Development of CD137 (4-1BB) receptor occupancy assay using fluorescently labeled *Bicycles*®

ABSTRACT#

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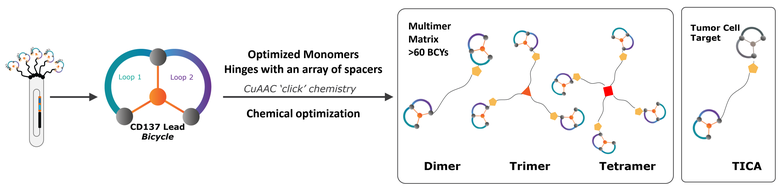
ABSTRACT

- Receptor occupancy (RO) assays are designed to quantify the binding of therapeutics to their cell surface targets and are frequently used to generate both pre-clinical and clinical pharmacodynamic biomarker data. Flow cytometry is a commonly used technique to measure receptor occupancy on immune cell populations within fresh blood specimens. RO assays are subject to numerous technical and logistical challenges. To ensure that reliable and high-quality results are generated from receptor occupancy assays, careful assay design and key reagent selection, characterization, and utilization are of critical importance. Antibodies are commonly conjugated with fluorophores and used in receptor occupancy assays for related therapeutics.
- *Bicycles*® are a new therapeutic modality - fully synthetic, constrained bicyclic peptides with high affinity and excellent target selectivity.
- Here, we describe a novel utility for fluorescently-labeled *Bicycles* in the development of a CD137 (4-1BB) receptor occupancy assay. CD137 is a member of the TNFR superfamily involved in stimulation of several immune cell types, including T cells and NK cells. CD137 is well validated pre-clinically, as agonism with anti-CD137 antibodies is effective in vivo, however, clinical utility to date has been limited by dose dependent hepatotoxicity.
- We have demonstrated that tumor-targeted immune cell agonists (TICAs™) comprised of CD137 binding *Bicycles* coupled to tumor antigen binding *Bicycles* exert anti-tumoral properties with a favorable safety profile.
- Here, we demonstrate another functionality for the *Bicycle* platform, as a measurement of CD137 TICA target engagement and receptor occupancy. Using fluorescently-labeled CD137 *Bicycles*, we measured RO on immune populations in isolated human peripheral blood mononuclear cells (PBMCs), as well as in whole blood.
- The assay shows high specificity as no *Bicycle* binding was observed in CD137 negative cell populations. Additionally, RO was shown to be dependent on the presence of CD137-binding *Bicycles*. Together, this novel approach of measuring receptor occupancy may be used to inform on both the pre-clinical and clinical therapeutic properties of *Bicycle* TICAs

INTRODUCTION



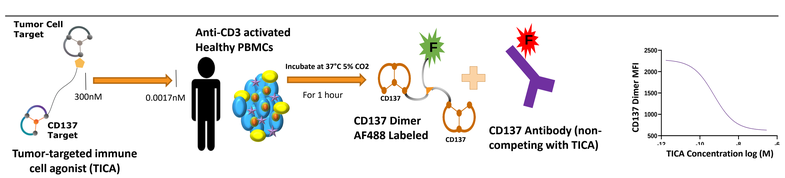


Figure 2: **CD137 receptor occupancy assay workflow. Cryopreserved healthy human PBMCs were activated with anti-CD3 antibody overnight. Activated PBMCs were incubated with variable concentrations of TICA for 1 hr. Treated PBMCs were stained with CD137 dimer and a non-competing CD137 antibody. CD137 receptors on immune cells unoccupied by TICA are labelled with the CD137 dimer and total CD137 levels were determined by the non-competing CD137 antibody. As the concentration of TICA increases, CD137 dimer binding decreases giving an inhibition curve.**

DATA ANALYSIS

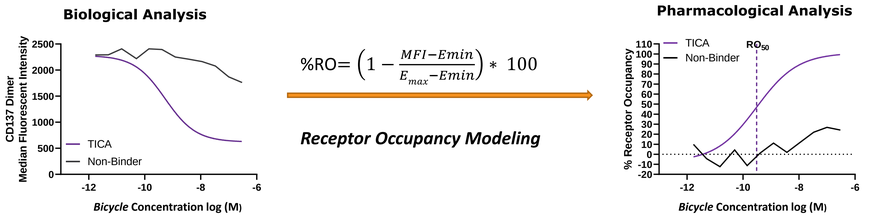


Figure 3: **Calculation of receptor occupancy (RO) from binding data measured using flow cytometry and expressed as the median fluorescence intensity (MFI)[1]. Variability in the measured MFI arise from instrumental, experimental, and sample specific sources and therefore MFI values were normalized using E_{min} and E_{max} values. Percent RO is calculated from the MFI using an inverse relationship shown above.**

