Indonesia International Institute for Life Sciences

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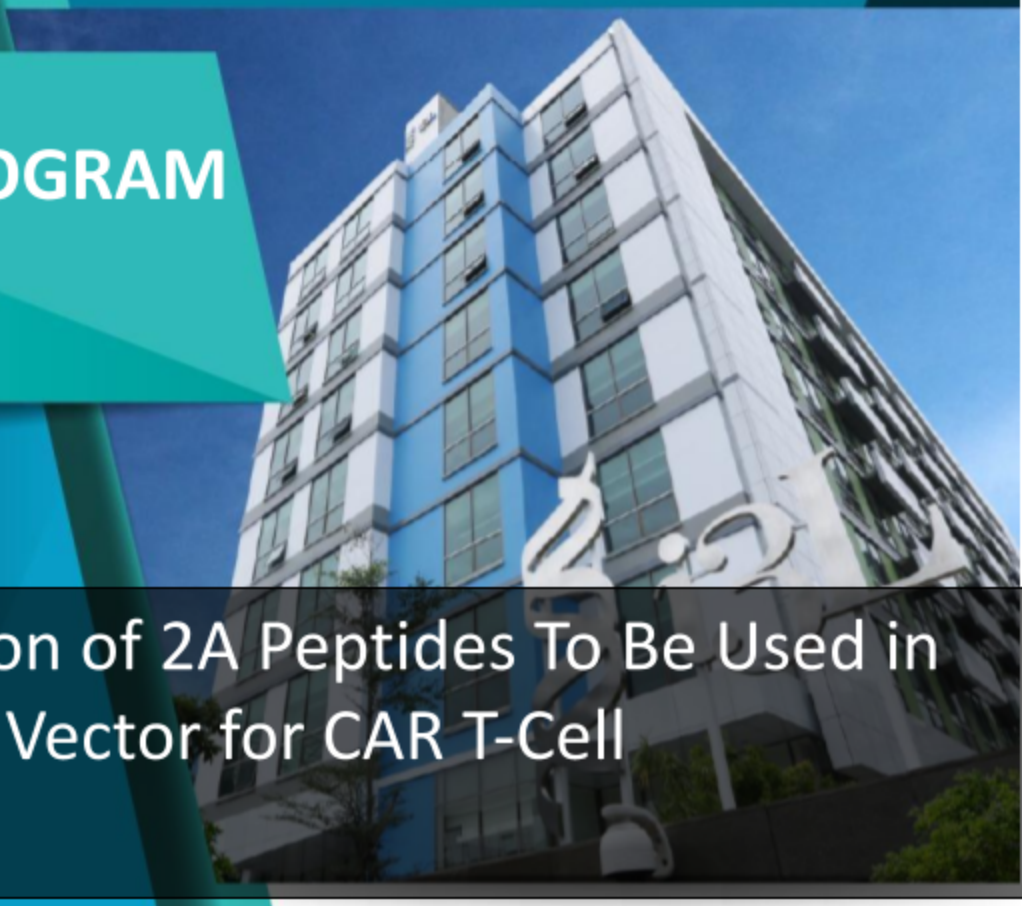

Investigation of 2A Peptides To Be Used in Bicistronic Vector for CAR T-Cell

STUDY PROGRAM
Biomedicine

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RESEARCH REPORT

INVESTIGATION OF 2A PEPTIDES TO BE USED IN BICISTRONIC VECTOR FOR CAR T-CELL

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We hereby declare that this EP project is from student's own work. The EP Report has been read and presented to i3L's Examination Committee. The EP has been found to be satisfactory and accepted as part of the requirements needed to obtain an i3L bachelor's degree.

Approved by,

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22 January 2025

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ABSTRACT

The development of multi-directional promoter-driven chimeric antigen receptors (CARs) has great potential to enhance the effectiveness of antigen-targeting CAR-T therapies. In order to express two proteins or genes simultaneously, a 2A peptide site is added between the target proteins. However, based on research, there are many different types of these cleaving sites. Therefore, to determine the best 2A peptide to be utilized for the vector construction, a proper and detailed systematic review was conducted. Recent studies involving vector design were extracted, where the frequency in which different 2A peptides were used relative to one another, as well as the cleavage efficiencies of these peptides were observed. Based on these factors, it was found that P2A, with its high usage and cleavage efficiency, was the ideal 2A peptide. Thereafter, a bicistronic vector for CAR T cells involving a P2A cleaving site was constructed through PCR and ligation methods. The process outlined will provide further opportunities for the expansion of the development of bicistronic CARs.

Keywords: 2A peptide, CAR T-cell, bicistronic vector

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1.1. Background

A novel kind of immunotherapy called chimeric antigen receptor (CAR) T-cell therapy genetically modifies a patient's T cells so that they express CARs, artificial receptors made to identify particular antigens in cancerous cells (Sadelain et al., 2014). There are now several approved CAR T-cell therapies for patients with regressed lymphoma, and a majority of those treatments focus on targeting the CD19 antigen (Turtle et al., 2016; Vairy et al., 2018). The first generation of CARs typically targets a single tumor-associated antigen. However, when failure to bind to other antigens occurs, the effectiveness of this type of CAR is greatly diminished. A possible solution to combat this would be to target multiple molecules. Instead of using a monocistronic vector for CAR, this could be done through a bicistronic vector instead (Schwirz et al., 2020).

