Design and characterization of C3d targeted fusion proteins for tissue localized inhibition of complement activation

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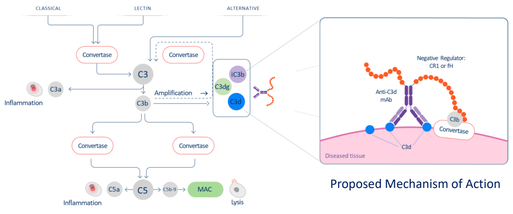
INTRODUCTION

Aberrant complement activation is thought to be an underlying pathological driver in acute and chronic inflammatory and autoimmune diseases, many of which involve significant tissue damage. Currently approved therapies are directed at systemic inhibition of complement. Given complement's essential role in innate and adaptive immunity, unmet need exists for novel therapies that inhibit complement in disease tissue without systemic blockade. One approach to this is development of novel fusion proteins that enable tissue-targeted delivery of complement negative regulatory proteins. The ability to direct fusion proteins to the tissue is accomplished by binding to the complement fragment C3d, which is deposited on tissues where complement is active. C3d targeting is achieved using an anti-C3d mouse monoclonal antibody (mAb) clone 3d8b, which was raised against human C3d and found to bind mouse and human cleavage fragments C3d, iC3b and C3dg[1].

To establish proof of mechanism, we generated a library of bifunctional fusion proteins that include the mouse anti-C3d tissue targeting mAb fused to one of several complement negative regulatory effector domains, including the first 5 short consensus repeats of factor H (fH_{1-5}) and the first 10 or 17 short consensus repeats of Complement Receptor 1 ($CR1_{1-10}$ and $CR1_{1-17}$). Design of these anti-C3d mAb fusion proteins is intended to deliver high local concentrations of these complement negative regulators to tissue bound complement fragments.

Biochemical and *in vitro* characterization of this collection of bifunctional fusion proteins identified several lead-like constructs with superior binding, functional activity and developability attributes.

Rationale for Tissue Targeted Complement Inhibitors



Complement Activation Pathways

Proposed Mechanism of Action

OBJECTIVES

Assess a range of prototype molecule designs to identify targeted complement regulators with therapeutic properties. To do this, we determine:

1. Whether orientation of the complement regulator relative to targeting antibody impacts complement inhibitor function or affinity of the antibody to C3d
2. Rank order potency of prototype targeted complement inhibitors.
3. Whether fusion of complement regulators preserves inhibitory activity and specificity for the Classical and Alternative Pathways
4. Manufacturability and developability of bifunctional fusion proteins
5. Binding of targeted complement regulators to injured cells/tissues where C3d is deposited

LIBRARY DESIGN

Fusion Protein Components

Targeting antibody formats

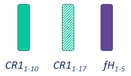
Monoclonal antibody (mAb)

Bispecific antibody (bsAb)

antigen binding fragment (fAb)

single chain fragment (variable) (scFv)

Effector domains



Gly4Ser Linker

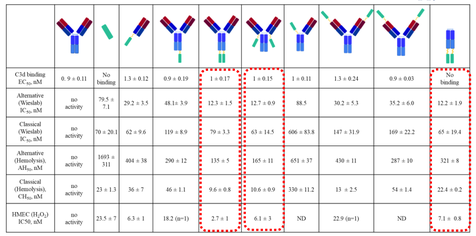
METHODS & RESULTS

- Fusion proteins transiently expressed in Chinese hamster ovary (CHO) cells
  - Proteins were affinity purified yielding material with greater than 95% purity and endotoxin levels below 0.5 EU/mg.
  - Constructs with only one heavy and one light chain had the highest titer and yield
- Fusion proteins were evaluated for binding potency in a C3d ELISA as compared to the C3d mAb alone.
- Complement inhibitory activity was assessed in three *in vitro* orthogonal assays
  - Wieslab assay – formation of C5b–9 by complement activation in human serum
  - Hemolysis assay - lysis of rabbit or sheep red blood cells by human serum
  - HMEC assay - human serum-mediated complement activation on the surface of injured endothelial cells (predominantly CP driven).
- Top-ranked fusion proteins were evaluated in convertase stabilizing assays (CSA) to test their ability to disrupt the stabilizing activity of Alternative Pathway (C3CSA) or Classical Pathway (C4CSA)
  - Inhibition of C3 Convertase activity on sheep red blood cells co-incubated with either C3 or C4 Nephritic Factor, C3Nef or C3Nef, stabilizing IgGs.
- Validation of target expression, tissue targeting, and distribution of anti-C3d mAb and mAb-fusions were evaluated in a mouse model of Epidermolysis Bullosa Acquisita (EBA).

