Novel Bioluminescent Bioassays for the Discovery and Development of Molecular and Cellular T-Cell Redirecting Cancer Therapy

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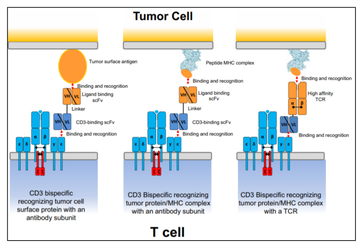
1. Introduction

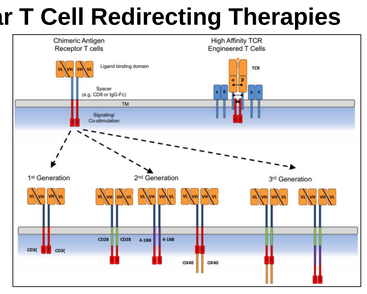
1. Molecular T Cell Redirection: CD3 Bispecifics



Sasu B et al, *Current Cancer Drug Targets (*2016)

- CD3 bispecific molecules can simultaneously engage CD3 on T cells and a tumor antigen on tumor cells.
- The dual binding induces T cell activation and proliferation, cytokine release and specific lysis of tumor cells.

2. Cellular T Cell Redirecting Therapies

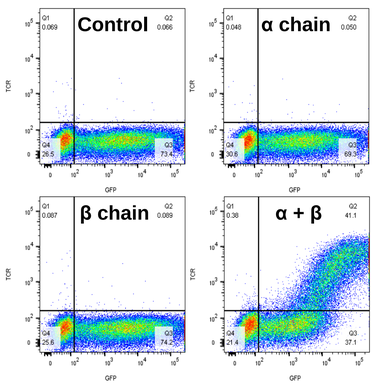


- Adaptive T cell therapy applies engineered T cells that are originally harvested from the patient or normal donors.
- T cells were engineered with a chimeric antigen receptor (CAR) or a new T cell receptor to recognize specific tumor antigens.
- The modified T cells, once binding to the antigens on the cancer cells, can induce T cell activation and proliferation, cytokine release and specific lysis of tumor cells

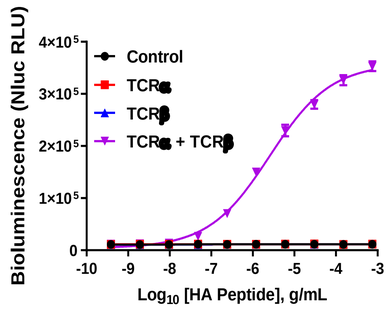
2. TCRαβ-deficient Reporter T Cell Lines for the Screening and Characterization of Therapeutic TCRs

Development of a CD4+ TCRαβ-deficient Reporter T cell line

- Stable integration of a luciferase reporter that responds to TCR signaling.
- Knockout of endogenous TCR alpha and beta chains via CRISPR to prevent recombination of endogenous and transgenic TCR chains.

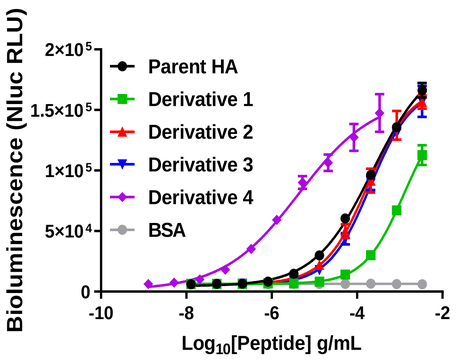


Confirmation of TCR alpha and beta chain knockout by phenotypic assay.
TCRαβ-KO (CD4+) Reporter Cells were transiently transfected with Control DNA, TCR α chain, TCR β chain, or α + β plasmids (with GFP transfection ctrl). TCR surface expression was analyzed by flow cytometry (anti-TCR Ab clone IP26).



Re-introduction of HA1.7 TCR chains to TCRαβ-KO (CD4+) Reporter Cells leads to hemagglutinin peptide-dependent activation.
Expression of antigen-specific TCR α and β chains results in dose-dependent reporter activation by influenza HA peptide presented by MHCII-positive cells.

3. TCRαβ-KO (CD4+) Reporter Cells Re-introduced with HA1.7 TCR Show HA Peptide Specificity

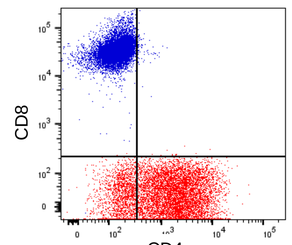


Peptide	EC50 (g/mL)
Parent	2.3E-04
Derivative 1	~1.3E-03
Derivative 2	1.8E-04
Derivative 3	2.1E-04
Derivative 4	4.3E-06
BSA	-

TCRαβ-KO (CD4+) Reporter Cells transiently expressing HA1.7 TCR were incubated with MHCII-positive cells, and parent HA peptide or derivatives of the HA peptide with amino acid substitutions. BSA was used as a control.