With the exploration of different types of multiple antigen-expressing CARs, the coexpression of multiple proteins is an important factor. This can be done by incorporating a 2A self-cleaving site, which enables ribosomes to bypass the 2A site's peptide bond formation during translation. In addition, 2A peptides are usually compact and smaller in size, which helps preserve the proteins' functionality and integrity (Shibuta et al., 2019). Typically, the insertion of 2A peptide sites allows for high expression levels of both target proteins, while also reducing the development of uncleaved fusion proteins due to its high cleavage efficiency. 2A peptides are integral to bicistronic vector designs. However, because there are numerous types of 2A peptides it is important to determine the best 2A peptide in order to theoretically yield the best cleavage efficiency for the bicistronic vector construct (Liu et al., 2017; Schwirz et al., 2020).

1.2. Objectives

This research aims to address two main objectives:

(1) Evaluate the activity of different splitting peptides in multicistronic vectors in published papers and research articles

(2) Construct a bicistronic plasmid for CAR T-Cell

1.3. Hypothesis

The hypothesis for this study is that P2A self-cleaving peptide would be the ideal 2A peptide to be used to construct a bicistronic vector for CAR T-cells.

II. LITERATURE REVIEW

2.1. Chimeric Antigen Receptors

Chimeric antigen receptors, or CARs for short, are genetically engineered T cells that have been developed to improve T cells' capacity to identify and combat cancer cells. These receptors are able to both bind to an antigen and activate T cells, which allows T cells to target particular antigens found on tumor cells (Lindner et al., 2020). CARs are composed of three main domains, all of which play important roles in managing the CAR's functions (**Figure 1.1**). The antigen binding domain allows for specific binding of the T cell to the target antigen on the respective cancer cells. Upon binding, the intracellular signaling domain initiates the activation of the T cell. The transmembrane domain serves as an anchor that allows the chimeric antigen receptor to stay connected within the T cell membrane (Cappell & Kochenderfer, 2021; Zhang et al., 2021).

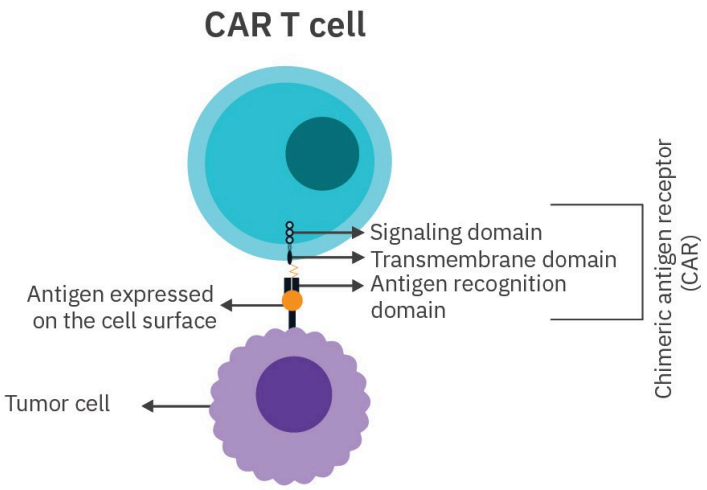

Figure 1.1. General overview of CAR T-cell

Compared to traditional therapy, CARs have been found to serve additional benefits. Firstly, they are able to target a wider variety of malignancies. In some cases, they may provide long-term protection against cancer recurrence, as these modified CAR T cells can remain in the body for an extended period of time (Sadelain et al., 2014; Zhang et al., 2021). CARs are typically considered a

revolutionary development in the treatment of cancer, as they can utilize and boost the body's T cells' innate ability to target and destroy cancer cells (Leyfman, 2018).

2.2. CAR T-cell Therapy

CARs are mainly developed for CAR T-cell therapy, which is an alternative treatment to cancer. This treatment focuses on immunotherapy, as a way to utilize and strengthen the body's immune system to fight against cancer. It involves drawing the patient's blood to obtain the individual's T cells. From here, the gene(s) for the CAR are genetically inserted into T cells in the laboratory and are cultured afterward to grow the CAR T cells into the millions. Once a sufficient amount has been achieved, the CAR T cells grown are infused back into the patient. There, they will be able to bind to an antigen on the cancer cells and kill them (**Figure 1.2**; Milliotou & Papadopoulou, 2018; Sterner & Sterner, 2021).

