as a drawback to many immunotherapies.

More importantly, the TMEs inhibit immune checkpoints. CTLA-4 and PD-1/PD-L1 are common checkpoints used to regulate T-cell activity. However, the TME uses these

checkpoints, such as CTLA-4 and PD-1, to its own advantage, promoting tumor growth, as seen with PD-1 in Figure 3.[9,10] Evidence of solely CAR-T cell therapy using CTLA-4 suggests that the T-cell accumulation was at extreme lows, reasoning that the TME can inhibit these regulatory checkpoints removing their functions.[12] Without those checkpoints functioning properly, no tumor cell suppression will occur because nothing stops it. In addition, patients who relapsed or were refractory to CAR-T cell therapy lymphomas had high expression of PD-L1 on tumor cells or TME.[9] These trials indicate many challenges *in vivo*, including regulatory checkpoints and loss of function due to the TME. Besides, immune checkpoint cytokines play a role in maintaining an immunosuppressive tumor microenvironment. They tend to contain immunosuppressive cytokines such as TGF-β, which inhibits T-cell activation and function.[12] The inhibition of T-cells makes CAR-T cell therapy remission difficult and faces many challenges.[9,11,12]

The second reason that CAR-T therapy faces many problems is T-cell exhaustion. T-cell exhaustion is a state in which the T-cells are dysfunctional.[13] When this occurs, numerous events occur, such as the loss of function and loss of T-cells.[13] The immunosuppressive tumor microenvironment is a prominent cause of T-cell exhaustion because of its capabilities.[14] The result of exhausted T-cells includes the increased expression of PD-1 and CTLA-4, which can harm the T-cell functions and result in greater tumor growth; in CAR-T cell therapy specifically, T-cell exhaustion plays an important role in reducing the limiting therapeutic success.[15]

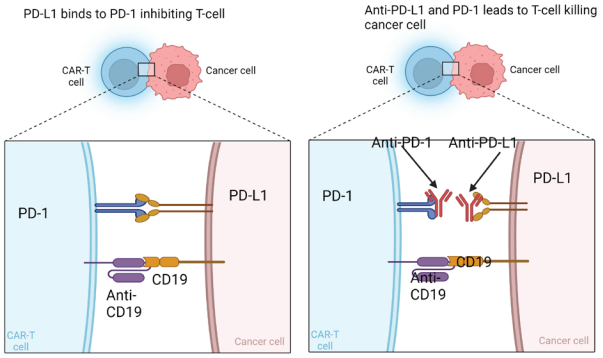

Figure 3: Made with BioRender. PD-1 and PD-L1 interaction with and without an inhibitor. The anti-PD-L1 and PD-1 inhibitors allow the T-cell to kill the cancer cell.

Specifically, Clinical studies show that a prominent reason for CAR-T cell therapy failure is a high T-cell exhaustion state. A trial found that CAR-T cells in the wild-type form expressed as high as 60% of CTLA-4 and PD-1 combined with a median value in the mid-40% in comparison to other integrative models discussed in the review.[16] That indicates that CAR-T cell therapy could be improved because it expresses high amounts of these T-cell inhibiting receptors.[16] Other studies using 4T1-HER2 also found similar results with substantially higher levels of PD-1 in the simple single-administered CAR-T cell.[11] Those results suggest that CAR-T cell therapy increases T-cell exhaustion because when T-cell inhibitors such as PD-1 and CTLA-4 are expressed at higher rates, there will be less T-cell activation.

Finally, when administering CAR-T cell therapy, tumor size and growth should also be noted. An *in vivo* study on mice targeting ovarian cancer found that typical CAR-T cell therapy yielded extremely low survival rates compared to other integrative models.[12] The lack of beneficial tumor-reducing results can be observed in the context of tumor growth. These unsuccessful results have prompted researchers to move from bedside to bench to develop more effective forms of therapy involving checkpoints, cytokines, and many others not limited to this discussion.