IN VITRO FUNCTIONAL ACTIVITY

- Assessment of functional activity in Wieslab, hemolytic and HMEC assays show that potency is dependent on the identity, orientation, and number of negative regulatory proteins fused to the antibody
- Binding of the C3d targeting antibody remains unaffected by the addition of a negative regulatory protein, regardless of the orientation, multiplicity or identity.
  - This is likely due to the extended, flexible structure of the effector domains
- **The identity of the complement regulator drives potency in the classical and alternative pathways in the following manner $CR1_{1-17}$ >> $CR1_{1-10}$ >>fH_{1-5}**
  - **Fusion of the negative regulatory protein to the C-terminal end of the antibody heavy chain imparts better potency than the N-terminal**
  - The addition of 2 negative regulatory proteins provides additive inhibitory activity relative to fusion proteins with a single negative regulatory protein

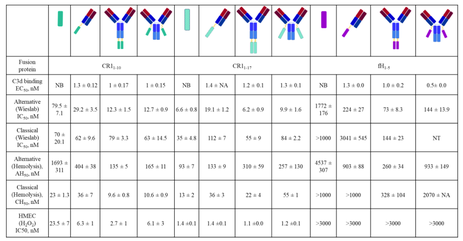
Example Fusion Protein Designs: anti-C3d (mIgG1) - $CR1_{1-10}$



C3d binding EC_{50}, nM	0.9 ± 0.11	No binding	1.3 ± 0.12	0.9 ± 0.19	1 ± 0.17	1 ± 0.15	1 ± 0.11	1.3 ± 0.24	0.9 ± 0.03	No binding
Alternative (Wieslab) IC_{50}, nM	no activity	79.5 ± 7.1	29.2 ± 3.5	48.1± 3.9	12.3 ± 1.5	12.7 ± 0.9	88.5	30.2 ± 5.3	35.2 ± 6.0	12.2 ± 1.9
Classical (Wieslab) IC_{50}, nM	no activity	70 ± 20.1	62 ± 9.6	119 ± 8.9	79 ± 3.3	63 ± 14.5	606 ± 83.8	147 ± 31.9	169 ± 22.2	65 ± 19.4
Alternative (Hemolysis), AH_{50}, nM	no activity	1693 ± 311	404 ± 38	290 ± 12	135 ± 5	165 ± 11	651 ± 37	430 ± 11	287 ± 10	321 ± 8
Classical (Hemolysis), CH_{50}, nM	no activity	23 ± 1.3	36 ± 7	46 ± 1.1	9.6 ± 0.8	10.6 ± 0.9	330 ± 11.2	13 ± 2.5	54 ± 1.4	22.4 ± 0.2
HMEC (H_2O_2) IC_{50}, nM	no activity	23.5 ± 7	6.3 ± 1	18.2 (n=1)	2.7 ± 1	6.1 ± 3	ND	22.9 (n=1)	ND	7.1 ± 0.8

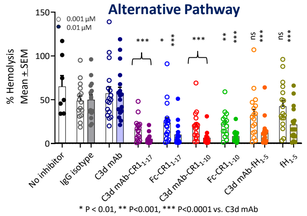
Symmetrical C-terminal fusions rank highest in: potency, expression titer & purification yield

Top Ranked Fusion Protein Designs (C-terminus)



Fusion protein												
	$CR1_{1-10}$				$CR1_{1-17}$				fH_{1-5}			
C3d binding EC_{50}, nM	NB	1.3 ± 0.12	1 ± 0.17	1 ± 0.15	NB	1.4 ± NA	1.2 ± 0.1	1.3 ± 0.1	NB	1.3 ± 0.0	1.0 ± 0.2	0.5± 0.0
Alternative (Wieslab) IC_{50}, nM	79.5 ± 7.1	29.2 ± 3.5	12.3 ± 1.5	12.7 ± 0.9	6.6 ± 0.8	19.1 ± 1.2	6.2 ± 0.9	9.9 ± 1.6	1772 ± 176	224 ± 27	73 ± 8.3	144 ± 13.9
Classical (Wieslab) IC_{50}, nM	70 ± 20.1	62 ± 9.6	79 ± 3.3	63 ± 14.5	35 ± 4.8	112 ± 7	55 ± 9	84 ± 2.2	>1000	3041 ± 545	144 ± 23	NT
Alternative (Hemolysis), AH_{50}, nM	1693 ± 311	404 ± 38	135 ± 5	165 ± 11	93 ± 7	133 ± 9	310 ± 59	257 ± 130	4537 ± 307	903 ± 88	260 ± 34	933 ± 149
Classical (Hemolysis), CH_{50}, nM	23 ± 1.3	36 ± 7	9.6 ± 0.8	10.6 ± 0.9	13 ± 2	36 ± 3	22 ± 4	55 ± 1	>1000	>1000	328 ± 104	2070 ± NA
HMEC (H_2O_2) $IC50$, nM	23.5 ± 7	6.3 ± 1	2.7 ± 1	6.1 ± 3	1.4 ±0.1	1.4 ±0.1	1.1 ±0.0	1.2 ±0.1	>3000	>3000	>3000	>3000

