# Development and Characterization of Fusion Proteins for Identification of Cytokine-Optimized CAR-T Cells

Justin Fisher[1]; Scott Dessain, MD, PhD[2, 3]; William Kelly, PhD[1]
[1] Villanova University; [2]Lankenau Institute for Medical Research, [3]OCMS Bio, LLC

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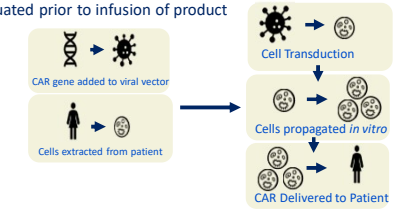
## INTRODUCTION

- Long-term CAR-T efficacy linked to long-term anti-tumor activity, an approach for improving clinical success of CAR-T is the selection of specific CAR-T subpopulations
- Single-cell cytokine expression studies have shown that CAR-T cytokine expression levels are highly variable- expression of cytokines by CAR-T cells reflects an activated state that correlates with anti-tumor activity in patients
- If 'activated' CAR-T cells, determined by high cytokine expression) give improved efficacy then future manufacturing need to efficiently create and deliver a product enriched in such cells.
- Current technologies do not allow CAR-T cell cytokine expression profiles to be evaluated prior to infusion of product




## BACKGROUND & MOTIVATION

**How Can You Examine A Polyclonal Population of CAR-T Cells and Identify Those Expressing Cytokines At The Desired Level?**

Previous Attempts to Develop polyclonal cell culture methods where mAbs are associated with cells that secrete them:

- Secretion Capture Report Web (SCRW)
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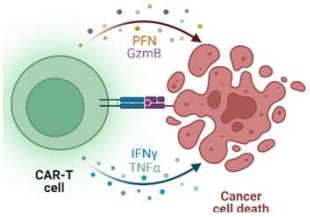
- Semi-Solid Medium

Methods designed to directly attach mAbs to hybridomas that secrete them via secondary mAb, but these do not prevent mAbs from binding to nearby cells that did not secrete them




**To overcome these challenges, we developed an IFN-γ scFv:CD19 extracellular domain (ECD) fusion protein which when incubated with anti-CD19 CAR expressing cells will facilitate membrane capture of secreted IFN-γ.**

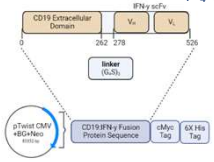
## OBJECTIVES

- Aim #1: Evaluate Fusion Protein IFN-γ scFv Binding to Recombinant IFN-γ
- Aim #2: Evaluate anti-CD19 scFv binding to Fusion Protein CD19 Extracellular Domain
- Aim #3: Evaluate Fusion Protein Binding to anti-CD19 CAR Expressing Cells *in vitro*

## FUSION PROTEIN CHARACTERIZATION

### 1. Fusion Protein Production

Transient transfection of HEK293T cells with plasmid containing an IFN-γ scFv linked to the CD19 extracellular domain via flexible $(G_4S)_3$ linker driven by the CMV promotor.




### 2. Enzyme-Linked Immunoassay

***Recombinant IFN-γ Binding:***
MaxiSorp plates coated with 500 ng Fusion Protein overnight was incubated with various concentrations of rIRN-γ for 1hr following blocking and washing. 500 ng of biotinylated mouse anti-human IFN-γ mAb was added and binding was evaluated using Streptavidin-HRP with TMB substrate.

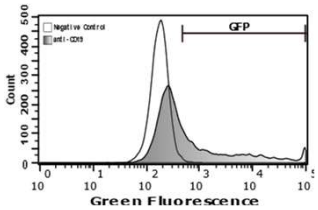
***Competitive Binding Assay:***
MaxiSorp plates coated with 500 ng IFN-γ overnight were incubated with either 500 ng rabbit anti-human IFN-γ polyclonal or PBS following blocking and wash. After wash, 500 ng biotinylated fusion protein was added with binding evaluated using SA-HRP and TMB substrate.

***FMC63 Binding:***
MaxiSorp plates coated with 500 ng FMC63-Fc (transiently expressed using pcDNA3.1(-) FMC-63 scFv-Fc (huFc) (AddGene #183252)) overnight were incubated with 500 ng of biotinylated fusion protein following wash and blocking. Binding was evaluated using SA-HRP and TMB substrate.

## *IN VITRO* FUSION PROTEIN BINDING

HEK293T cells were transiently transfected with pSLCAR-CD19-BBz (Addgene #135992) which contains a GFP reporter via P2A. Transfection efficiency was 38.43 ± 0.56% (Figure Below).

Two concentrations of biotinylated fusion protein (5 ng/uL or 0.05 ng/uL) were incubated with CAR-HEK293T after blocking with PBS +1% BSA. Binding was evaluated via 1:200 PE-labelled streptavidin.

