Tumor-antigen 5T4-dependent Activation of the CD137 Costimulatory Pathway by Bispecific 5T4 x CD137 TRIDENT™ Molecules

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Abstract

Introduction: Trophoblast glycoprotein 5T4 is expressed on the cell surface of multiple cancers yet sparingly on normal adult tissues. CD137 (4-1BB) is a co-stimulatory molecule expressed by activated T and NK cells that support cell activation, proliferation and survival. Previously we described bispecific tumor associated antigen x CD137 DART® molecules limiting CD137 mediated immunostimulation to the tumor microenvironment (AACR2017) as an approach to reduce the unwanted systemic CD137 effects associated with agonistic anti-CD137 mAbs. We subsequently identified a bispecific TRIDENT format bearing bivalent CD137 and monovalent tumor antigen engagement that provides maximal CD137 activation in a tumor antigen anchored manner. Here we apply that format to generate a 5T4 x CD137 TRIDENT molecule that promotes CD137 activation in proximity to 5T4-expressing tumor cells leading to enhanced T-cell co-stimulation, proliferation and tumor cell killing activity when combined with CD3-engaging tumor-targeting bispecific DART molecules.

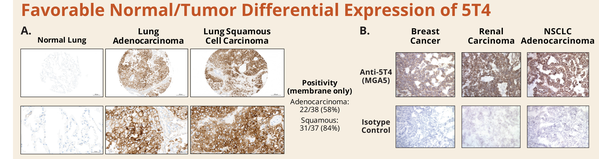
Methods: TRIDENT molecules were constructed comprising bivalent CD137 binding and monovalent 5T4 binding. Binding properties were evaluated by surface plasmon resonance (SPR) and flow cytometry. Signaling was assessed using a NF-kB luciferase reporter cell line expressing CD137. Co-stimulatory activity was characterized with primary human or cynomolgus monkey T-cells. T cells were incubated with or without antigen-expressing cells and submaximal concentrations of either anti-CD3 bead or CD3-engaging tumor-targeting bispecific DART molecules.

Results: SPR and flow cytometry analyses demonstrate that 5T4 x CD137 TRIDENT molecule binds human and monkey target antigens. The 5T4 x CD137 TRIDENT molecule induces CD137 signaling in a reporter cell line and promotes cytokine release in primary human & cynomolgus monkey T-cell assays in the presence of 5T4^{+} tumor cells. In the absence of 5T4^{+} tumor cells, the TRIDENT molecule lacks agonistic activity. Furthermore, 5T4 x CD137 TRIDENT molecule enhances T-cell proliferation and tumor cell cytolysis induced by CD3-targeted DART molecules. Consistent with preferential induction of CD137 by CD8 T cells, 5T4 x CD137 TRIDENT increases the fraction of CD8^{+} central memory and effector memory T cells in the presence 5T4^{+} tumor cells.

Conclusions: 5T4 x CD137 TRIDENT molecule promotes T-cell co-stimulation in a tumor antigen-dependent manner offering an opportunity to target CD137 immunostimulation, while potentially limiting non-specific systemic T-cell activation and related side effects.

Introduction

Favorable Normal/Tumor Differential Expression of 5T4

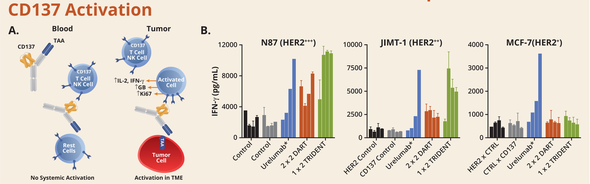


A) IHC of FFPE tissues on independent TMAs with anti-5T4 Ab (EPR5529, Abcam®) confirmed favorable normal tumor differential and high penetrance of expression in lung cancer. **B)** Evaluation of a panel of anti-5T4 mAbs generated at MacroGenics demonstrating exhibited broad tumor reactivity identified MGA5, the parental mAb humanized and incorporated into 5T4 x CD137 TRIDENT molecule.