Checkpoint Inhibitors as Improvements to CAR-T Cell Therapy:

The immunosuppressive tumor microenvironment and T-cell exhaustion are significant barriers to CAR-T cell therapy. Recent advances present a different solution from typical CAR-T cell therapy involving the integration of immune checkpoint inhibitors to increase CAR-T cell efficacy.[9] Certain immunotherapies, such as pembrolizumab and Nivolumab, can aid CAR-T cell therapy by reducing the impact of T-cell exhaustion and the tumor microenvironment. Pembrolizumab is an anti-PD-1 immune checkpoint inhibitor that can reduce T-cell exhaustion.[9] Pembrolizumab (Figure 4) was in phase 1/2a study for patients with relapsed or refractory B-cell lymphomas.[9] All patients had already undergone anti-CD19 CAR-T cell therapy, but the results failed due to T-cell exhaustion.[9] Approaches combining CAR-T cell therapy with checkpoint inhibitors have shown promising results in many clinical and preclinical models in B-cell lymphomas and Glioblastoma.[9]

For example, the clinical trial for pembrolizumab, aimed at patients with B-cell lymphomas, mitigated the aforementioned issues with promising results. 25% of the patients achieved a clinical response, and 33% had clinical benefits from the trial.[9] The study also found that 75% of these patients also had increased CD19 expression levels detected by PCR allowing for the CAR-T cells to kill the cancer cells more easily.[9] The research found that ten of the twelve patients in the trial experienced a proliferation of CAR-T cells in peripheral blood.[9] Furthermore, deep immune profiling with the help of CyTOF displayed less CAR-T cell exhaustion while its activation and growth increased.9 More findings suggest that the patients treated with pembrolizumab had an increased proportion of CAR-T cells, according to CyTOF.[9]

However, CAR-T cell therapy clinical trials without pembrolizumab suggest T-cell exhaustion, but an approach including pembrolizumab resulted in a buildup of CAR-T cells, leading to increased therapy efficacy.[9] These results indicate that CAR-T cell therapy is not efficient while using checkpoints such as PD-1 and pembrolizumab can increase efficiency. The results of pembrolizumab have been conveyed as promising and safe, giving hope for a future with an integrated treatment.[9]

Glioblastoma (GBM) is another example of how CAR-T cell therapy is being used with checkpoint inhibitors. CAR-T

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cell therapy has been promising in hematologic malignancies but faces similar challenges as the aforementioned studies, such as T-cell exhaustion.[15] As previously stated, there is a relation between CAR-T cells' reduced function, the expression of many surface inhibitory receptors, and the upregulation of inhibitory T-cell enzymes. There are many strategies for integrating checkpoints with CAR-T cell therapy.

One of these strategies, a potential solution to T-cell exhaustion and TME issues, is the co-administration of CAR-T cell therapy with another drug. This approach uses immune checkpoint-blocking agents in parallel to CAR-T cell therapy.[15] It would entail providing checkpoint inhibitors such as anti-PD-1 or anti-CTLA-4 to block the interaction between the receptor and the ligand.[15] The inhibition would allow the CAR-T cell to function normally and not get silenced by the inhibitors. Some preclinical examples are in HER2-expressing breast cancer models and IL-13Rα2-expressing GBM.[15] Both studies found that the delivery of checkpoint-inhibiting agents resulted in the amplification of the CAR-T cells.[15] Though this is still being researched, a current phase one clinical trial (NCT03726515) is ongoing regarding co-delivery. It aims to see how safe and effective combining EGFRvIII-targeting CAR-T cells with PD-1 monoclonal antibody pembrolizumab in patients with GBM.[15] EGFRvIII is a mutated wild-type receptor expressed on GBM and other tumors specifically and can be found in approximately 30% of GBM patients.[15] It is useful for CAR-T cell therapy because it is easily identifiable and can't be found in normal tissues.[15] Patients received both EGFRvIII-targeting CAR-T cells and pembrolizumab because pembrolizumab can limit T-cell exhaustion.[15] No results were disclosed from the study, but the data will greatly enhance the research and develop viable treatments.[15]

Another integrative solution proposed for GBM is the additional genetic engineering of CAR-T cells. The approach relies on CAR-T cell's ability to secrete checkpoint-blocking agents themselves.[15] By allowing the CAR-T cells to immediately inhibit the checkpoint molecules, helping eliminate the tumor, it could be safer and more potent boosting effectiveness. An example includes engineering CAR-T cells to use membrane-bound, secreted antibodies or secrete a smaller blocking scFv.[15]

