The Binding Nature of TGF-beta Chimeric Antigen Receptors, With Mutant Transforming Growth Factors

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ABSTRACT

A crucial aspect of the immune system is its maintenance adaptivity, this is done through the transforming growth factor beta (TGF-b). Transforming growth factors (TGF-b), which affect all cell types and regulate cell activity, are cytokines. A family of transmembrane protein kinases is how TGF-b communicates. TGF-b maintains the homeostasis of T cells in the periphery by activating TGF-b through TGF-b binding proteins in the extracellular matrix. TGF-b can only carry out its several tasks once it transitions from a latent to an active state, during which the latency-associated peptide no longer protects the mature peptide's receptor binding site. TGF-b has been proven to impair cell maintenance in the absence of this bondage, which limits its ability to promote homeostasis, differentiation, cell development, and tolerance in the face of dysregulation. Therefore, when TGF-b is altered, it prevents cell cycle progression and encourages apoptosis to create tumor-suppressive effects. As a result, malignancies become more aggressive and spread more widely in their later stages.

Lab-designed receptors called chimeric antigen receptors (CAR) bind to specific proteins on cancer cells. As antigen receptors attach to transforming growth factors to create the protein TGF-b CAR, anti-immunosuppressive effects are engaged when TGF-b binds to CAR. Consequently, it possesses the question of what results from mutant TGF-b binding to TGF-b CAR? However, it is essential to determine whether the interaction is even conceivable before exploring its effects on proliferation. As a result, the question of whether TGF-b CAR binds to TGF-b mutant exists.

VISUAL ABSTRACT:

Figure 1. visual abstract of a future implication of the presented method.
Literature review

The immune system's ability to maintain its adaptivity is one of its most important functions, and transforming growth factor beta is responsible for this (TGF-b).

Transforming growth factors (TGF-b) are cytokines that act on all cell types and mediate cell function. TGF-b signals through a family of transmembrane protein kinases (Altman, 2002). TGF-b is activated through TGF-b binding proteins in the extracellular matrix (Immunol, 2014), through which TGF-regulates the homeostasis of T cells in the periphery. “TGFβ-1 can only exert its many functions after going from this latent to an active state, in which the binding site of the mature peptide for its receptor is no longer shielded by the latency-associated peptide” (Janssens, 2003). Without this bondage, TGF-β has been shown to inhibit cell maintenance, limiting its role in homeostasis, differentiation, cell development, and tolerance during dysregulation. Therefore, when mutated, TGF-inhibits cell cycle progression and promotes apoptosis to produce tumor-suppressive effects, therefore in the later stages, cancers become more aggressive and spread.

Understanding the ins-and-outs of chimeric antigen receptors will increase the efficiency of my experiment and my research. For example, “There are normally three primary areas in chimeric antigen receptors. First, the CAR region of an antibody's single chain variable fragment (scFv) is an extracellular ligand-binding domain” (Ahmad, 2022). Participating authors within the paper have been featured on publication domains which serves as a credible platform and search engine when researching medicine and science related topics as all articles and posted journals are peer reviewed by experts before publication. Independent of the peptide-HLA complex, this area is in charge of recognizing particular target antigen. A transmembrane domain and a hinge/extracellular spacer are found in the second section of a CAR construct. This area connects the other two CAR parts and improves the construct's overall flexibility, stability, and dimerization. A cytoplasmic signaling domain and costimulatory molecules make up the third area. This section is in charge of signal transmission, and here is where most CAR construct modifications are made to increase a particular CAR's capability for signal transmission.

Conducting this experiment presents my research gap as this experiment has never been conducted before. This allows for new conclusions and research findings to add onto, and support this area of knowledge. Experimenting with the abilities of TGF-b CAR to bind with TGF-b mutant will help patients that express the mutated protein through the interference of TGF-b CAR as “TGF-βRI mutation has been detected in ~19% of HNSCC [(Head and neck squamous cell carcinoma)] patients with metastasis” (Wu, 2018). Additionally, testing the binding tendency of TGF-b CAR to mutated TGF-b will be a good model to test the extent of the functions of Chimeric antigen receptors as Chimeric antigen receptors play a significant role in eliminating cancer, for example, assisting t-cells in targeting and eliminating cancer cells that have specific proteins with specific receptors to bind to (Curran, 2012).