CD137 Introduction

- CD137 (4-1BB) is a potent co-stimulatory molecule expressed by activated T, NK and DCs
  - Inducible expression on TILs following antigen engagement
  - Inducible expression on NK cells following interaction with mAb-opsonized tumor cells
  - Expressed by tumor-associated vascular endothelium
- CD137 interaction with CD137 ligand or CD137 agonistic Ab results in:
  - ↑ Immune-cell proliferation and anti-tumor cytolytic activity, countering of immune cell exhaustion and apoptosis
  - ↑ Expression of endothelial adhesion molecules and chemokines that facilitates ↑ CD8 T cells homing to tumor site
  - ↑ ADCC by NK cells; synergy with rituximab, trastuzumab or cetuximab in mouse models
- Systemic CD137 agonistic interventions may present systemic safety concerns
  - Urelumab associated with dose-dependent hepatitis (some fatal)
- Localized, conditional CD137 activation in the tumor microenvironment may diminish the probability of systemic effects
  - Bispecific CD137 DART proteins were previously designed for proof-of-concept[1]
  - A bispecific CD137-engaging TRIDENT format was then identified that provides maximal conditional CD137 activation (see companion AACR 2019 abstract #3042, Poster #29, 2019, April 1st 8:00 am – 12:00 pm)

HER2 x CD137 TRIDENT Molecule Induces HER2-dependent CD137 Activation



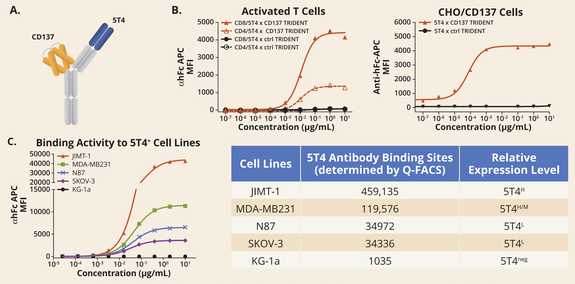
A) Proposed mechanism of action. In peripheral blood, bivalent TAA x CD137 TRIDENT engagement of CD137 insufficient to provide costimulatory signal; in TME clustering of TAA x CD137 TRIDENT molecule on tumor cells supports multivalent engagement of CD137 and costimulation.

B) Antigen-dependent maximal enhancement of T-cell activation by HER2 x CD137 TRIDENT molecule. IFN-γ production by T cells treated with HER2 x CD137 DART or TRIDENT molecules as indicated or control molecules in the presence of N87 (HER2^{+++}) cells, JIMT-1 (HER2^{++}) cells and MCF-7 (HER2^{+}) cells following a 72-hr culture under suboptimal stimulation condition. TAA: tumor-associated antigen; TME: tumor microenvironment; GB: Granzyme B; Urelumab*: Urelemab replica.

- **Here we report the characterization of 5T4 x CD137 TRIDENT molecules for conditional CD137 agonism in the tumor microenvironment**

Results

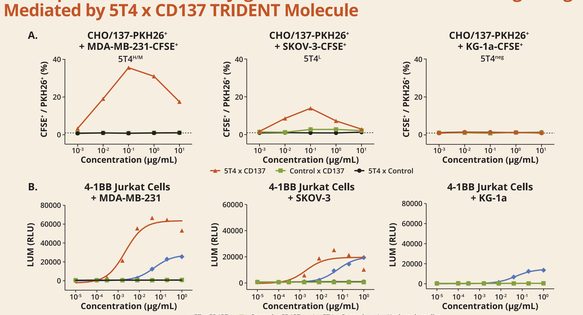
5T4 x CD137 TRIDENT Molecule Binding Activity to CD137- and 5T4-positive Cells



Cell Lines	5T4 Antibody Binding Sites (determined by Q-FACS)	Relative Expression Level
JIMT-1	459,135	5T4^{Hi}
MDA-MB231	119,576	5T4$^{Hi/M}$
N87	34972	5T4^{L}
SKOV-3	34336	5T4^{L}
KG-1a	1035	5T4^{neg}