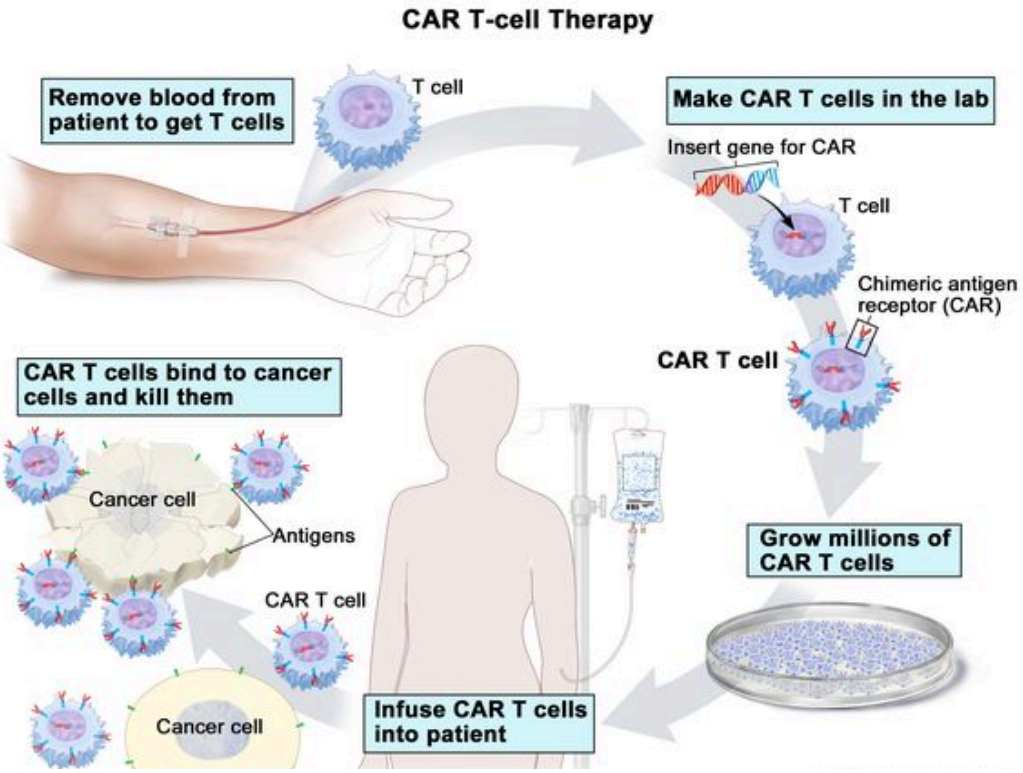

Figure 1.2. General overview of CAR T-cell Therapy

2.3. CAR T-cells

2.3.1. Single Antigen-Directed CAR T-cells

The first generation of CAR T cells revolves around targeting a single specific tumor-associated antigen. Each CAR T-cell is programmed to target a single antigen, such as CD19 or BCMA, which is typically seen on certain types of cancer cells, particularly in hematological malignancies. This generation of CAR T-cells has demonstrated effectiveness in treating a variety of blood malignancies (Vairy et al., 2018). However, despite its success, they have been observed to come with some potential limitations. Over the years, there have been some observations of disease relapse, or the loss of target antigens in cancer cells and failure to bind to other specific antigens, following single antigen-directed CAR T-cells. A possible solution to combat this includes targeting multiple molecules instead of just one. Therefore, it is important to research ways to develop multiple antigen-expressing CARs (Milone et al., 2021).

2.3.2. Dual Antigen-Directed CAR T-cells

Dual antigen-directed CAR T cells are similar to their single antigen-directed CAR T cell counterparts, with the only difference being that they are able to recognize and target two distinct tumor-associated antigens, instead of just a single antigen. These dual antigen-directed CAR T cells utilize a bicistronic vector to allow two proteins to be expressed through a single transcript. While dual antigen-directed CAR T cells have great potential, their development is still new and quite limited. There is a lot of room for improvement, therefore, it is important to optimize its creation through detailed reviews and construction (Globerson, 2020; Xie et al., 2022).

Figure 1.3. An example schematic of how a dual antigen-directed CAR T-cell would be designed

2.4. 2A Peptides

The 2A self-cleaving site is a short peptide sequence that allows several proteins to be expressed from a single open reading frame (ORF) in genetic constructs. They are widely utilized in CAR T cell design, especially in the context of bicistronic / multicistronic vectors. The 2A peptide causes a condition known as "ribosomal skipping" during translation. It specifically instructs the ribosome to skip the creation of a peptide bond between glycine (G) and proline (P) at its C-terminus. As a result, the protein upstream of the P2A sequence is generated properly, whereas the downstream protein contains an additional proline residue. This technique enables the simultaneous creation of two different proteins from a single mRNA transcript without the use of additional cleavage enzymes or sophisticated splicing procedures (Liu et al., 2017).

2A sites are frequently included in constructions that express several elements required for T-cell activity in CAR T-cell treatment. A 2A peptide sequence between the CAR and a reporter protein or co-stimulatory molecule, for instance, may be present in a CAR design. By guaranteeing that all necessary components are created concurrently, this configuration makes it possible to express both proteins from a single vector, simplifying the design and possibly increasing the therapeutic efficiency of the CAR T-cells. There are numerous types of 2A peptides utilized for the purpose of creating bicistronic vectors. However, it is still unclear which of these peptides is best used today. Therefore, a follow-up analysis of the different peptides, such as which has a greater efficiency in stimulating cleavage, will be useful for better insight into the topic (Liu et al., 2017; Shibuta et al., 2019).

2.5. Systematic Review

A systematic review is a more methodological approach to reviewing and studying various literature for a particular subject. While traditional narrative studies or reviews tend to take a more general

approach based on availability, a systematic review has to be conducted with a fixed and detailed plan in mind. By properly identifying, inspecting, and analyzing all applicable studies on a specific topic of interest, a systematic review is able to reduce unnecessary biases that may emerge as with traditional literature reviews (Aromataris & Pearson, 2014). There are various methods to organize a systematic review, however, they tend to follow a similar structure: identification, screening, and analysis.

Research identification can typically be split into two main parts. First, is the identification of the main 'question' or 'goal' of the systematic review. It is important to have a rooted understanding of the definite area of study while avoiding topics that are too broad or general. One way to particularize a scope of interest is to ask distinct questions within the subject area. This can be done using several methods, for example, the PICO or PICOs method for more medical research (Pollock & Berge, 2018). From this point, the question(s) selected should help identify the main goal. Subsequently, the second research identification refers to deciding the details of the plan to aid in conducting the review. Typically, this begins with identifying the (academic) databases to be used, as well as the initial keywords on the field of interest. The entire process of research identification is of significance, as it paves the way for research screening.