The last proposed integrative therapy with the goal of checkpoint inhibition is another combination therapy. This approach combines CAR-T cell therapy with multiple types of checkpoint inhibitors.[15] For example, a combination therapy could include anti-PD-1 antibodies with anti-CTLA-4 antibodies because they are checkpoint inhibitors but have different functions and targets. This approach has its benefits because it can address multiple issues and challenges that CAR-T cell therapy has, such as T cell exhaustion and the TME, by using multiple checkpoint inhibitors, creating a more diverse defense against cancer and adding to the performance of the therapy.[15] Currently, there is a 60-patient GBM clinical trial (NCT04003649) going on in Phase I, aimed to compare the effects of a group that is receiving IL-13Rα2 CAR-T cells with nivolumab and ipilimumab and a group that receives only IL-13Rα2 CAR-T cells.[15] The two different arms of the study aim to determine the efficacy and safety of integrative combination therapy.[15] It is believed that combination therapies using nivolumab and ipilimumab can attack cancer and tumors in the body and mitigate tumor growth.[15] Due to the ongoing status of the trial, it is yet to be determined if IL-13Rα2 and the two monoclonal antibodies will interact and work together beneficially.[15]

Similar studies surrounding CTLA-4 (Figure 4) found that checkpoint inhibition can reduce T-cell exhaustion, making it potentially a viable integrative approach. CTLA-4 is an immune checkpoint receptor that can inhibit T-cell activation. Specifically, a study aimed to use CRISPR-Cas9-mediated deletion of CTLA-4 in a preclinical model to see if the deletion of those receptors could boost T-cell function and reduce T-cell exhaustion.[16] The preclinical model examined how the expanded CART19 compared with WT CART19, CTLA4 knockout CART19, and others.[16] The study looked at the population doubling of the CAR-T cells, which measured the growth of the CAR-T cells *in vitro* before being put into the patient.[16] The result tends to vary once the CAR-T cell is given to samples obtained from patients. The study measured T-cell exhaustion by measuring the amount of inhibitory receptors present when each of the different versions of T-cells was given.[16] Results indicate that inhibitory receptors such as LAG3, TIM3, CD38, and CD39 were reduced when CTLA-4 was knocked out compared to WT CART19.[16] As fewer inhibitory receptors were expressed in the CTLA-4 knock-out CAR-T cells, T-cell exhaustion also reduced alongside it according to the findings.[16] Those findings suggest that performing CTLA-4 knockout checkpoint molecule in CART19 reduces T-cell exhaustion, making CAR-T cell therapy more efficient. Though the T-cell exhaustion may be lower, the findings suggest no substantial difference in the percentage of specific killing when using CTLA-4 knockout.[16] In addition, the population doubling of T-cells when stressed is higher when using CTLA-4 knockout, indicating that the number of T-cells increases, mitigating the problem of T-cell exhaustion.[16] Furthermore, there is an exponential decrease in the amount of tumor cells in all the versions of CTLA-4 knockout used in the experiment.[16] The results are significant because they indicate that the integrative solution of the CAR-T cell combination therapy substantially reduces tumor size *in vivo* (mouse) and has the potential to work in clinical trials. In addition, the presence of Granzyme B, the substance that T-cells secrete to kill the tumor, was observed.[16] By knocking out CTLA-4, the percentage of Granzyme B secretion skyrocketed compared to the wild-type CART19.[16] This caused a higher rate of tumor cells being destroyed, resulting in an overall increase in the elimination of cancer.[16] It is evident how knocking out CTLA-4 while doing CAR-T cell therapy is useful because it reduces T-cell exhaustion, reduces tumor size, and increases Granzyme B secretion.

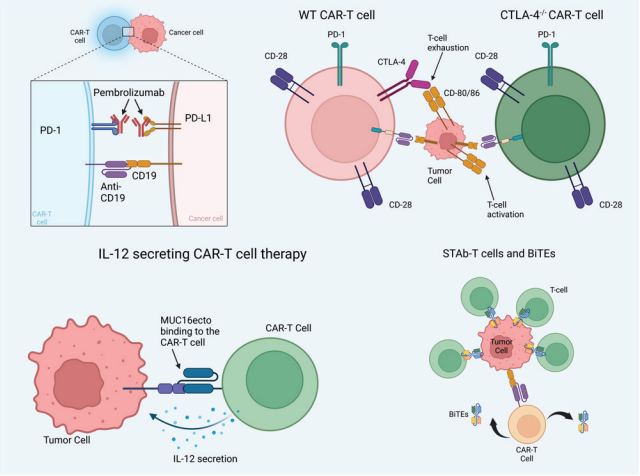

Figure 4: Made with BioRender. The mechanism of various cancer treatments using integrative therapies for CAR-T cell therapy. Treatments include checkpoint inhibition, cytokines, and BiTEs.