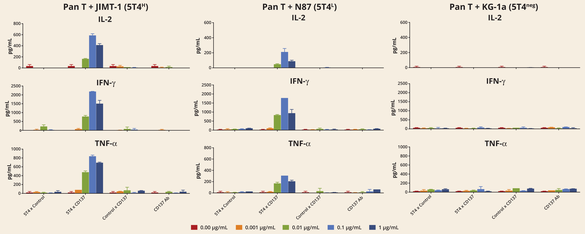
A) 5T4 x CD137 TRIDENT molecule comprising bivalent CD137 binding and monovalent 5T4 binding. **B)** 5T4 x CD137 TRIDENT molecule binding titration on primary activated CD4, CD8 T cells expressing CD137, and engineered CHO/CD137 cells expressing CD137 determined by flow cytometry analysis. **C)** 5T4 x CD137 TRIDENT molecule binding titration on tumor target cell lines expressing 5T4 at various levels. 5T4 Ab binding sites on tumor cell lines shown were determined by Q-FACS.

5T4-dependent Cell-cell Conjugation and Activation of CD137 Signaling Mediated by 5T4 x CD137 TRIDENT Molecule



A) 5T4^{+} cell - CD137^{+} cell conjugation by 5T4 x CD137 TRIDENT or control TRIDENT molecules determined by flow cytometry following incubation of PKH26-labeled CHO/CD137 cells with CFSE-labeled tumor target cells as indicated at 37oC for 2 hours. **B)** NF-kB activation by 5T4 x CD137 TRIDENT or control TRIDENT molecules as indicated in CD137 reporter cell line (Jurkat-NF-kB-Luc) co-cultured with either MDA-MB231 (5T4^{Hi}), SKOV-3 (5T4^{L}) or KG-1 (5T4^{neg}) cells. The luminescent signal (RLU) was used as a relative measure of CD137 pathway activation. Urelumab replica used as a positive control demonstrating 5T4-independtent activation of CD137 signaling.

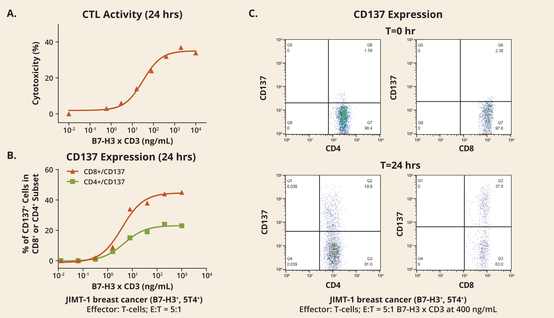
5T4-dependent Conditional T-cell Co-stimulation Mediated by 5T4 x CD137 TRIDENT Molecule



IL-2 (top), TNF-α (middle) and IFN-γ (bottom) production by T cells treated with 5T4 x CD137 TRIDENT or control TRIDENT molecules in the presence of JIMT-1 (5T4^{Hi}), N87 (5T4^{L}) or KG-1 (5T4^{neg}) cells following a 72-hr culture under suboptimal stimulation condition. The TRIDENT molecule concentrations used were as shown.

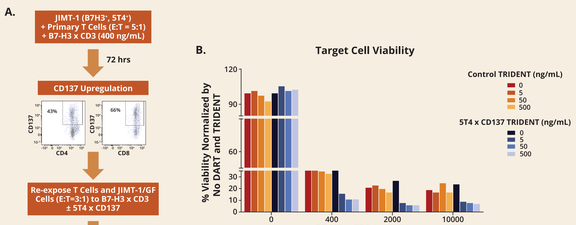
5T4 x CD137 TRIDENT Molecule Enhances Redirected T-cell Killing of 5T4-positive Tumor Cells In Vitro