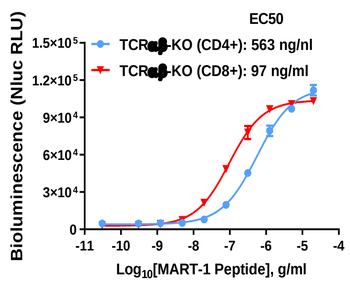
4. TCRαβ-KO (CD8+) Reporter Cells Re-introduced with DMF5 TCR Responded to MART-1 More Efficiently Than TCRαβ-KO (CD4+) Reporter Cells

TCRαβ-KO (CD8+) Reporter Cells were generated by CD4 knockout from TCRαβ-KO (CD4+) Reporter Cells and exogenous CD8 engineering via random integration.



TCRαβ-KO (CD4+) and TCRαβ-KO (CD8+) Reporter Cells were analyzed for CD4 and CD8 expression by flow cytometry.

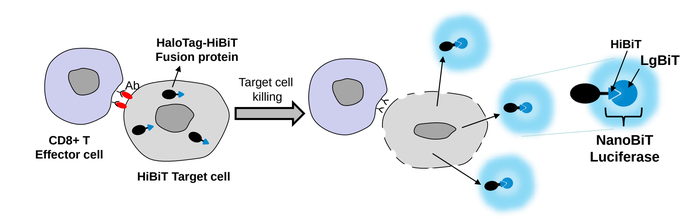
Red: TCRαβ-KO (CD4+) Reporter Cells
Blue: TCRαβ-KO (CD8+) Reporter Cells



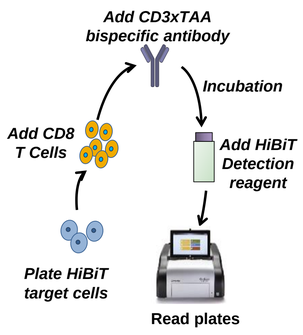
- TCRαβ-KO (CD4+) and TCRαβ-KO (CD8+) Reporter Cells transiently expressing the α and β chains of the DMF5 (MART-1-specific) TCR were incubated with A375 cells (HLA-A2+) and a titration of MART-1 peptide (26-35).
- Similar TCR surface expression was observed in both cell lines (data not shown)

TCRαβ-KO (CD8+) Reporter Cells expressing DMF5 TCR showed more potent response to MART-1/HLA-A2 than its counterpart CD4+ Reporter Cells, demonstrating the CD8-dependence for efficient antigen stimulation with this TCR/peptide pairing.

5. T Cell-Dependent Cytotoxicity (TDCC) Assay for Target Cell-specific Killing



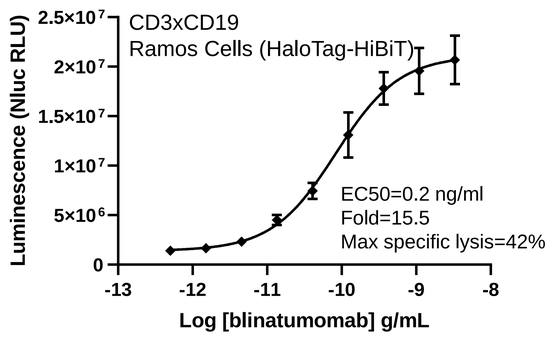
- Incubation of CD8 T cells, CD3xTAA (tumor-associated antigen) bispecific molecules and TAA+ target cells expressing HaloTag-HiBiT leads to T cell activation and target cell lysis which releases HaloTag-HiBiT protein into the medium.
- HiBiT binds to cell-impermeable LgBit in the HiBiT detection reagent and forms functional NanoBiT™ luciferase and emits lights
- Simple and fast detection in endpoint or kinetic formats
- Homogenous, sensitive, no medium transfer required

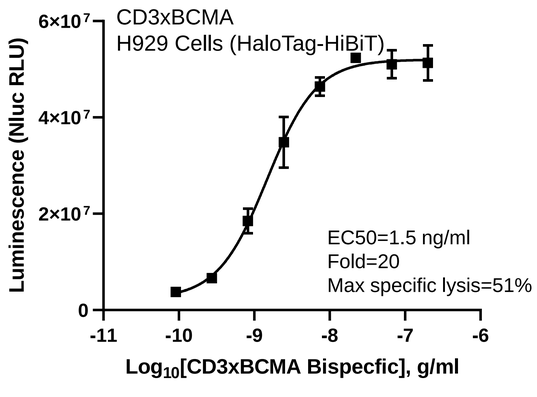


Assay procedure
1. Plate HaloTag-HiBiT target cells
2. Add TDCC-prequalified CD8 T cells
3. Add CD3xTAA bispecific molecules
4. Incubate
5. Add HiBiT detection reagent
6. Read plates

