A CTLA-4-targeted ETB for Treg depletion shows favorable preclinical efficacy and safety

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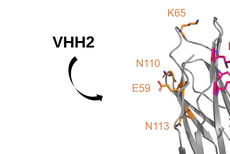
CTLA-4 Targeted ETBs Designed to Deplete Regulatory T cells

Engineered Toxin Bodies (ETBs) are fusion proteins consisting of an antibody fragment genetically fused to a proprietarily de-immunized (DI) form of the Shiga-like toxin A subunit (SLTA). Once the antibody fragment portion of the ETB binds its target, the SLTA portion of the ETB induces internalization into the cell, routing to the cytosol, and cell kill through enzymatic and irreversible ribosomal destruction.

CTLA-4 targeted ETBs are designed to deplete *CTLA-4* positive regulatory T cells in the tumor microenvironment (TME) through:

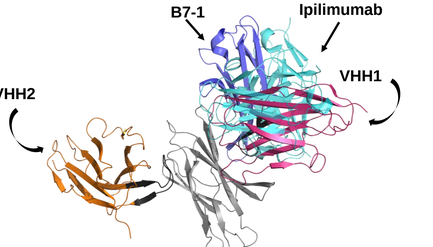
- **Specificity for CTLA-4**: Preferential activity against Tregs vs CD8+T-cells based on receptor density
- **Potency**: Direct cell-kill of Tregs via enzymatic and irreversible inactivation of ribosomes
- **Small size (55 kDa)**: Increased tumor penetration



CTLA-4 Candidate ETB
- Ability to **deplete Tregs in TME independent of effector function**
- Short half-life may offer reduced time for irAE resolution following treatment

In Vitro Binding Properties

CTLA-4 ETB Candidate Biparatopic Design and CTLA-4 Engagement



Binding ELISA Species Cross-reactivity EC50 [nM]

ETB	Human	NHP	Mouse
VHH1 ETB	1.2	0.53	No binding
VHH2 ETB	19.1	7.4	No binding
CTLA-4 Candidate ETB	0.59	0.38	>50

The CTLA-4 Candidate ETB is of biparatopic nature, composed of two unique llama single domain antibody fragments, or VHH domains, in tandem. Each VHH binds human and NHP CTLA-4 with similar affinities (within 3-fold) by ELISA. The candidate ETB binds with higher affinity compared to the single VHH ETBs

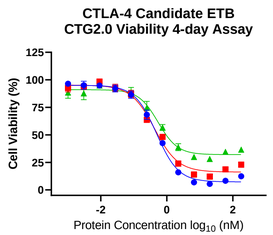
ETB	EC50 (nM)	Fold Induction Max
VHH1 ETB	No Blocking	1.7
VHH2 ETB	No Blocking	1.2
CTLA-4 Candidate ETB	12.9	14.51

CTLA-4 Blockade Bioassay (Promega) was used to measure the ability of ETB to block the interaction of CTLA-4 with its ligands in a cell system. ETB was added to CTLA-4-Jurkat cells, aAPC/Raji cells were added, then signal readout after an 8h incubation.

Left: VHH1 (Red) and VHH2 (orange) critical CTLA-4 contact residues identified through shotgun mutagenesis and high-throughput flow cytometry (work performed at Integral Molecular) Right: Docked structure is superimposed on crystal structure of complex of CTLA-4 with Fv of ipilimumab (PDB: 5TRU, cyan) and B7-1 (PDB: 1I8L, blue). CDR3 loop of VHHs are colored black. Docking supports that VHH1 competes with Ipilimumab for a similar epitope region, while VHH2 binds in a distinct epitope region.

CTLA-4 ETB Efficacy in Vitro is Tuned by CTLA-4 Receptor Levels

CTLA-4 Candidate ETB Potency on Gain-of-function Cell Lines Expressing Different CTLA-4 Levels

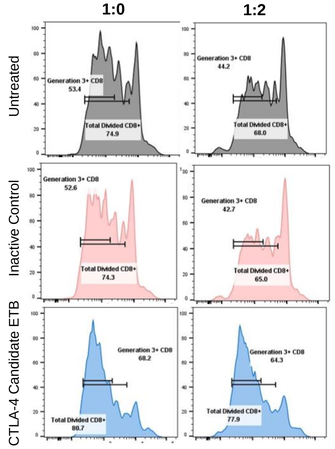


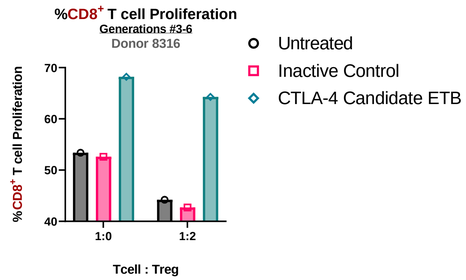
Cell Line	Approximate receptors/cell	ETB IC50 [nM]	% viability at max dose
High	4510	0.49	12.4
Med	2617	0.42	22.9
Low	1262	0.54	36.5

Viability of various cell lines was measured 96 hours after ETB addition to cells using Cell Titer-Glo®(Promega). IC50 values reported in nM. The cell lines represent different subclones of the same parental hCTLA-4-CHOK1 monoclonal cell line; each subclone was selected to represent a different range of CTLA-4 Expression.