Redirected T-cell Killing Is Associated with CD137 Upregulation



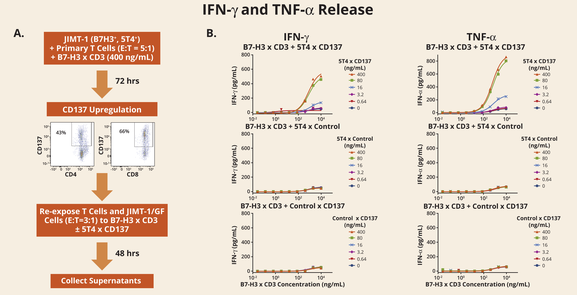
JIMT-1 (B7-H3^{+}) breast carcinoma cells were mixed with freshly isolated human T-cells at E:T ratio = 5:1 in the presence of increasing concentrations of B7-H3 x CD3 DART. **A)** The level of JIMT target cell cytotoxicity mediated by B7-H3 x CD3 DART determined by evaluation of LDH release at 24 hrs. **B)** The surface expression level of CD137 on the effector T-cell (CD4^{+}, CD8^{+}) population at 24 hrs measured by flow cytometry. **C)** Representative flow cytometry plots showing basal level and upregulated CD137 expression in CD4 and CD8 T cells pre- and 24-hr post-treatment with B7-H3 x CD3 DART at 400 ng/mL in the mixture of human T cells as effectors and JIMT-1 as target cells at E/T=5:1.

Redirected T-cell Killing



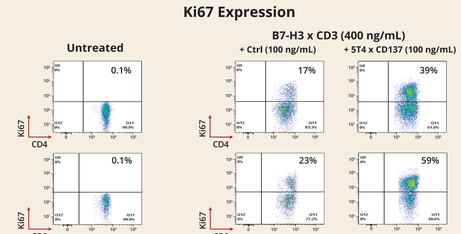
A) Flow chart showing B7-H3 x CD3 DART-mediated CD137 upregulation on T-cells following B7-H3 x CD3 DART treatment, with T-cells then re-exposed to in second round CTL assay to evaluate combo effect of 5T4 x CD137 TRIDENT with B7-H3 x CD3 DART in T-cell cytolytic killing of tumor target cells. **B)** B7-H3 x CD3 DART mediated cytolysis of B7-H3 target cells was assessed in the presence of 5T4 x CD137 TRIDENT or control molecule and JIMT-1/GF breast cancer cells (B7H3^{+} and 5T4^{+}, stably transfected with constitutive luciferase reporter gene) mixed with pre-activated T-cells at E/T ratio of 3:1. Cell viability was determined by evaluation of luciferase levels at 48 hrs. Shown is one representative assay with B7-H3 x CD3 as a model system.

IFN-γ and TNF-α Release



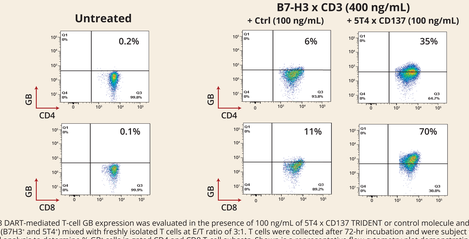
A) Flow chart showing B7-H3 x CD3 DART-mediated CD137 upregulation on T-cells following treatment, with T-cells then re-exposed in second round assay to evaluate combo effect of 5T4 x CD137 TRIDENT with B7-H3 x CD3 DART (as a model system) in T-cell cytokine release. **B)** B7-H3 x CD3 DART mediated T-cell cytokine release, exemplified by IFN-γ and TNF-α, was evaluated in the presence of 5T4 x CD137 TRIDENT (top) or control TRIDENT molecules (middle and bottom) and JIMT-1/GF breast cancer cells (B7H3^{+} and 5T4^{+}, stably transfected with constitutive luciferase reporter gene) mixed with pre-activated T cells at E/T ratio of 3:1. Supernatants collected after 48-hr incubation were subject to ELISA assay to determine IFN-γ and TNF-α levels.