Following this, the second stage of the systematic review is termed the 'screening phase'. This is where researchers define the specifics of the inclusion and exclusion criteria: parameters that will be applied to the prior selection of papers, in order to narrow the scope of research (Booth et al., 2021). The framework includes elements such as year of publication, duplicates, language context, how the paper is reviewed, and so forth. The screening process aims to obtain a set of specific and relevant studies directly related to the initial 'question' or 'goal' identified. Since the research data has been focused and refined, they will be ready for further analysis.

Research analysis is the last phase of the process, which focuses on studying and compiling information from the final papers/data selected. How the author chooses to conduct the research analysis is highly dependent on the ‘goal’ or ‘question’ they initially aimed to address. Regardless, skimming and reading through each research paper is necessary, while noting down any interesting findings to be explored further at a later stage.

III. MATERIALS & METHODS

3.1. Systematic Review

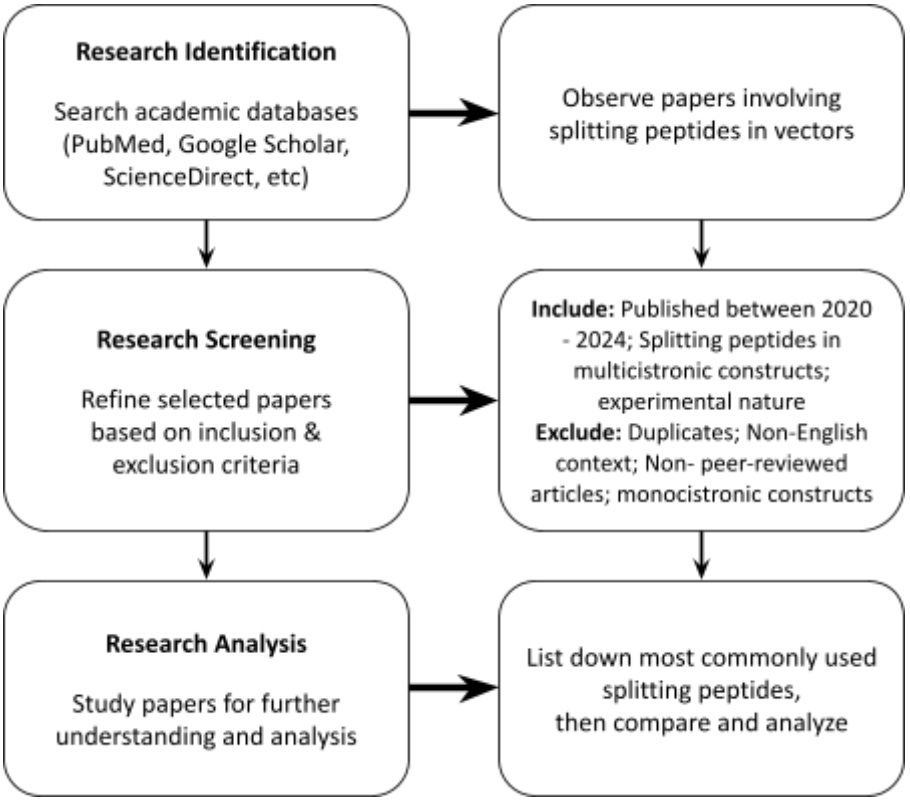

Figure 2. Overview plan for conducting the systematic review

3.1.1. Research Identification

The first step to conducting the systematic review was to determine the specific goal. In this case, it was decided that the main goal was to evaluate the activity of different splitting peptides in multicistronic vectors. Recent studies involving the use of 2A peptides in vector design will be analyzed, and a comparison of the frequency of use of each 2A peptide relative to one another will be conducted. The PICO(T) framework was used to determine a specific goal by identifying the problem/exposure/intervention, comparison, outcomes, and timeframes, as shown in **Table 1**. By combining these factors, the research question developed was: "In recent studies involving vector

design, how frequently are different 2A peptides used relative to one another, and which of these peptides demonstrates the highest cleavage efficiency?"

Table 1. PICO(T) framework analysis

Problem/Exposure/Intervention	Usage of 2A peptides in vector design
Comparison	Frequency/Efficiency
Outcomes	Determine the most suitable 2A peptide
Timeframe/Type of Study	Recent research studies

The second step is to decide the specific academic databases to be used. The academic databases utilized included: Google Scholar, PubMed, ResearchGate, ScienceDirect, Plos One, and Springer. These databases were used to first observe papers that involved splitting peptides into vectors.

3.1.2. Research Screening

For the screening process, specific inclusion and exclusion criteria needed to be stated firsthand. The details of these criteria can be viewed in **Table 2**.

Table 2. Inclusion and exclusion criteria applied

Inclusion Criteria	**Exclusion Criteria**
Published between 2020-2024	Duplicates
Splitting Peptides in Multicistronic Constructs	Non-English Context
	Non-peer-reviewed articles
	Monocistronic constructs

3.1.3. Research Analysis

The research analysis was done by analyzing the final selected papers. This was done by understanding the paper’s abstract and overall study while noting down any interesting points that

were relevant to the aforementioned goals and questions. A more comprehensive review was done in the **Discussion** section below.

3.2. Construction of Bicistronic Vector for CAR T-Cell

The construction of the bicistronic vector focuses on three main parts: Polymerase chain reaction (PCR), restriction digestion, and HiFi Assembly. In order to ensure the steps have been successfully conducted, a gel electrophoresis check will be done after each phase.