CTLA-4 Candidate ETB kills Tregs ex-vivo and Antagonizes T cell suppression

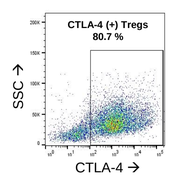
CTLA-4 Candidate ETB Reduces Treg-mediated T cell suppression in an SLTA-dependent manner



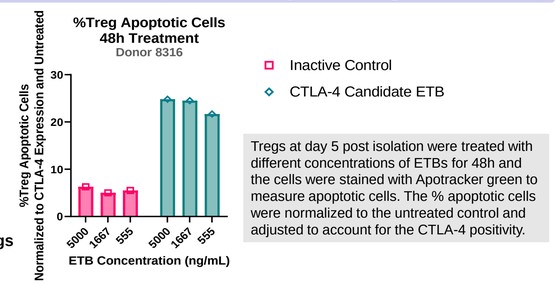


CTLA-4 Candidate ETB reduced Treg-mediated T cell suppression in an SLTA-dependent manner. Tregs and T cells were magnetically isolated from peripheral blood mononuclear cells (PBMC) of a healthy donor. The T cells were stained with **Violet proliferation dye** and then co-cultured with autologous Tregs at various ratios and were either treated with or without CTLA-4 Candidate ETB for 4 days. The cells were plated with antiCD3/CD28 beads to stimulate the proliferation of T cells. After 4 days of incubation, the antiCD3/CD28 beads were removed from the cells and the cells were surface stained with antiCD4 and antiCD8 antibody and acquired on a flow cytometer. CD8+ T cell proliferation is shown. CD4+ T cell proliferation was affected similarly with active ETB treatment, but with a less marked change.

CTLA-4 Candidate ETB Induces Apoptosis in Primary Human Tregs



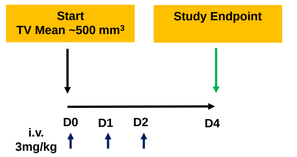
A subset of ex-vivo cultured Tregs are CTLA-4 Positive



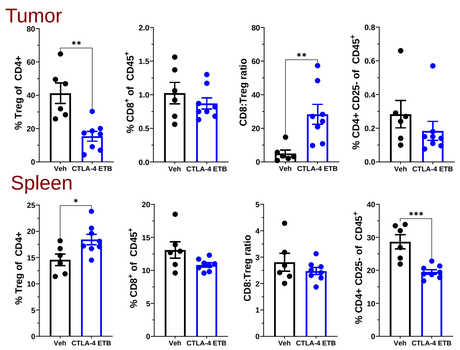
Tregs at day 5 post isolation were treated with different concentrations of ETBs for 48h and the cells were stained with Apotracker green to measure apoptotic cells. The % apoptotic cells were normalized to the untreated control and adjusted to account for the CTLA-4 positivity.

CTLA-4 Candidate ETB depletes Tregs of the TME in a Mouse MC38 Model

T cell Immunophenotyping in a Tumor-bearing Syngeneic Human Knock-In Mouse Model



Human CTLA-4 knock-in HuGEMM mice (Biocytogen) were inoculated with MC38 tumors. When the tumors reached 500 mm3, ETB was dosed at 3 mg/kg for 3 consecutive days. On day 4, the tumors and spleens were harvested and processed for immunophenotyping. The % CD4+ effectors, CD8+ CTLs and Tregs from the tumor and spleen are displayed on the graphs.



CTLA-4 Candidate ETB is Well-tolerated in NHPs

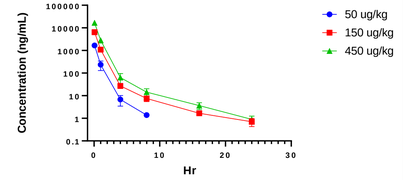
Study Design

Group	Test Article	Dose (µg/kg)	Dosing Days	N (Female)
1	Vehicle	0	1, 8, 15, 22	3
2	CTLA-4 ETB	50	1, 8, 15, 22	3
3	CTLA-4 ETB	150	1, 8, 15, 22	3
4	CTLA-4 ETB	450	1, 8, 15, 22	3

Toxicology Summary

- **No mortality was observed.**
- Clinical observations included: mild flaking/sloughing of the skin of the face, of the right hindlimb, and of both hindlimbs in one animal at 150 µg/kg; mildly decreased food consumption in all animals at 450 µg/kg.
- Minimally decreased albumin and mildly to markedly increased C-reactive protein in animals administered ≥50 µg/kg.
- The tolerated dose was 450 µg/kg, the highest dose administered in the study.

PK Analysis



Group	Test Article	Dose (µg/kg)	C_{max} (ng/mL)	AUC_{inst} (hr*ng/mL)	$T_{1/2}$ (hr)
2	CTLA-4 ETB	50	1660	999	0.47
3	CTLA-4 ETB	150	6400	4290	3.71
4	CTLA-4 ETB	450	16700	11000	4.05

CONCLUSIONS

- CTLA-4-targeted ETBs are designed to preferentially deplete regulatory T cells in the TME to improve efficacy and reduce the toxicity associated with CTLA-4 targeted antibodies
- CTLA-4 ETB Candidate has been identified with the ability to bind human and cynomolgus CTLA-4 in a biparatopic fashion, and to induce cytotoxicity of cell line models in a manner that is responsive to CTLA-4 receptor levels.
- CTLA-4 ETB Candidate induces apoptosis in ex-vivo cultured Tregs that express CTLA-4.
- CTLA-4 ETB Candidate antagonizes Treg-mediated CD8+ T cell suppression ex-vivo in a manner dependent on SLTA enzymatic activity.
- In a transgenic mouse model expressing human CTLA-4 and bearing syngeneic subcutaneous tumors, we observed that ETB treatment depletes Tregs in the TME, supporting our overall hypothesis.
- Initial tox assessment was performed in a NHP model. CTLA-4 ETB Candidate was well tolerated up to 450 µg/kg. No changes to the peripheral T cell numbers were observed in this study.
- Overall, these preclinical data support the use of ETB technology to deplete immune repressive regulatory T cells to allow immune reactivation to tumor.

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