Ki67 Expression



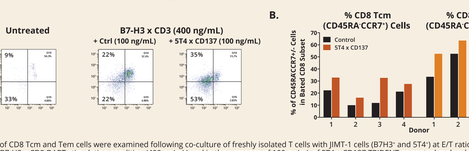
B7-H3 x CD3 DART-mediated T-cell proliferation was evaluated in the presence of 100 ng/mL of 5T4 x CD137 TRIDENT or control molecule and JIMT-1/GF breast cancer cells (B7H3^{+} and 5T4^{+}) mixed with freshly isolated T-cells at E/T ratio of 3:1. T cells were collected after 72-hr incubation and were subject to flow cytometry staining and analysis to determine % Ki67^{+} cells in gated CD4 and CD8 T-cell subsets. Shown is a representative flow cytometry plot demonstrating % Ki67^{+} cells increase in both CD4 and CD8 subsets following co-treatment with B7-H3 x CD3 DART (as a model system) & 5T4 x CD137 TRIDENT.

Granzyme B (GB) Expression



B7-H3 x CD3 DART-mediated T-cell GB expression was evaluated in the presence of 100 ng/mL of 5T4 x CD137 TRIDENT or control molecule and JIMT-1/GF breast cancer cells (B7H3^{+} and 5T4^{+}) mixed with freshly isolated T cells at E/T ratio of 3:1. T cells were collected after 72-hr incubation and were subject to flow cytometry staining and analysis to determine % GB^{+} cells in gated CD4 and CD8 T-cell subsets. Shown is a representative flow cytometry plot demonstrating % GB^{+} cells increase in both CD4 and CD8 subsets following co-treatment with B7-H3 x CD3 DART (as a model system) & 5T4 x CD137 TRIDENT.

Upregulation of Memory T Cells



Percentage of CD8 Tcm and Tem cells were examined following co-culture of freshly isolated T cells with JIMT-1 cells (B7H3^{+} and 5T4^{+}) at E/T ratio of 3:1 under suboptimal B7-H3 x CD3 DART stimulation condition (400 ng/mL) and in the presence of 100 ng/mL of 5T4 x CD137 TRIDENT or control molecule. T cells were collected after incubation for 3-7 days and were subject to flow cytometry staining and analysis to determine Tcm (CCR7^{+}CD45A) and Tem (CCR7^{-}CD45A) in gated CD8 T-cell subset. **A)** A representative flow cytometry plot showing % Tcm and % Tem increase in CD8 subset following co-treatment with B7-H3 x CD3 DART and 5T4 x CD137 TRIDENT. **B)** Percentage of Tcm, Tem cells in gated CD8 subset from 4 independent donors following co-treatment with B7-H3 x CD3 DART (as a model system) and 5T4 x CD137 TRIDENT or B7-H3 x CD3 DART and control molecule.

Conclusions

- A 5T4 x CD137 TRIDENT molecule demonstrated:
  - Conditional 5T4-dependent activation of CD137 signaling
  - Induction of 5T4-dependent co-stimulatory function: enhanced IL-2, IFN-γ and TNNF-α release
  - Potentiation of T-cell mediated cytolytic activity driven by a bispecific CD3-engager (B7-H3 x CD3 DART molecule), accompanied by:
    - Enhanced T-cell activation and proliferation with relative expansion of CD8 central/effector memory cells

5T4 x CD137 TRIDENT molecules may offer an opportunity to maximize conditional CD137 costimulation with limited systemic toxicity

References

1. Liu L, et al., *Cancer Research* 77(13 Supplement, 2017):3642-3642. **2.** Kohrt HE, et al., *European Journal of Cancer* 54 (2016) 112-119. **3.** Ignacio Melero, et. al., *European Journal of Immunology* 46 (2016) 513-522. **4.** Ye Q, et al., *Clin Canc Res* 20(1) (2014) 44-55. **5.** Palazon A, et al., *Cancer Discovery* 2 (2012) 608–623. **6.** Kohrt HE, et al., *J Clin Invest* 122 (3) (2012) 1066-75.

Acknowledgements

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