3.2.1. Polymerase Chain Reaction (PCR)

Firstly, primers provided by the institution were received and diluted to an optical density (OD) of 10µM. After this, a further 90% of nuclease-free water (NFW) was added in as well. The PCR components were then mixed thoroughly. This included 5µl Q5 reaction buffer, 0.5µl dNTPs, 0.5µl of both forward and reverse primers, 0.5µl of the template DNA, 0.25µl of High-Fidelity DNA polymerase, 5µl of Q5 high GC enhancer, and lastly 12.75µl of NFW, totaling up to 25µl in final volume. The polymerase chain reaction (PCR) settings consisted of the pre-denaturation phase at 98℃ for 30 seconds, the denaturation phase at 98℃ for 10 seconds, the annealing phase at 72℃ for 30 seconds, the extension phase at 72℃ for 60 seconds, and lastly, the post-extension phase at 72℃ for 60 seconds.

3.2.2. Gel Electrophoresis

The first gel electrophoresis check process began by creating 30mL of the agarose gel template by dissolving 0.3g of TAE buffer in water. This mixture was then heated in a microwave for a brief moment. Then, the mixture is allowed to cool for a while. 2µl of SYBR Safe was added to the solution, then swirled to mix. The gel was then poured onto the mold, and the wells were added in as well. The solution was covered with aluminum foil while waiting for it to cool. Once the gel was ready, the solutions, including the PCR product, SB CAR19 template, 1kb DNA ladder, and 100bp ladder, were all

carefully added into the wells. The gel was then run for 40 minutes. Once the process was complete, the final gel electrophoresis agar was carefully removed and brought to a GelDoc machine. Then, the machine scanned and a final result was shown.

3.2.3. Restriction Digestion

For the restriction digestion process, an SB-CAR19 plasmid (provided by the institution) was digested using NcoI enzyme. The components mixed for the solution were 5µl of rCut buffer, 1µl of NcoI, 3µl of the template, and 41µl of NFW, totaling up to 50µl in the final volume. This was incubated at 37℃ for 90 minutes. Then, another gel electrophoresis check was conducted in a similar manner to the first gel electrophoresis check.

3.2.4. DNA Extraction from Agarose Gel

The previous gel electrophoresis results were kept for the use of DNA extraction from the agarose gel. Firstly, the gel containing the band of interest was cut and placed in a microtube. Using a kit, an NT1 binding buffer was added, and left in a water bath of 50℃ for 10 minutes, while vortexing occasionally. While conducting this, the column and collection tube were prepared for the following steps. 700µl sample was added into the column. Then, the sample was centrifuged at 17,000 x g for 60 seconds. The flow-through was discarded, and the column was washed with 700µl of NT3 buffer. This was then centrifuged again at 17,000 x g for 60 seconds. The flow-through was discarded, and the column was washed with 200µl of NT3 buffer. The sample was centrifuged at 17,000 x g for 60 seconds, with the removal of flow-through afterwards. A follow centrifugation with the same settings was done for drying, with a duration of 120 seconds. After this, the collection tube was discarded and replaced with a microtube. 20µl of Buffer NE was added, and then the sample was incubated at room temperature for 90 seconds. A final centrifugation was done at 17,000 x g for 60 seconds, and the sample was stored in a freezer.

3.2.4. HiFi Assembly

After the extracted DNA was prepared, a HiFi assembly kit was used alongside the vector and insert prepared previously. In this case, 10µl of HiFi assembly master mix, 3µl of the vector plasmid, and 7µl of the inserted plasmid were carefully combined and then incubated at 50℃ for 1.5 hours. When the process has been completed, the master mix could then be stored in a freezer for future use.

IV. RESULTS AND DISCUSSION

4.1. Results

4.1.1. Systematic Review

For the first phase of the systematic review, by utilizing the academic databases to first observe papers that involved splitting peptides into vectors, a total of 13,500 papers were identified. In the research screening, inclusion and exclusion criteria were applied to the initial papers gathered. After applying the inclusion criteria, a total of 3,300 papers were noted down. Moreover, following the exclusion criteria, a total of 347 papers were considered eligible for brief analyses, and a few further select papers were used for continued discussion (**Figure 3.1**).

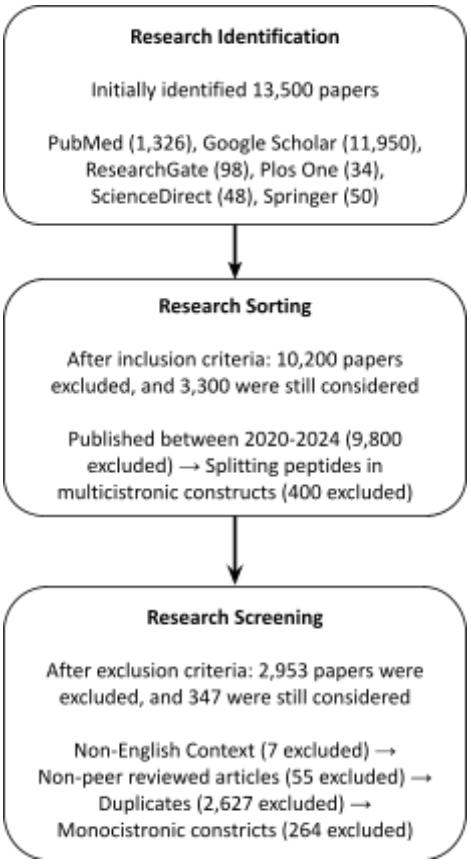

Figure 3.1. Systematic review progress involving research identification and research screening

Looking into these papers further, a list of data involving the usage of different 2A peptides was gathered and compiled. It was found that a total of 36 papers utilized E2A in their construct, 27 papers included F2A, 129 papers for the inclusion of T2A; and lastly 155 for P2A (**Figure 3.2**).

