Design of Chimeric Gasdermin Proteins: Enhancing Combination Immunotherapies via Oncogene-Targeted Pyroptosis

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ABSTRACT

Gasdermin D (GSDMD) and gasdermin E (GSDME) are two-domain proteins composed of a large N-terminal domain and smaller C-terminal domain, joined by a flexible linker region [1]. GSDMD and GSDME are natively cleaved by caspase-4/5/11 and caspase-3, respectively, in the flexible linker separating the N- and C-terminal domains [1]. Upon caspase cleavage, the N-terminal domain of GSDMD/E oligomerizes to form pores in the cell membrane; this pore formation leads to cell death via pyroptosis, an inflammatory form of cell death [1]. Craspase (CRISPR-guided caspase) is an RNA-activated CRISPR system in which TPR-CHAT/Csx29 cleaves a protein called Csx30 upon binding of the guide RNA to an RNA sequence [2]. We demonstrate that by inserting Csx30 into the linker region of gasdermins, we produce chimeric gasdermins that can be cleaved by Craspase upon binding of the guide RNA. By designing the guide RNA complementary to an oncogenic mutation, we develop a system to induce pyroptosis selectively in cancerous cells. Oncogene-driven pyroptosis through Craspase cleavage of the chimeric gasdermins may represent a method of stimulating antitumor immune response to improve the efficacy of adjuvant immunotherapies.

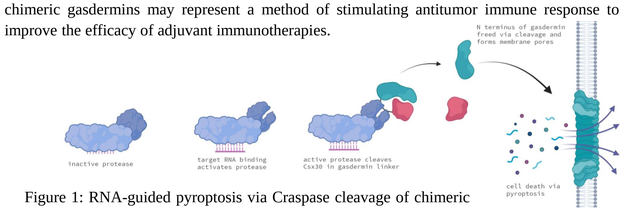

Figure 1: RNA-guided pyroptosis via Craspase cleavage of chimeric gasdermins

INTRODUCTION

Cancer immunotherapy, which aims to harness a patient's immune system to fight cancer cells, has been one of the most promising advancements in cancer therapeutics in the last decade. However, the success of cancer immunotherapies is often stifled by an immunosuppressive tumor microenvironment. In recent years, a new emphasis in cancer research has been the use of programmed cell death to induce an antitumor immune response. By releasing immunomodulatory molecules from tumor cells, immunosuppression within the tumor microenvironment can be reduced [3]. As a result of programmed immunogenic cell death, immunotherapies that currently fail to elicit an anticancer immune response in immunosuppressive tumors may demonstrate improved efficacy [3].

Craspase (CRISPR-guided caspase) is an RNA-activated protease CRISPR system used by bacteria as a defense against bacteriophages [2]. The Craspase system is composed of a guide RNA bound to gRAMP/Cas7-11, a CRISPR-Cas type III RNA-targeting complex, with associated protease complex TPR-CHAT/Cxs29 [2]. Upon binding of the guide RNA to invading bacteriophage RNA, a conformational change in Cas7-11 opens the cleavage domain of Csx29, which cleaves Csx30, a protein endogenous to bacterial cells [2].

Gasdermins are a family of two-domain proteins, composed of a large pore-forming N-terminus and smaller autoinhibitory C-terminus, separated by an intrinsically disordered linker region [1]. As a response to signals caused by bacterial infection and other dangers to human cells, inflammatory caspases cleave gasdermins in the linker region, separating the N- and C-terminus domains [1]. The N-terminus of gasdermins oligomerizes in the cell membrane to form membrane pores, inducing cell death via pyroptosis [1]. Pyroptosis is an inflammatory form of lytic cell death in which cell contents are leaked through gasdermin-formed membrane pores [1]. Cell death through pyroptosis recruits lymphocytes through the release of IL-18 and IL-1β [4], thereby improving antitumor immune response. By inserting the Csx30 linker natively cleaved by Craspase into the linker region of gasdermins, we create a chimeric gasdermin protein that can be cleaved by Craspase upon recognition of a specific RNA sequence. Designing the Craspase guide RNA complementary to an oncogenic RNA sequence leads to cleavage of the chimeric gasdermins and consequent pyroptosis only in cancer cells. This Craspase-chimeric gasdermin system holds potential as an in-vivo therapeutic approach to reduce immunosuppression in cold tumors. As a proof of concept, we illustrate the design of four fusion proteins for GSDME and GSDMD, respectively, for expression in an *S. cerevisiae* cancer model, which expresses the KRAS G12D oncogene.