Roberts and Sporn went on to describe TGF- as a secreted polypeptide that can stimulate collagen and fibroblast growth. TGF- was quickly discovered to also suppress cell proliferation, leading to the recognition of its dual function. The human genome encodes 33 TGF-beta family members, including TGF-beta itself, as well as activin, nodal, growth, and differentiation factors, and bone morphogenetic protein (BMP) (GDFs) (Rothfels, K (2017-05-24). To trigger SMAD (Suppressor of Mothers against Decapentaplegic)-dependent and SMAD-independent signaling, this superfamily of ligands typically binds as dimers to hetero-tetrameric cell-surface receptor serine/threonine kinases (reviewed in Morikawa et al, 2016; Budi et al, 2017). TGF-beta initiates signaling by the TGF-beta receptor complex. When TGF-beta (TGFβ1), which is released as a homodimer, interacts to TGF-beta receptor II (TGFBR2), it causes the latter to dimerize and create a stable heterotetrameric complex with the homodimer of TGF-beta receptor I (TGFBR1). The internalization of the heterotetrameric TGF beta receptor complex (TGFBR) into clathrin-coated endocytic vesicles and the recruitment of cytosolic SMAD2 and SMAD3, which function as R-SMADs for TGF-bR, are both triggered by TGFBR2-mediated phosphorylation of TGF-bR1. This is significant as it relates directly to my experiment when analyzing the bonding structure of receptors and the interaction between the two proteins.
Research Strategy

To determine if TGF-b CAR binds to mutant TGF-b, mouse cell lines will be used, specifically, NKT (Natural killer T) cell lines. This is because the CAR gene is already expressed, additionally, TGF-b is a key regulator of the NKT cell line (Doinse, 2009). However, the TGF-b needs to be inhibited beforehand in each cell line in order for the results to be controlled. Firstly, the blocking of the expression of TGF-b in all cell lines while in culture through siRNA (small interfering RNA molecules) (Wang, 2014) this will inhibit the function of transforming growth factors, and therefore affirming a mutation. Next, each siRNA paired cell line will be infused with TGF-b CAR in order to begin the experiment. Finally, the cell line will undergo incubation in order to be eligible for co-immunoprecipitation which will be used in order to assess the interaction between the TGF-b CAR protein with the mutated TGF-b cell line. The positive control of this experiment was set as the use of an unmutated TGF cell line in order to assess binding without a mutation and affirm the possibility of the hypothesis. And the negative control will be the suppression of TGF-b through pseudo-receptor (BMP) and activin membrane-bound inhibitor (BAMBI), which will be responsible for inhibiting the factor from binding (Lin, 2006). This will affirm the null hypothesis that negates the theory that there will be any binding at all between the two growth factors.

Experimental Design

With the specific aim of concluding whether TGF-b CAR is able to bind to mutant TGF-b, this experiment was designed in order to pave the easiest way for these results. This is briefly done through using mutated TGF-b cell lines and injecting TGF-b CAR into the cell lines in order to assess binding through bio-fluorescence complementation. TGF-responsiveness and tumor-targeting specificity must be successfully combined in order for TGF-CAR-T cells to be clinically translated for cancer therapy. Additionally, it is important to carefully consider the possibility that contaminating, TGF-producing regulatory T (Treg) cells may preferentially increase during the production of TGF-CAR-T cells and repress effector T (Teff) cells. The anti-tumor effectiveness of nearby cytotoxic T cells is greatly increased by the presence of GF-CAR-T cells. Furthermore, neither TGF-CAR-Treg cells nor TGF-CAR-Treg cells cause CAR-mediated suppression of Teff cells when TGF-CARs are introduced into mixed T-cell populations. These findings confirm the value of using TGF-CARs to provide adoptive T-cell therapies for cancer which will be significant in my rationale and purpose.