Cytokines as Improvements to CAR-T Cell Therapy:

A different approach to integrative treatments for CAR-T cell therapy is through cytokines. Cytokines are small signaling proteins in the body that carry messages between cells and can play a role in a cell's immune defense.[17] They are secreted primarily by T-cells, depending on the cytokine.[17] Cytokines such as Interleukin-12 (IL-12) and IL-2 can enhance T-cell function and help overcome its inhibitory environment.[17] The integration of cytokines into CAR-T cell therapy has shown promising results.

For instance, in glioblastoma (GBM), CAR-T cell therapy, integrated along with cytokine therapy, has been ongoing, and various kinds of research have been conducted to support it. The most straightforward integration model is co-administration, which has pointed towards promising effects in other diseases, such as melanoma, where it increases the treatment efficacy and reduces the TME.[18] Many other recent efforts have been seen, such as a potential treatment where they administered IL-2 with EGFR-directed CAR T-cells.[18] However, it struggled after the preclinical trials due to toxicity and lack of effect on humans.[18] Eventually through continued effort and modification, the treatment results were prevalent, and the T-cell exhaustion, TME, and other challenges of CAR-T cell therapy were mitigated.[18] In addition, another example engineered the CAR-T cells to secrete cytokine against GBM. by using NK cells, IL13Rα2, among many others, to manipulate the secretion of cytokines.[18]

Another method that makes it possible for cytokines to aid CAR-T cells is through coexpression and designing the CAR to include cytokine receptors.[18] Those cytokine receptors can change a cytokine's response, boosting the CAR or even specifically, the NK cell.[18] This results in improved functionality of the CAR and the destruction of the tumor. An abundance of action has been taken, including clinical trial NCT01811992, which introduces IL-7 into EGFRvIII-targeted CAR T-cells with the goal of proliferating a greater amount of T-cells and increasing expression of memory markers in those cells.[18] The trial has indicated promising results, aiding an encouraging future of research regarding many modes of using and engineering cytokines into CAR-T cell therapy.[18]

Furthermore, IL-12-secreting CAR-T cells against ovarian cancer (Figure 4) were also studied. The study modified MUC16 ecto-specific CAR-T cells to secrete IL-12, whose role was to boost the function of CD8+ T cell to enable it to overcome its "inhibitory environment" and allow CAR-T therapy to perform better.[12] The trial was designed based on previous research on injecting IL-12 into the subjects, which found some efficacy.[12]

The data from the *in vitro* study were encouraging as they suggested that the 4H11-28z/IL-12 performed better because they could express a higher value of IFNγ than the 4H11-28z.[12] Although there was more IFNγ expressed, the 4H11-28z and 4H11-28z/IL-12 CARs both killed their targets equally.[12] Another result found *in vitro* was that the IL-12 secretion increased four-fold in the 4H11-28z/IL-12 and EGFRt/4H11-28z/IL-12 CARs compared to the 4H11-28z CAR.12 Additionally, the IFNγ levels in the 4H11-28z/IL-12 and EGFRt/4H11-28z/IL-12 were 27-fold higher than the 4H11-28z CAR T cells.[12] The increase of cytokines secreted in this therapy is important to help the defense and to destroy the tumor cells better.[12] The amount of cytokine secretion thus causes the results to be noticed *in vivo* in mice. The *in vivo* results concluded in the analysis that the mice treated with 4H11-28z/IL-12 and EGFRt/4H11-28z/IL-12 performed better and had a higher rate of survival than the mice not treated with IL-12.[12] It was also clear that when treated with IL-12, the mice's tumors were cleared.[12] It is also important to note that the blood samples drawn from the mice with 4H11-28z/IL-12 had elevated T-cell persistence levels compared to those with 4H11-28z.[12] The mice with 4H11-28z CARs had hardly any CAR-T cells remaining, while those with IL-12 had more T-cells.[12] The results from an *in vitro* and an *in vivo* study indicate that when cytokines are combined with CAR-T cell therapy, there are benefits such as elevated T-cells, more secretion of tumor-killing cytokines, and the eventual elimination of the tumor.