METHODOLOGY

Four gasdermin-Csx30 (GDx30) fusion proteins were designed for GSDMD and GSDME, respectively. Native Csx30 has a length of 565 amino acids; in the original paper [2] characterizing the Craspase system, it was revealed that Csx29 cleaves Csx30 between residues 427 and 429 at the "MKK" sequence (shown in orange to the right). Furthermore, the paper [2] demonstrated that only amino acids 396 to 565 are necessary for cleavage of Csx30. Therefore, we chose to use a truncated Csx30 linker (amino acids 396 to 565) in the chimeric gasdermins. This is advantageous because the original Csx30 linker is too large to fit properly in the flexible linker region of GSDMD/E.

In order to determine whether the Csx30 linker could be inserted into gasdermins, the AlphaFold model of GSDMD was aligned with the crystal structure of truncated Csx30 in PyMOL to visualize the scale and orientation of the Csx30 linker in relation to the GSDMD linker. Luckily, the C- and N-terminal domains of the truncated Csx30 aligned with the linker region of GSDMD and appeared to be of appropriate size to fit in the GSDMD linker. Conveniently, the alignment of Csx30 with GSDMD placed the "MKK" Csx29 cleavage site (circled on the PyMol structure below) in an exposed position to be cleaved by Csx29.

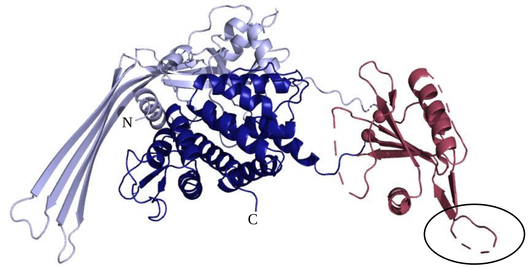

Figure 3: AlphaFold structure of GSDMD aligned with the crystal structure of truncated Csx30 (amino acids 396-565) with exposed Csx29 cleavage site of Csx30 (circled). N-terminus of GSDMD is shown in dark blue, C-terminus of GSDMD in light blue, and truncated Csx30 in red.

Csx30 was connected to the GSDM linker region, on each side, by a GSS linker [5]. In each of the gasdermins, the native caspase cutsite was removed by mutating a single amino acid (D → A) in the caspase cut site to prevent cleavage by native caspases. Caspase is a cysteine-dependent aspartate-directed protease [6], so we reasoned that mutating out the D at the cleavage site would prevent Caspase cleavage. Each of the fusion proteins was cloned into yeast expression vector pYES2 using Gibson assembly. Native GSDMD/E with removed caspase cutsite was first cloned into pYES2 using Gibson assembly, then the Csx30 linker was introduced into the GSDM at each of the four sites using Gibson assembly. Each of the fusion proteins is expressed constitutively under the GAP promoter and FLAG-tagged to check for protein expression in downstream experiments. Because the first validation of this system will be performed in yeast, a yeast Kozak sequence and start codon precede each fusion protein for proper protein translation in yeast.

Figure 4: Plasmid design for the expression of fusion proteins. GAP constitutive promoter, GSS linker, Kozak + start codon, FLAG-tag, GSDMD N-terminus, truncated Csx30 linker, GSS linker, GSDMD C-terminus.