In order to introduce the experiment, research has presented two hypotheses and the following controls:

Hypothesis: TGF-b CAR can be activated by TGF-b mutant.

Null Hypothesis: there will be no activation of TGF-b

Positive control: the usage of an unmutated TGF-b

Negative control: the suppression of TGF-b through pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI). Which will inhibit the factor from binding. According to the hypothesis and controls, the following predictive observations have been made.
Table 1. Potential observations of experiment.

<table>
<thead>
<tr>
<th>Potential Observations</th>
<th>Interpretations</th>
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<tbody>
<tr>
<td>A  Positive control has more fluorescence than the negative mutant TGF-b and gives just as much signal as positive binding as mutated TGF-b.</td>
<td>Indicates the receptor is effective and active to the extent of the mutated TGF-b binding to the TGF-b CAR.</td>
</tr>
<tr>
<td>B  Mutant TGF-b doesn’t have as much fluorescence as the positive control.</td>
<td>Indicates the receptor is active but to as much as using unmutated TGF-b.</td>
</tr>
<tr>
<td>C  Failure of TGF-b CAR to bind to mutant TGF-b to bind as per the negative control through the suppression of TGF-b.</td>
<td>Indicates the suppression of TGF-b mutant through pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI). Which will inhibit the factor from binding.</td>
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The following are the results being sought out by this experiment: potential observation A, which affirms the hypothesis in which the positive control has more fluorescence than the negative mutant TGF-b. And gives just as much signal as positive binding as mutated TGF-b. This would indicate the receptor is effective and active to the extent of the mutated TGF-b binding to the TGF-b CAR. Furthermore, potential observation B, which would be interpreted as the mutant TGF-b has less fluorescence then the positive control. Which would indicate that the receptor is active but to as much as using unmutated TGF-b. This would affirm the null hypothesis, thus affirming the negation and introducing limitations to this experiment. Additionally, the observation of the failure of TGF-b CAR to bind to mutant TGF-b to bind as per the negative control through the suppression of TGF-b. Which would indicate the suppression of TGF-b mutant inhibiting the factor from binding, furthermore supporting the null hypothesis. The use of co-immunoprecipitation will be used to assess these results as it is the technique typically used to easily identify protein interactions. In order to pursue this experiment the following experiments have been purchased under a student license through Cell Signaling Technology.

Instruments:

- TGF-b CAR cell lines
- TGF-b mutant cell lines
- Solutions and Media: *SG-CAA Media* (Induction media) & *SD-CAA Media* (Growth media)

Additionally, to determine if TGF-b CAR binds to mutant TGF-b, mouse cell lines will be used. Specifically, NKT (Natural killer T) cell lines. This is because the CAR gene is already expressed, additionally, TGF-b is a key regulator of the NKT cell line (Doinse, 2009). However, the TGF-b needs to be inhibited beforehand in each cell line in order for the results to be controlled. To begin with, the blocking of the expression of TGF-B in all cell lines while in culture through siRNA (Wang, 2014). Next each cell line will be infused with TGF-b CAR, then, a co-immunoprecipitation will be used in order to assess the interaction between the TGF-b CAR protein with the TGF-b cell line. The positive control of this experiment will be the use of an unmutated TGF cell line, and the negative control will be the suppression of TGF-b through pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI), which will inhibit the factor from binding (Lin, 2006).
Following the purchase of the previous equipment, S.A Hunter’s procedure on Cell-Binding Assays for Determining the Affinity of Protein–Protein Interactions, posted in 2016, was manipulated and used to suit my research objectives.

**Procedure**

Determine the range of ligand concentrations.
Measure the OD600nm of each induced cell culture.
Resuspend in binding assay buffer for the final binding incubation volume (step 1).

Add the ligand to each sample. Do not add ligand to the control tubes for expression, nonspecific secondary binding, and cells only.