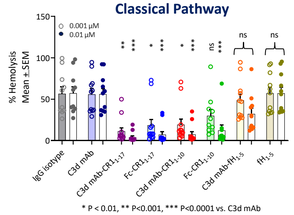
C3 nephritic factor (C3Nef) Convertase stabilizing assay (C3CSA) Alternative Pathway



* P < 0.01, ** P<0.001, *** P<0.0001 vs. C3d mAb

17 patient samples tested; each point represents 1 patient
16/17 patients have acquired C3Nef w/o mutations

C4 nephritic factor (C4Nef) Convertase stabilizing assay (C4CSA) Classical Pathway



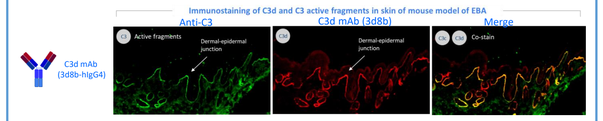
* P < 0.01, ** P<0.001, *** P<0.0001 vs. C3d mAb

12 patient samples tested; each point represents one patient
11/12 patients have acquired C4Nef w/o mutations

Lead molecules in *in vitro* translational studies demonstrate potent inhibition of convertase activity in C3G patient serum relative to untreated, isotype and parent C3d mAb controls, in a pathway dependent manner.

- All fusion proteins significantly reduce hemolysis
- CR1 fusions are more potent than fH_{1-5} fusions in both AP and CP assays, consistent with Wieslab, hemolysis and HMEC assays
- C4CSA is a CP driven assay where potency of CR1 differentiates from fH, an AP specific inhibitor

IN VIVO TISSUE TARGETING



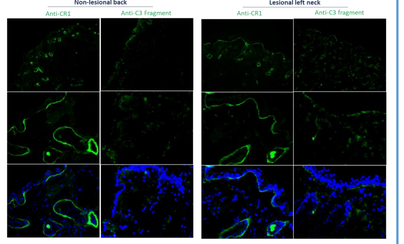
C3d and C3 active fragments are co-deposited in skin from a mouse model of epidermolysis bullosa acquisita (EBA)

Single IV dose of mAb-$CR1_{1-10}$ distributes to tissues of EBA mice and reduces C3 fragment deposition (below)

- anti-COL7 IgG administered IP into C57BL6/J mice on days 0, 2, 4, 8, 10.
- 50 mpk C3d mAb - $CR1_{1-10}$ (below) administered on day 12.
- Skin samples collected 24 hours after treatment
- Skin immunostained with anti-CR1 and anti-C3 fragment antibodies to evaluate distribution of the fusion protein to the Dermal/Epidermal Junction (DEJ) and measure complement inhibition, respectively

Day 0, 2, 4, 6, 8, 10: anti-mCol7 IgG
Day 12 : 50 mpk C3d mAb – $CR1_{1-10}$
Day 13: Skin biopsy & immunostaining

C3d mAb (mIgG1) – $CR1_{1-10}$



CONCLUSIONS

These data outline a range of novel therapeutic candidates that can effectively inhibit complement activation in a tissue-specific context. Depending on the negative regulator deployed, our therapies have the capability to potently inhibit either the alternative pathway (fH) or the classical, lectin and alternative pathways. We demonstrate that these fusion proteins can distribute in vivo to sites where C3d is deposited and inhibit complement activity. The unique profiles of these bifunctional proteins provide an opportunity to deliver pathway specific tissue targeted complement inhibitors. This is a novel approach to restore proper complement activity in diseased tissue in the absence of systemic complement blockade. This approach has the potential to provide increased potency, decreased dosing frequency, and improved safety.

REFERENCES

1. Thurman JM, Kulik L, Orth H, Wong M, Renner B, Sargsyan SA, et al. Detection of complement activation using monoclonal antibodies against C3d. J Clin Invest. 2013;123(5):2218-30.

CONTACT & ADDITIONAL DATA

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Fahnoe et al., Design and characterization of ADX-097: A C3d targeted antibody – fH1-5 fusion protein for the treatment of complement alternative pathway driven disease. **Poster B68**

Liu et al., C3d-Targeted fH Achieves Potent Tissue-Directed Complement Inhibition and Disease – Modifying Efficacy Without Affecting Systemic Complement. **Poster B24**