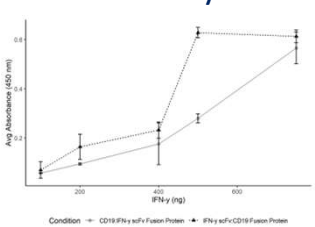



## RESULTS

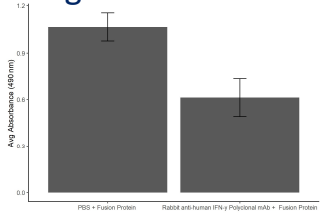
- Sandwich ELISA assays were used to assess the presence, and binding affinity of the IFN-γ scFv domain on both fusion protein orientations. Both fusion protein orientations bound to soluble IFN-γ in a dose-dependent fashion.
- Competitive Binding Assay revealed a 43% decrease in binding of the fusion protein to IFN-γ in presence of competitor.
- Both fusion protein orientations were captured by FMC63-Fc indicating that the CD19 ECD in fusion protein is functional. There was not a significant difference in binding between the two fusion protein orientations.

## RESULTS

### Aim #1: IFN-γ scFv Binding

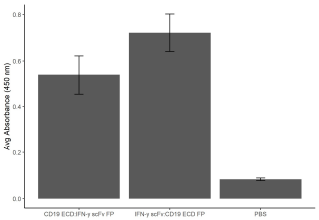



Sandwich ELISA revealed dose-dependent binding of IFN-γ to both fusion protein orientations




Binding of fusion protein to 500 ng IFN-γ reduced by 43% in presence of competitor.

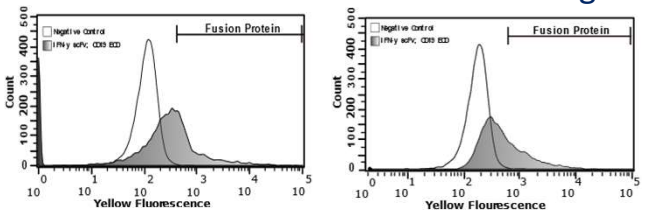
### Aim #2: FMC63 Binding




Both fusion protein orientations were captured by FMC63 indicating that the CD19 ECD is functional and available for anti-CD19 scFv capture.

There was not a significant difference in binding between CD19 ECD: IFN-γ scFv (M = 0.537, SD = 0.084) and IFN-γ scFv: CD19 ECD (M = 0.721, SD = 0.080); t(3.99) = -2.7399, p = .05205.

### Aim #3: *In* Vitro CAR-HEK293T Binding




- Higher concentration of fusion protein (5 ng/uL) led to higher binding to heterologous population (44.86%) compared to 0.05 ng/uL (35.12 ± 2.74%; see above histograms)
- Gating on GFP to identify only CAR-H binding revealed no difference in 5 ng/uL fusion protein (80.775%) compared to 0.05 ng/uL fusion protein (80.325%).

## CONCLUSIONS & FUTURE DIRECTIONS

- We have successfully designed and produced a fusion protein linking an IFN-γ scFv to the CD19 extracellular domain. This novel fusion protein can bind to IFN-γ in a dose-dependent fashion and can be recognized and bound by FMC63 indicating that both key domains are functional.
- The novel fusion protein can successfully identify CAR cells specifically; however, the concentration required to meet the minimum and maximum capture has yet to be determined.
- Future experiments will identify CAR expressing cells along with identification of IFN-γ expression levels with the goal of CAR isolation based on their IFN-γ secretion levels

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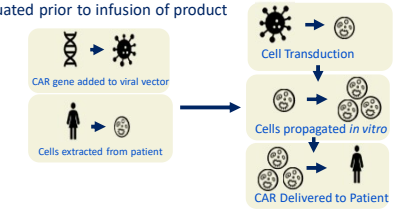


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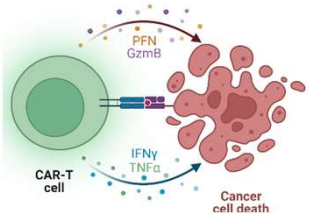


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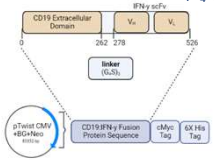


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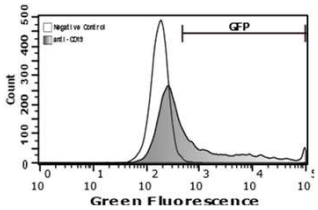


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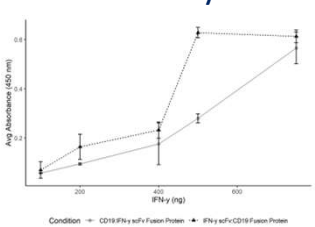


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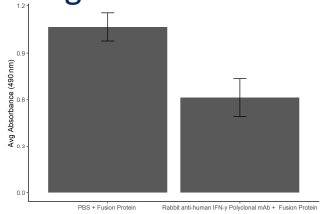


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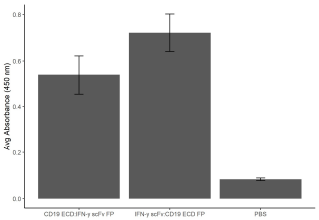


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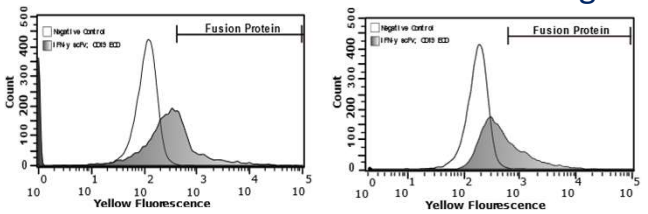


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