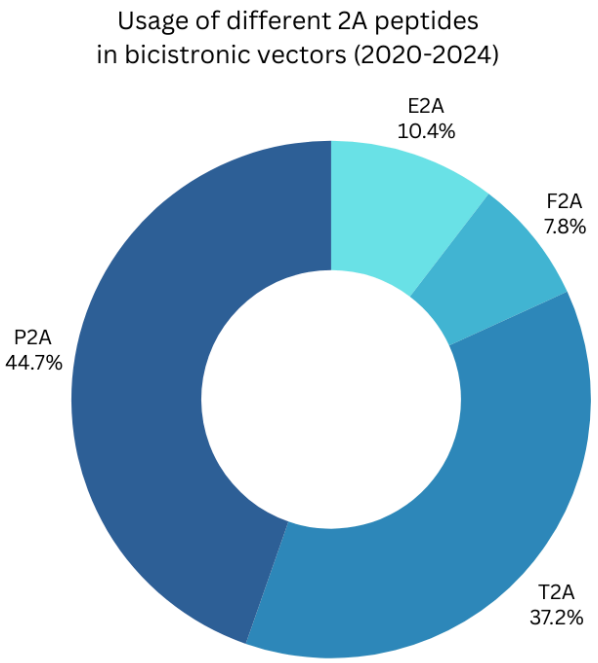

Figure 3.2. Usage of different 2A peptides in bicistronic vectors (2020-2024)

On the other hand, **Table 3** showcased the percentages for mean cleavage efficiency of the different 2A peptides across the viable papers, with P2A observed to have the highest mean cleavage efficiency (~±99.6%), and F2A having the lowest mean cleavage efficiency (~±75.1%).

Table 3. Comparison of 2A peptide cleavage efficiencies across viable papers

Type of 2A Peptide	Mean Cleavage Efficiency
F2A	~±75.1%
T2A	~±99.4%
E2A	~±98.2%
P2A	~±99.6%

4.1.2. Construct Schematic

For the bicistronic design, a promoter that drives the construct, along with the selected 2A peptide (P2A) placed in between the green fluorescent protein (GFP) and CD-CAR19, were included. Inverted terminal repeats (ITRs) are included as well (**Figure 3.3**).

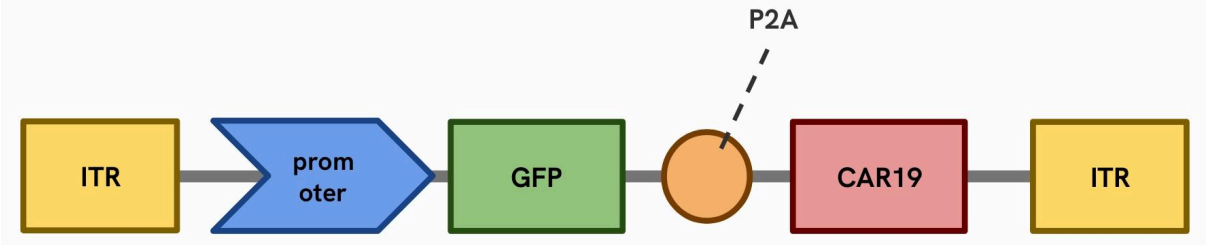

Figure 3.3. Bicistronic vector schematic. Included: a promoter, and P2A placed in between GFP and CD-CAR19

4.1.4. PCR and Restriction Digestion Schematics

In addition to the bicistronic vector design, a few additional plasmids (provided by the institution) were utilized, specifically for the PCR and restriction digestion methodologies. Firstly, a bidirectional CAR template, CAR20-P2A-GFP-CAR19, was used for PCR (**Figure 3.4**). In addition, an SB-CAR19 construct was used for the restriction digestion, as shown in **Figure 3.5**.