Lastly, another study addressing the issue of TME and CAR-T cell exhaustion, especially in solid tumors, involved co-engineering IL-2 with CAR-T cell therapy. The study aimed to improve the efficacy of CAR-T cell therapy against solid tumors by engineering the CAR-T cells to coexpress an IL-2 mutein (IL-2m).[11] The trial included *in vitro* and *in vivo* methods, including the use of mice as subjects. IL-2 aided the immune system by influencing many other cells in the immune system to combat the tumor.[11] The cytokine has the versatility of influencing many other cells because of its large number of receptor subunits. The study used HER-2-targeting CAR-T cells to compare the efficacy of co-engineering IL-2 into therapy.[11] The study results were promising and allowed the researchers to make a definitive conclusion about the integrative use of IL-2 in CAR-T cell therapy.[11]

In vitro, when the IL-2 cytokine was co-engineered and implemented in the lab, it performed better than the normal CAR-T cell.[11] It was evident that both IL2-HER2-CAR-T cells and IL2m-HER2-CAR-T cells exhibited significantly

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higher values of cell count and proliferation than the normal CAR-T cell.[11] The study also found that while using the IL2-HER2-CAR-T and IL2m-HER2-CAR-T cells, there was an upregulation of p-STAT5 expression, an activation marker for IL-2 located downstream of IL-2 signaling.[11] That is significant because there was a greater proliferation of T-cells to target the tumor and a greater amount of activation found in the study, indicating that the IL-2 can be expressed and enhance CAR-T cell therapy. More importantly, IL2m-HER2-CAR-T cells expressed far reduced levels of PD-1, previously defined as a marker indicating T-cell exhaustion.[11] According to the study, there were also higher levels of CD25, which is important because it relates to T-cell activation, signifying that the number of T-cells increased.[11] This suggests that integrating cytokines into CAR-T cell therapy reduces challenges such as T-cell exhaustion. In addition, while using IL-2, the efficiency at which the tumors were killed also increased due to the increase in Granzyme B percentage.[11]

Similarly, the *in vivo* results also aligned with the *in vitro* results. All CAR-T cell therapies worked in immunodeficient mice, but in immunocompetent mice, the results varied.[11] The findings only saw that IL2m-CAR-T inhibited tumor growth at a major level, while IL2-CAR-T and HER2-CAR-T cells had the same results.[11] These findings show that only specific versions of cytokine immunotherapy and certain modifications can work at a better rate than typical CAR-T cell therapy. In terms of T-cell exhaustion and TME, IL2m-CAR-T cell therapy worked the best in reducing PD-1 expression, and NK cell proportions were higher, similar to the *in vitro* studies implying that the cytokine integration allowed for the reduction of T-cell exhaustion and the improvement of the microenvironment.[11] This research can go far and can play a role for humans by countering solid tumors, especially among many others.

Secreted Factors as Improvements for CARs:

In addition to all the other treatments discussed, an up-and-coming new treatment uses T cell-redirecting bispecific antibody (STAb) T cells.[19] The idea behind this approach is to find a treatment that enhances the function of CAR-T cells by recruiting other endogenous immune cells to inhibit tumor growth (Figure 4).[19] In addition, it involves the recruiting of endogenous immune systems.[19] Compared to CAR-T cells engaging with the tumor cells and targeting the tumor by itself, an alternative treatment uses STAb-T cells to kill the tumor while recruiting other T-cells to perform the same job after they are secreted.[19] The STAb-T cells secrete T cell-redirecting bispecific antibodies that allow CD3 to engage with the tumor-associated antigen (TAA).[19] The T cell-redirecting bispecific antibody helps the T-cells engage with the tumor, leading to more interactions. Using these STAb-T cells has many benefits.[19] Some include the increase in the polyclonal recruitment of T-cells more than what CAR-T therapy itself does.[19] In addition, because it is secreted *in vivo*, fewer concerns revolve around storing the bsAb therapeutic itself, but more research is necessary because certain limitations may be present.[19]

Outlook of CAR-T Cell Therapy Improvements:

Though these integrative treatments have demonstrated promising results, side effects, and toxicity are key issues. In terms of side effects, for a clinical trial, neutropenia was observed, and side effects as harmful as severe autoimmune complications were exhibited.[9] It is important to note that CAR-T cell therapy has side effects, and integrating another treatment method may add unforeseen side effects. In addition, Cytokine Release Syndrome (CRS) may occur as it is already a side effect of CAR-T cell therapy in addition to neurotoxicity.[15] IL-2 also has toxic aspects leading to side effects, drawing many questions and discussion about its use. IL-2 at high doses can be associated with severe conditions such as vascular leak syndrome or capillary leak syndrome, leading to oliguria, ischemia, and confusion, cautioning against high-dose treatments.[22] Overall, the nature of the side effects is not fully known making it necessary for further research.