Post Incubation:
*Keep samples at 4°C. Spin down samples at 14,000×g for 30 s at 4°C, and remove the supernatant.*
*Resuspend each tube with 50 μL of cold binding assay buffer. Wash each sample with 0.5 mL of cold binding assay buffer.*
*Resuspend each sample in 50 μL of secondary culture containing a binding assay buffer. Analyze the samples on a flow cytometer.*

Co-immunoprecipitation (Co-IP):
Preparation of the cell lysate.
Binding of the bait and prey by GFP-Trap.

Washing off unbound molecules, prey should remain bound to bait. Elution of bound bait and prey from the beads.
Analysis of bait and prey on SDS-PAGE, Western blot, or by mass spectrometry.
(S.A Hunter, 2016)

**Limitations**

- TGF-b CAR attaches to the competitive site instead of allosteric binding region
- Alternative approach: cross linking proteins-co-immunoprecipitation followed by western blot to determine protein to protein interaction.

There are still a number of difficulties in successfully using CAR T cells in solid tumors.

First, if the target is expressed on a tissue that is vital to life, the strength of these “living pharmaceuticals” can cause lethal on-target toxicity. Second, although these adverse effects are normally brief and reversible, active CAR T cell multiplication in the body can result in potentially dangerous side effects like CNS toxicity and cytokine release syndrome. Third, even though it can be difficult to identify a single tumor antigen that is uniquely expressed, targeting only one antigen may not be enough to produce long-lasting effects because antigen loss has been found to frequently result in tumor resistance to CAR T cell therapy.

Additionally, another limitation presented by this method is the possible inhibition of binding between both factors caused by competition which will be explained later in my conclusion. Furthermore, the absence of a proper scientific environment to conduct this experiment may have limited the proficiency of the conclusions presented as they could have been more accurate with a suitable environment.
As stated previously, while conducting this experiment would show promising results in concluding the success of the binding between both factors, there is a possibility that the TGF-b CAR attaches to a different molecule in the mutant TGF-b receptor. If the active site of the TGF-b receptor is shielded by the latency-associated peptide, then there will be a failure in the binding process. Therefore, as stated in my alternative approach, cross linking both the TGF-b CAR and the mutant TGF-b proteins can be conducted in order for direct protein-protein exchange through a covalent bond which will stabilize the proteins tertiary and quaternary structures for proper binding. Followed by a western blot to assess the success of the protein-to-protein interaction and provide the same aim as the original experiment.

Although chimeric antigen receptor (CAR)-T cells have shown very little efficiency against solid tumors, they are successful in the treatment of hematologic malignancies. Researcher K.E Noh, proves that the co-expression of a human anti-CD19-specific single-chain variable fragment (scFv) CAR (CD19 CAR) and a TGF-/IL-7 chimeric switch receptor (tTRII-I7R) in T cells inhibits the recurrence of B cell lymphoma (CD19 CAR-tTRII-I7R-T cells) (Noh, 2021). This is significant to my method as it explains the application of the results of my experiment in the real world. According to this source, understanding the implementation of TGF-b CAR is important as the anti-suppressor does not work on solid tumors.

**Research Conclusions**

After two days of incubation, results were deemed inconclusive of binding due to an inhibitor. This was concluded to be the cause of the inhibition caused by the mutant. The disulfide bond, which stabilizes mature proteins, allows the mutant TGF-b to remain inactive, preventing it from binding to its receptors. If this experiment was to be replicated, it would be advised to take a secondary approach of focusing binding the factor on eliminating the binding region of the mutant TGF-b and signaling the protein to bind to the allosteric site of the enzyme. Therefore, the binding would hypothetically avoid being inhibited.

![Figure 2. TGF-beta-activated kinase-1 (TAK1) process.](image)
The diagram presented on the right entails mitogen-activated protein kinase cascades are triggered by TGF-beta-activated kinase-1 (TAK1), a subunit of TGF-beta signaling. This is significant as it relates directly to my experiment when analyzing the bonding structure of receptors and the interaction between the two proteins. This diagram shows that through the inhibitory Smad6/7, TGF-beta/Smad signaling may be negatively regulated. The degree of aggressiveness of human gliomas is correlated with increased expression of TGF-beta 1-3. By directly promoting tumor growth, glioma initiating stem cells' capacity for self-renewal, and inhibition of anti-tumor immunity, TGF-beta may play a role in tumor etiology. In animal models, TGF-beta signaling inhibitors decrease the viability and invasion of gliomas, showing promise as novel, prospective anti-tumor treatments. This will be significant when compiling research in regard to the process between the binding of TGF-b CAR to TGF-b mutant in the experiment.