RESULTS

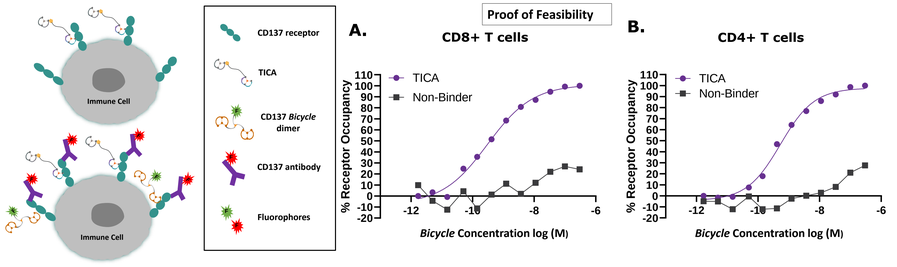


Figure 4: **CD137 *Bicycle* dimer as a probe to measure receptor occupancy on immune cells. Total and unoccupied CD137 on activated PBMCs were measured using a non-competing CD137 antibody or labeled dimer using the general method described in Fig 1. The percentage of receptors occupied by TICA increases with concentration and but not for a closely-related non-binder *Bicycle*. A) *Bicycle* target engagement to CD137 receptors on CD8+ cytotoxic T cells. B) *Bicycle* target engagement to CD137 receptors on CD4+ naïve T cells.**

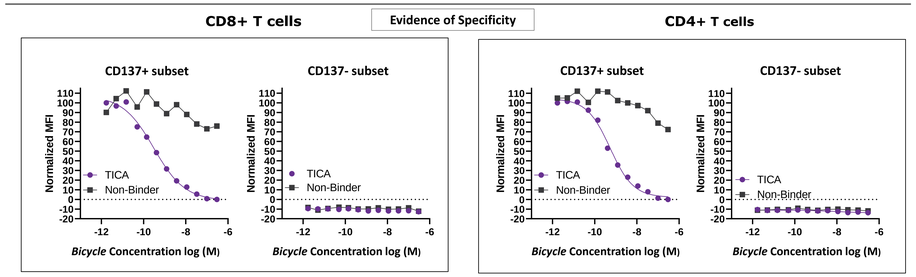


Figure 5: **The CD137 *Bicycle* dimer selectively binds to CD137 positive T cell populations. Following a 1 hour treatment of TICA (or non-binding TICA), the level of labelled CD137 dimer was measured within the both the CD137 positive and negative populations. The MFI for each TICA concentration in each cell sub-population is graphed above.**

RESULTS

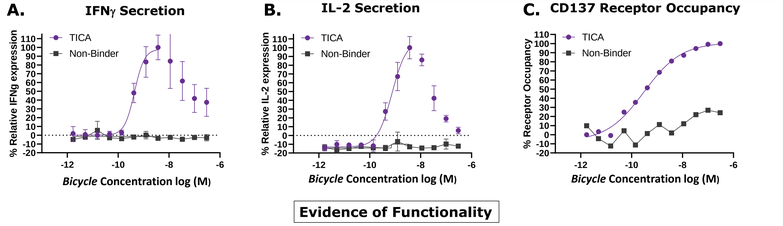


Figure 6: **TICA treatment enhances the secretion of cytokines at concentrations that afford occupancy on CD137 in co-cultures of PBMCs and tumor cells. IFNγ (A) and IL-2 (B) secretion increases with increasing *Bicycle* TICA. At high concentrations, a "hook" effect is observed consistent with blocking of simultaneous binding of tumor antigen and CD137. No cytokine secretion was induced CD137 non-binding *Bicycle* TICA (non-binder). C) CD137 receptor occupancy on activated PBMCs from same donor used in the co-culture assay was measured as described in Fig 1.**

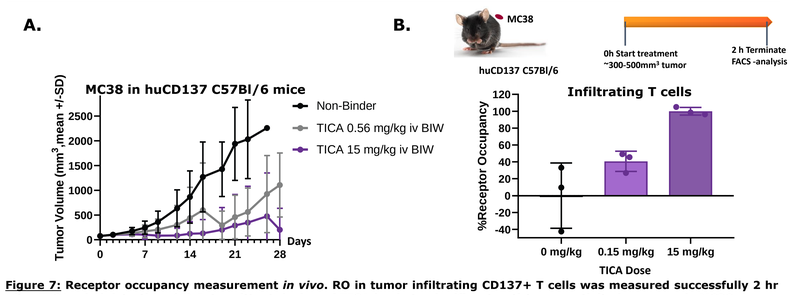


Figure 7: **Receptor occupancy measurement *in vivo*. RO in tumor infiltrating CD137+ T cells was measured successfully 2 hr after TICA administration. (A) Intermittent dosing of TICA leads to significant anti-tumor activity in syngeneic MC38 mouse model and informed doses selected for the RO study. The TICA is specific for human CD137 and does not bind mouse CD137 and therefore human CD137 transgenic C57BL/6 mice were used. Treatment was initiated when the tumor volume was ~60mm3. Different doses of TICA were administered twice weekly (BIW) over 22 days. (B) CD137 RO on tumor-infiltrating CD137 positive T cells 2 hr after administration of TICA determined by flow cytometry using labelled CD137 dimer and a total CD137 antibody essentially as described in Fig. 1. As in the *in vitro* studies, no binding of labelled CD137 dimer was observed on CD137 negative cells.**

CONCLUSION/SUMMARY

We have created a novel fit-for-purpose reagent to enable the study of CD137 target engagement in both *in vitro* and *in vivo* systems and shown that it can be used to dissect the relationship between functional activity and receptor occupancy.

REFERENCES

[1] Liang et al. Cytometry. Part B, Clinical cytometry 90: 117-27 :(2016)

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