The overall results of the studies allow many ideas for reflection and improvement. For example, the results of the pembrolizumab study found that overall efficacy is just 25% in response to the integrative approach.[9] Though the result is promising, it hints at the need for improvement and modifications to improve overall efficacy.[10, 20] The only way to create these improvements is by conducting more research and more clinical trials with a larger patient size greater than the 12 in the pembrolizumab study.[9] Doing so will enhance CAR-T cell treatment in integrative models and cancers where CAR-T cell therapy is ineffective. For instance, the ovarian cancer study found a lack of responsive therapy to CAR-T cell therapy with a Phase I clinical trial, indicating no positive results.[12] More research, studies, and modifications in this study would be integral to enhancing CAR-T cell therapy, especially by utilizing other mechanisms such as checkpoint receptors, cytokines, and BiTEs.

However, none of these trials discuss the costs of integrating CAR-T with other mechanisms, which can be up to a staggering $500,000 per infusion.[20] In addition to those high costs, monitoring the effects of the therapy can cost approximately an extra $80,000.[20] With integration or coadministration, there are chances that the price may be affected, making it harmful to patients who can't afford the treatment or patients whose insurance providers don't cover the full costs of the treatment. It is pertinent to observe the effect of price when developing a potential treatment for cancer to see its financial impact on patients. Thankfully, some treatments, such as the CTLA-4 study, have described increased feasibility in engineering therapy, which could have cost implications because the development process will be shortened demonstrating how the field is rapidly changing along with costs.[16] Overall, there is hardly any mention in the studies conducted about the additional costs of the treatment and the impact that it would have on patients and treatment accessibility.

The integration of CAR-T cell therapy has benefits, but it also has drawbacks. Future research may answer many of these questions and allow modifications.

Conclusion

The pursuit of finding a viable, safe cancer treatment continues. Over the years, many creative approaches have surfaced, including integrative methods for CAR-T cell therapy. CAR-T cell therapy is an outstanding immunotherapy with some challenges, including T-cell exhaustion and the tumor-microenvironment, which rationalizes combining other treatments. Much early research has been put into place, including integrating CAR-T cell therapy with checkpoint inhibitors, cytokines, and BiTEs. For example, checkpoint inhibitors like pembrolizumab can aid patients who have relapsed from cancer by combining the treatment of a certain CAR-T cell therapy and the drug to act as an immune checkpoint inhibitor. CTLA-4 does a similar job but has not yet been developed for clinical trials. In addition, cytokines can aid CAR-T cell therapy by secreting cytokines that can target the cancer cells in many ways. The engineering of the CAR-T cell allows the secretion of cytokines in a specific way. Lastly, an integrative therapy that can boost CAR-T cell efficiency is T cell-redirecting bispecific antibodies because they allow for a greater value of T cells to target the tumor by increasing the rate of T-cell activation. These methods have been promising, and studies have proven that they can have a beneficial impact in the future. Still, many challenges exist, including side effects, lack of research, and feasibility. Other treatments, such as LAG3, may be used with CAR-T cell therapy, such as CRISPR-Cas9 mediated gene editing, to knock out those checkpoints. Research about LAG3 has been increasing recently, and in the future, it will likely be researched more on a clinical scale.

Similarly, T-cell immunoglobulin and mucin-domain-containing TIM3, a transmembrane protein, have not been researched enough because they are newer. TIM3 inhibition can reduce the tumor by decreasing the immunosuppressive environment and yielding more T-cells.[21] In addition to LAG3 and TIM3, many other treatments, such as a CRISPR-influenced treatment, other cytokines not mentioned in the paper, and other checkpoint molecules, are in the earliest stages of research. In the future, more efficient, feasible, and safe treatments can pave the road for further research, especially with less-researched treatments that could revolutionize cancer immunotherapy.

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Author

Pranit Rao is a Junior in the Class of 2026 at Lincoln-Sudbury Regional High School. He enjoys playing tennis, playing basketball with his friends, and spending time with his family. He aspires to study something around biology or pre-medicinal studies.