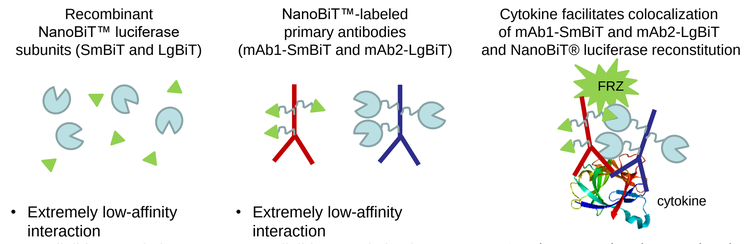
Features
- Low spontaneous release (<10% maximum release)
- Signal comes from target cells only
- Simple, fast and sensitive

6. TDCC Assay Measuring the Potencies for CD3xTAA Bispecific Molecules





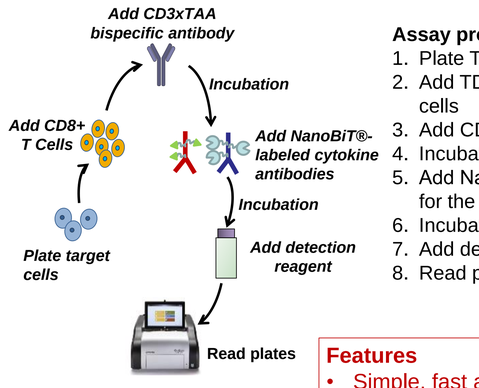
7. Homogenous NanoBiT™ Immunoassays for Cytokine Detection



- Extremely low-affinity interaction
- Negligible association in solution

- Extremely low-affinity interaction
- Negligible association in solution
- Very low assay background

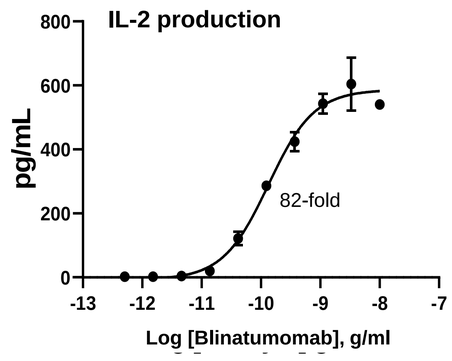
- Luminescent signal proportional to the levels of cytokine
- Homogeneous assay format

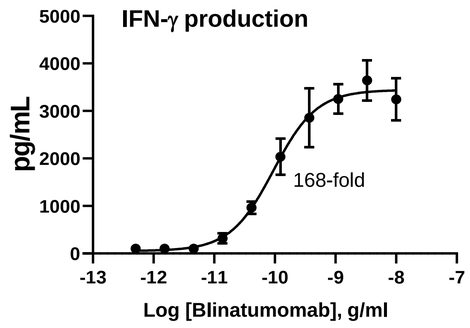


Assay procedure
1. Plate TAA+ target cells
2. Add TDCC-prequalified CD8 T cells
3. Add CD3xTAA bispecific molecules
4. Incubate 24 hours
5. Add NanoBiT™-labeled antibodies for the cytokine of interest.
6. Incubate for one hour
7. Add detection Reagent
8. Read plates

Features
- Simple, fast and sensitive
- Homogenous, no washing steps

8. NanoBiT™ Immunoassays Measuring IL-2 and IFN-γ Production from Activated CD8 T Cells





9. Conclusions

We have developed a platform of bioluminescent T Cell bioassays for the discovery and development of T Cell redirecting cancer therapy.

- **TCRαβ-deficient Reporter T Cells for the screening and characterization of antigen-specific therapeutic TCRs**
  - TCRαβ-deficient reporter T Cells can prevent the recombination of endogenous and transgenic TCRs.
  - CD4+ and CD8+ variants enable the screening of therapeutic TCRs designed against MHCI- or MHCII-dependent antigens
- **HiBiT-based T Cell-Dependent Cytotoxicity (TDCC) Assay**
  - The CD8 TDCC assay applies HiBiT complementation technology and can measure target cell-specific killing in mixed culture.
  - Primary CD8 T effector cells are prequalified in TDCC assay and can be used in Thaw-and-Use format without the need of cell culture.
  - The assay is homogenous, sensitive and fast.
- **NanoBiT™ Immunoassays for Cytokine Detection**
  - The assay applies NanoBiT™ complementation technology and uses NanoBiT-labeled antibodies for the cytokine.
  - The assay is homogenous, fast and sensitive.
  - It shows linear correlation between assay signal and cytokine level.

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