This diagram was posted on sino-biology which presented educators and students with biology resources, therefore making this diagram reliable as it has been referenced in several linked peer reviewed works. And is also made credible by educators.

**Future directions**

Allocating the exact cause and hypothesizing an accurate solution will not be successful unless multiple trials have been conducted in order to conclude the certain source of inhibition. While the suggested source was the blockage of binding due to the disulfide bond, sources prove that the reasons could vary. For example, as stated by Decha Pinkaew, and fellow authors, who are research professors/assistant professors with Phds in internal medicine, working in several top schools such as the University of Washington and Michigan State University, “Here we report that fortilln specifically interacts with TGF-β1 and prevents it from activating the TGF-β1 signaling pathway” (Pinkaew, 2022). A multifunctional protein of 172 amino acids called fortilln is found in both intracellular and extracellular regions. Fortilln controls and binds a variety of cellular proteins, although it is yet unclear what physiologic function extracellular fortilln serves. This research will be used as a limiting reactant to my experiment in order to test the negative control, suppression of TGF-b through pseudo-receptor BMP and activin membrane-bound inhibitor (BAMBI). Which will inhibit the factor from binding.

Therefore, through multiple trials with extensive variables, this experiment could be improved upon in order to exhibit true implications. Through those accurate implications, testers would be able to come up with the appropriate solutions to manifest a successful outcome.

The significance of the success of this experiment is extensive, this is shown through Katrien Janssens research on the process of mutated TGF-b in Camurati-Engelmann Disease, a disease that because of mutated TGF causes deformations in the anatomical structure of human bones. The success of this experiment would mean the expression of a healthy growth factor would lead to increased signaling in the transduction pathways, inducing the role of homeostasis, differentiation, and regulation within the cell. Therefore, denoting the expressed disease.

Therefore, promoting the expressed disease. Researching the effects of mutated TGF-b on auto-immune diseases has paved the way of knowledge on the growth factor, allowing me to understand the change in function of the growth factor caused by the mutation. This is significant in my research as it insinuates a significance to the medical field, experimenting with the abilities of TGF-b CAR to bind with TGF-b mutant will help patients that express the mutated protein through the interference of TGF-b CAR.

Authors Andrew J Hou and ZeNan L Chang both share over 14 research works with over 300+ citations in the scientific field claim that, TGF-responsiveness and tumor-targeting specificity must be successfully combined in order for TGF-CAR-T cells to be clinically translated for cancer therapy. Additionally, it is important to carefully consider the possibility that contaminating, TGF-producing regulatory T (Treg) cells may preferentially increase during the production of TGF-CAR-T cells and repress effector T (Teff) cells.

The anti-tumor effectiveness of nearby cytotoxic T cells is greatly increased by the presence of GF-CAR-T cells. Furthermore, neither TGF-CAR-Treg cells nor TGF-CAR-Treg-Treg cells cause CAR-mediated suppression of Teff cells when TGF-CARs are introduced into mixed T-cell populations. These findings confirm the value of using
TGF-CARs to provide adoptive T-cell therapies for cancer which will be significant in my rationale and purpose. This emphasizes the role of TGF-b in cancer cells.

According to Dr. DeBeradinis, Chief of Pediatric Genetics and Metabolism at UT Southwestern and the director of the Genetic and Metabolic diseases program in the CRI, cell proliferation requires nutrients, fuel, and biosynthetic activity to split and reproduce during the cell cycle. Therefore, proving the importance of metabolism and the role it plays in producing healthy cells, and eliminating unhealthy ones. Since healthy products are demanded in order to create a healthy lifestyle, they are becoming more affordable and available. So are technological advances and medications used to speed up the metabolic process in the human body. With this in mind I will also be able to gain a thorough understanding of embryogenesis growth and tumorigenesis which are both powered by cell proliferation.