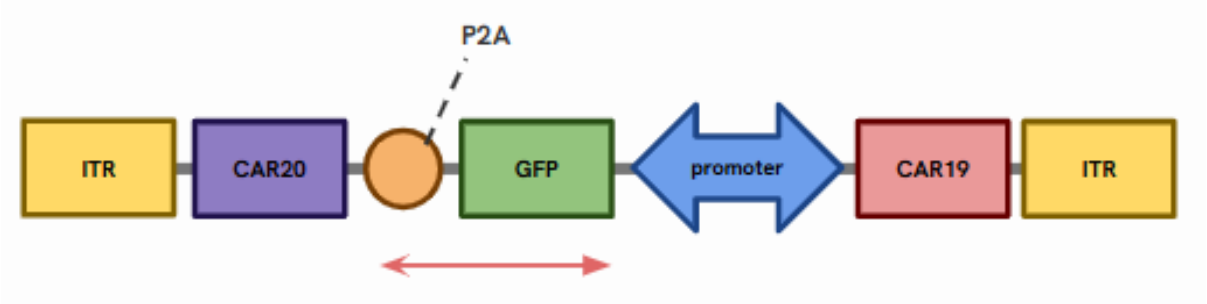

Figure 3.4. Bi-directional CAR template for PCR; Highlighted: GFP-P2A

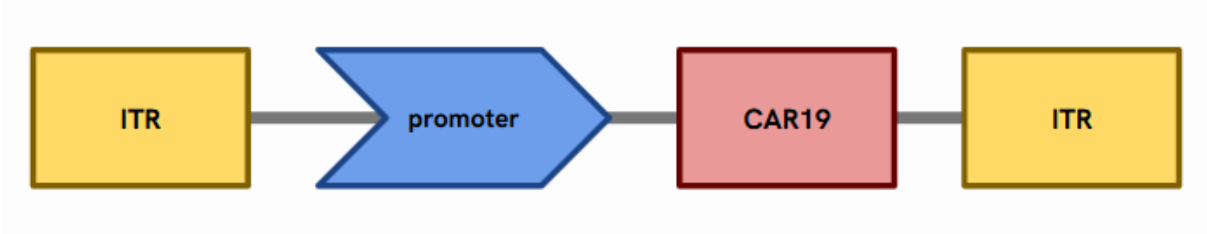

Figure 3.5. Single antigen-directed CAR template for restriction digestion

4.1.3. Gel Electrophoresis Results

Figure 3.6 shows the gel electrophoresis results after completing the PCR process. The leftmost is the 'GFP-P2A', next is the 1kb DNA ladder, following that is the 100bp ladder, and rightmost is the 'SB-CAR19'. The bands appear to be mostly visible and unblurred.

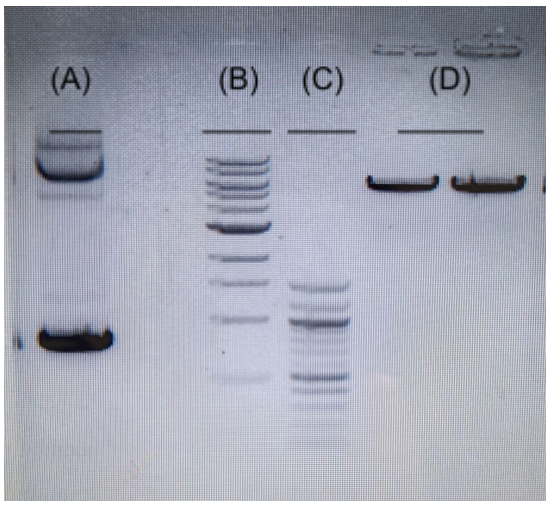

Figure 3.6. Gel electrophoresis results for PCR product;
(A) GFP P2A (~783 bp), (B) 1kb ladder, (C) 100bp ladder, (D) CAR19 (~>6000 bp)

Following this, **Figure 3.7** exhibits the gel electrophoresis results after the restriction digestion process. The leftmost is the 1kb DNA ladder, and the rest of the bands are from the plasmid sample. The bands appear to be visible and unblurred.

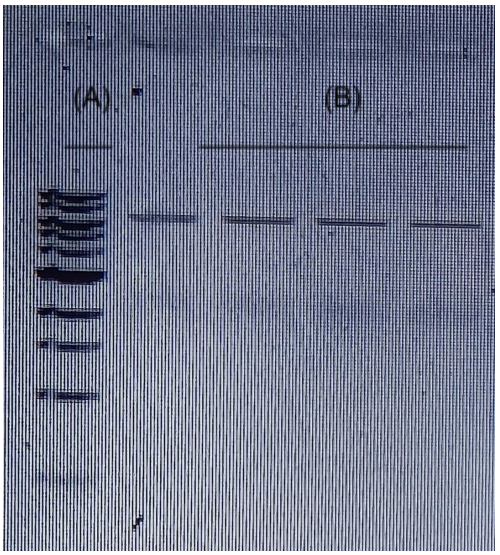

**Figure 3.7. Gel electrophoresis results for restriction digestion plasmid;
(A) 1kb ladder, (B) plasmid for digestion (~>6000 bp)**

4.2. Discussion

4.2.1. Analysis of 2A Peptides

Based on analyzing the final selected papers for the systematic review, 2A peptides used for multicistronic vectors included: foot-and-mouth disease virus 2A (F2A), thosea asigna virus 2A (T2A), equine rhinitis A virus 2A (E2A), and porcine teschovirus-1 2A (P2A). These 2A peptides are used for various purposes, with different ranges of applicability. Notably, many research papers mentioned the type of 2A used, however, many did not specifically include the exact expression levels of said peptides. Despite this, simply by knowing the type of 2A peptide used, approximating the amount of usage in a general sense is possible.

As shown in **Figure 3.2,** among the four peptides, F2A and E2A seemed to have the least amount of usage within the data obtained, while T2A and P2A were utilized the most frequently. Looking into this further, it was observed that outside of the inclusion criteria for the year published (2020 -2024), F2A was used more regularly in the past compared to the present. In a comparison study for cleavage efficiencies of different 2A peptides done by Zhu et al. (2023), the researchers observed that P2A yielded the highest efficiency, followed closely by T2A. On the other hand, F2A was observed to have the lowest efficiency. This may explain why the amount of F2A usage in multicistronic or bicistronic constructs decreased in recent years, despite the fact that it was the first discovered 2A peptide. T2A and P2A were found to have a similar cleavage efficiency, making them both suitable for multicistronic vectors. Additionally, a similar trend was also observed by compiling and obtaining the mean cleavage percentages of the different 2A peptides across viable papers, as shown in **Table 3**. Based on the data compiled, P2A was utilized for bicistronic vectors slightly more than T2A.