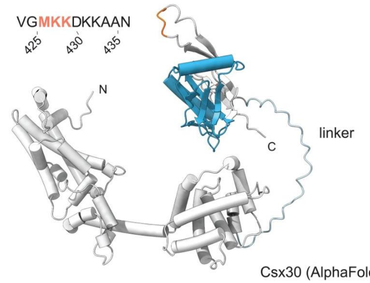

Figure 2: Csx30 AlphaFold structure. MKK Csx29 cleavage site is shown in orange. Adapted from [2]

For each of the fusion proteins, the truncated Csx30 was inserted into the linker region of GSDMD/GSDME. The design of the four fusion proteins for each gasdermin is as follows:

- Gasdermin native caspase cutsite replaced by Csx30
- 30% of GSDM linker replaced by Csx30
- Csx30 insertion in the N-terminal end of the GSDM linker
- Csx30 insertion in the C-terminal end of the GSDM linker

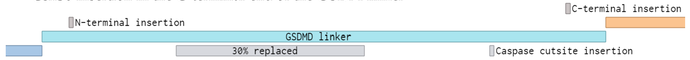

Figure 5: Schematic of Csx30 insertion points in the GSDMD linker. In blue on the left is the GSDMD N-terminus, in orange on the right is the GSDMD C-terminus, and in turquoise in the center is the GSDMD linker region.

RESULTS

Completed Gibson assemblies of each of the fusion proteins were transformed into chemically competent E. coli. Colony PCR was performed on several colonies for each fusion protein to confirm the successful transformation of the plasmid. PCR products were run using agarose gel electrophoresis to verify the fragment size produced by the colony PCR. The results of the gels are shown below, confirming the successful construction of all eight fusion proteins. The successful colonies were liquid cultured, miniprepped to extract plasmid DNA, and sent for sequencing to further validate successful fusion protein construction. Sequencing results confirmed eight successful constructs.

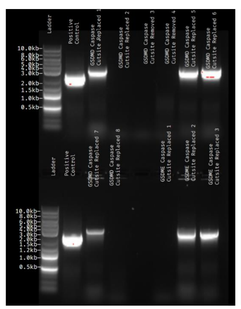

Figure 5: GSDMD caspase cutsite replaced by Csx30 and GSDME caspase cutsite replaced by Csx30

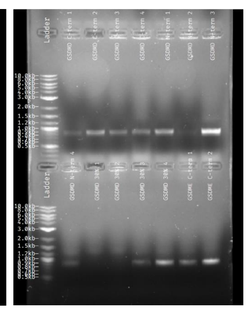

Figure 6: GSDMD C-terminal insertion of Csx30, GSDMD N-terminal insertion of Csx30, GSDMD 30% of linker replaced by Csx30, and GSDME C-terminal insertion of Csx30

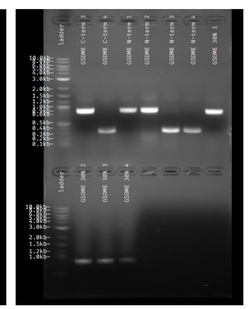

Figure 7: GSDME C-terminal insertion of Csx30, GSDME N-terminal insertion of Csx30, and GSDME 30% of linker replaced by Csx30

DISCUSSION AND CONCLUSIONS

Pancreatic ductal adenocarcinoma (PDAC) most commonly originates from a mutation in the Kirsten rat sarcoma virus (KRAS) oncogene [7]. The most common KRAS oncogenic mutation is a G → D mutation at the 12th codon (KRAS G12D) [7]. The original proof of concept of this system will therefore be performed in a BY4741 *S. cerevisiae* budding yeast strain expressing KRAS G12D as a model for pancreatic cancer. Control experiments will be performed to ensure that cell death occurs only in yeast expressing the entire Craspase system, the GSDM fusion proteins, and the mutated form of KRAS. In yeast expressing the unmutated KRAS, we do not expect to see cell death. Further validation of this system will be performed in HKP1 cell lines, again targeting KRAS G12D. PDAC was chosen as the proof of concept for this system due to its highly immunosuppressive tumor microenvironment; recent research into strategies for treating pancreatic cancer has highlighted the use of immunogenic cell death to improve combination immunotherapies. We predict that the induction of pyroptosis in PDAC using the Craspase-chimeric gasdermin system would be beneficial in converting the naturally "cold" pancreatic tumor microenvironment into a "hot" environment to allow for the penetration of lymphocytes and immunotherapies such as immune checkpoint inhibitors.

Although PDAC is our current area of research, the strength of this system lies in its modularity; by changing the guide RNA that activates Craspase, the chimeric gasdermin fusion proteins have potential applications in treating other types of cancers.

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