Researching the effects of mutated TGF-b on auto-immune diseases has paved the way of knowledge on the growth factor, allowing me to understand the change in function of the growth factor caused by the mutation. This is significant as it insinuates a significance to the medical field, experimenting with the abilities of TGF-b CAR to bind with TGF-b mutant will help patients that express the mutated protein through the interference of TGF-b CAR. Some may argue that as this article addresses a transforming growth factor that is not coupled with a chimeric antigen receptor, then the information presented can’t be applied to this experiment.

However, this publication addresses the expression of mutant TGF-b and its effects on Camurati-Engelmann Disease. This information serves as a foundation to the basis of the experiment, that TGF-b CAR will be an addition to.

**Conclusion**

Soyoung A. Oh and Ming O. Li address the gap between transforming growth factor beta, and immune T-cells. T-cells are important in cancer as they are used as antibodies to mutated cancerous cells. They are also often used during forms of immunotherapy treatments. Oh and Li uncover the role of TGF-b in regulating the body’s immune system. Soyoun A. Oh and Ming O. Li are very popular on PubMed, with over eight hundred and twenty-eight combined publications. They are both increasingly known in the immunology field as they are both senior scientists in the cancer immunology department. Furthermore, Ming O. Li is a Ph.D holder in immunology from Columbia University. Both authors present a great level of experience and knowledge in the field of science and cancer immunology.

Researching the role TGF-b plays in regulating T-cells applies a direct solution to the research question of does TGF-b CAR bind to TGF-b mutant? And how does this binding enhance cancer treatment? This is because “In the periphery, TGF-β regulates T cell homeostasis by promoting IL-7-dependent survival of low affinity T cells (resulting from thymic conditioning of IL-7R expression) and by inhibiting TCR-driven activation of autoreactive/high affinity T cells” (Oh, 2013). Therefore, insinuating that TGF- has been demonstrated to be crucial for the growth of traditional, regulatory, and innate-like T cells.

Additionally, researching the significance of chimeric antigen receptors throughout history, in relation to T cells explains the importance of coupling both TGF-b and CAR in order to pave the way for the functioning of all cells. Historically proven, in the 1990s, gene-transfer techniques were created to use CARs or T-cell receptors to reroute the specificity of T cells. CARs are synthetic receptors that provide a specificity to immune effector cells, usually T cells, and improve T-cell performance (June, 2018). CAR T cells are then injected into the patient, where they engraft and undergo considerable growth. Each CAR T cell has the capacity to kill multiple tumor cells. Additionally, CAR T cells may support immune surveillance to prevent tumor recurrence through the release of antigens, by assisting tumor-infiltrating lymphocytes in their tumor-attacking efforts, or by maintaining their own persistence. The understanding of the effects of chimeric antigen receptors on surrounding cellular components paves the way to questioning if it can aid in reversing mutations in proteins such as transforming growth factors. However, in the case of TGF-b coupled with CAR, chimeric antigen receptors were not able to enable the binding of a healthy factor to mutant due to the inhibition of the active site. Nevertheless, this does not deem the hypothesis disaffirmed. Instead,
these results suggest that we reject the null hypothesis and alter it to state that: CAR coupled TGF-b does not bind to the active site of mutant TGF-b. This will allow future testers to tweak the experiment in order to suit the new hypothesis, null hypothesis, and find a possible answer to the presented question.

Citations


Noh KE; Lee JH; Choi SY; Jung NC; Nam JH; Oh JS; Song JY; Seo HG; Wang Y; Lee HS; Lim DS; “TGF-β/IL-7 Chimeric Switch Receptor-Expressing Car-T Cells Inhibit Recurrence of CD19-Positive B Cell Lymphoma.” *International Journal of Molecular Sciences*, U.S. National Library of Medicine, 2022, https://pubmed.ncbi.nlm.nih.gov/34445415/.