Regardless, both 2A peptides showed satisfactory performance, allowing them to be suitable to be included in the construct design.

4.2.2. Construction of Bicistronic Vector for CAR T-Cell

The systematic review determined that P2A was the ideal splitting peptide to be used in a bicistronic vector for CAR T-cell. Before starting, it was important to choose the kind of construct to be made. While there are a number of CARs and the specific antigens they are able to target, ultimately, CD19 CAR was chosen due to its consistent expression levels, as well as availability for this research. Ideally, another antigen would be included in the bicistronic vector for CAR T-cell. However, this research chose to prioritize the consistency of the genes or proteins chosen to serve as a more general approach to the bicistronic vector design. Therefore, a green fluorescent protein (GFP) was utilized as the first target in the vector, followed by the CD19 CAR. The overall structure construct could also be shown as: “GFP-P2A-CAR19” (**Figure 3.3)**.

In order to create the GFP-P2A-CAR19 construct, PCR was performed to amplify the GFP-P2A section from the bidirectional CAR, with an expected size of 783bp. Following the checking using GelDoc, the results, as shown in **Figure 3.6**, compared the leftmost ‘GFP-P2A’, as well as the ‘SB-CAR19’, with the 1kb DNA ladder and 100bp ladder in the middle. Both of the bands produced were clear enough to be observed, and the size of the bands was as expected, which meant the PCR process went smoothly.

With the correct sizes of the samples secured from the PCR, the sample plasmid ‘SB-CAR19’ was to be digested with NcoI enzyme. After mixing and incubation, the sample was run through another agarose gel electrophoresis. **Figure 3.7.** shows the obtained results, it can be seen that the plasmid was of the expected size (~>6000 bp). Therefore, the construction of the bicistronic vector up to this point has been properly established.

4.2.3. Future Prospects

Despite the progress achieved thus far, the current research presents several avenues for improvement, such as by implementing follow-up experiments to address additional objectives. Firstly, a more comprehensive systematic review could be conducted, as the absence of a bias check or review limits the robustness of the findings. For future reference, bias assessment surrounding the protocols and specific areas of multicistronic vector construction can be taken into account for further observation, in order to properly classify the data. Moreover, following the restriction digestion process, this research failed to conduct the HiFi assembly protocol due to time constraints. In addition to this, the development of the bicistronic vector may require additional steps to be deemed complete. It may be of additional importance to evaluate the efficacy of the construct utilizing various methodologies, including cell culture, peripheral blood mononuclear cell (PBMC) isolation, transfection, and follow-up analyses. Additionally, the development of alternative bicistronic vectors that incorporate P2A should be pursued to facilitate comparisons regarding their effectiveness within CAR T-cell contexts. Finally, a deeper investigation into the splitting peptides is warranted to explain the mechanisms that allowed the notable efficiency of P2A. This investigation has not fully addressed the subject matter due to its focus solely on fundamental bicistronic vector designs. A subsequent experiment aimed at accurately transfected and propagated the generated CAR T-cell construct holds potential promising results. Furthermore, conducting validation experiments, such as assessing the actual efficacy of the constructed vectors, would be advantageous. Exploring innovative CAR designs tailored specifically for CAR T-cell therapies utilizing appropriate resources may also present a viable avenue for continued exploration. Nonetheless, many approaches remain open for expanding the scope of this study.

V. SELF REFLECTION

Engaging in this Enrichment Program has been an exceptional opportunity that has greatly enhanced my research and academic skills. Throughout this experience, I have received substantial support from supervisors, mentors, colleagues, and friends, with my prior semesters at i3L being instrumental to my success. The integration of theoretical and practical coursework has provided me with a solid grounding in fundamental concepts. I was able to explore further into the world of genetic engineering and its many capabilities. Through this program, I learnt about CAR T-cells and its practical uses; a topic I was completely unfamiliar with beforehand. In addition to this, I have effectively applied hard skills such as literature reviews and data analysis, while also developing essential soft skills like communication, teamwork, and professionalism through a variety of experiences. This program has prompted me to reflect on my personal growth, allowing me to recognize my strengths—such as a strong eagerness to learn, adaptability, and perfectionism—while embodying the values of role modeling and integrity promoted by i3L. Moreover, I have become aware of certain weaknesses, including tendencies to overthink, which have sometimes hindered my progress. This realization has underscored the importance of resilience; I have learned that mistakes and setbacks are a natural part of the learning journey, and what matters most is our dedication to perseverance and continuous self-improvement. I look forward to continue learning, and to be able to apply what I have learned to future endeavors.

VI. CONCLUSION

Through the process of a systematic review, it was determined that P2A would be the most suitable 2A peptide to be used in a bicistronic vector for CAR T-cell. P2A has been observed to have the most usage in bicistronic design in recent literature and research, as well as a consistent efficiency, making it reliable for usage. From there, a bicistronic CAR vector, involving P2A and CD19 CAR, was designed for further usage. By conducting PCR, restriction digestion, and purification protocols, alongside gel electrophoresis procedures to ensure established operation of the overall methodology, a base foundation of a bicistronic vector for CAR T-cell was properly obtained. There are still broad applications for this research, as well as follow studies to be done. For instance, this research has not covered the actual process of propagating and detailed analysis of the construct's efficiencies, therefore, these missing areas in the research could be valuable opportunities to further investigate the potential of bicistronic vectors for CAR T-cell.

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