MODE OF CYTOTOXIC ACTION OF T-CELL BISPECIFIC ANTIBODIES ON HEMATOLOGICAL MALIGNANCIES: A NOVEL *IN VITRO* APPROACH

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ABSTRACT

Objectives: Bispecific antibodies (BsAbs) act through the formation of an immunologic synapse between T-cells (CD3) and a tumor-associated surface antigen (TAA) leading to T-cell activation and serial lysis of tumor cells. The aim of the present study is to explore the mechanism of action (MOA) and the *in vitro* effect of BsAbs on acute myeloid leukemia (AML) samples with the PharmaFlow platform.
Methods: Thirty-one fresh whole bone marrow (BM) samples and two AML cell lines were tested with the CD3-CD123 BsAb in the PharmaFlow platform, an innovative proprietary method that uses flow cytometry (FCM) to efficiently count the number of tumor cells killed by each activated T-cells. We analyzed the populations of leukemic cells, activated T-cells, and residual normal cells. In addition, other key parameters were used to elicit the MOA after BsAb exposure at different time incubations (24h-144h), such as the effective E:T ratio (the number of T-cells that kill a number of leukemic cells), real basal E:T ratio, tumor antigen expression, T-cell expansion, expression of immune checkpoint proteins on target and effector cells before and after cell culture. For some experiments, fluorescence-activated cell sorting (FACS) was performed to evaluate T-cell cytotoxicity after BsAb exposure.
Results: Most of the samples demonstrated T-cell activation and effective lysis of tumor cells after BsAb exposure independent of TAA expression and in a dose-response manner. Once sorted, these T-cells could kill tumor cells in the absence of BsAb, as well as tumor cells that did not express the TAA target. Interestingly, these activated T-cells selectively killed tumor cells with low cytotoxicity in residual normal cells from the same patients. Moreover, differential T-cell cytotoxicity was observed between samples. We observed samples with leukemic resistance or no T-cell activity, as well as others with higher T-cell cytotoxicity and minimal number of activated T-cells. The integration of all the predictive parameters (E:T ratios, TSA expression, etc.) allowed us to generate an *in vitro* response model and select samples with higher T-cell cytotoxicity after the BsAb exposure.

METHODS

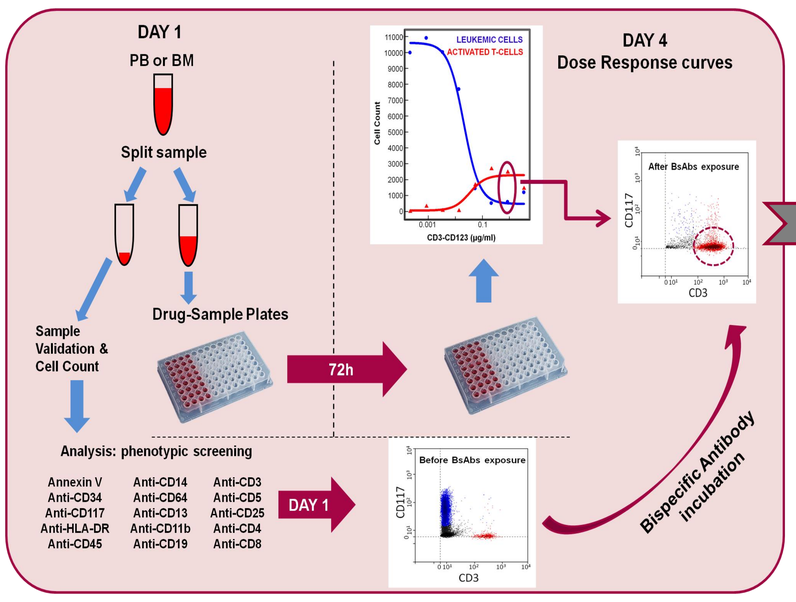


Figure 1. Screening set-up and Workflow

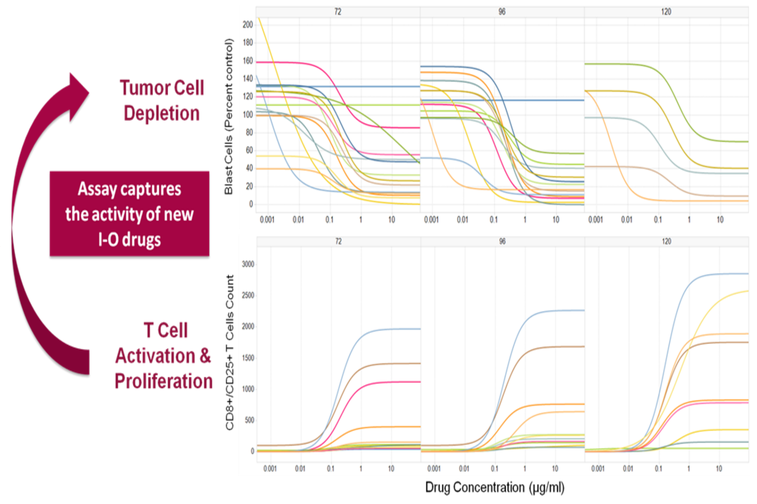


Figure 2. BsAb decrease leukemic cells and increase activated T-cells in a time and a concentration manner

Dose response curves to assess the CD3-CD123 bispecific antibody activity at different time points (72-96-120h) in AML samples. Upper panel displays leukemic cell depletion curves. The survival index (y-axis) ranges from 100% to 0% displaying the leukemic cell depletion after exposure to dose response CD3-CD123 bispecific antibody concentrations (x-axis). Bottom panel shows the simultaneous T-cell activation and proliferation along different time incubations. Absolute cell count of activated T-cells (y-axis) after CD3-CD123 bispecific antibody dose response concentrations (x-axis) is displayed.

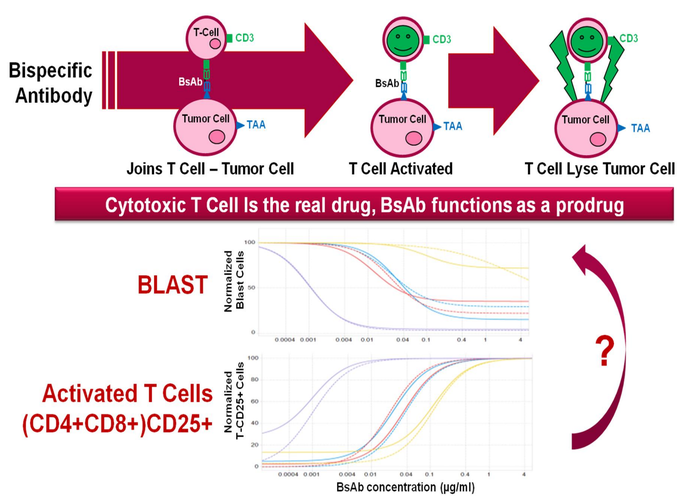
Quantitative Pharmacology for Bispecific Antibodies Activity In Patient Samples

1. **EC50 tumor depletion (same T Cell proliferation)**
   - When very low, predicts patient may respond at low doses
   - When very high, predicts resistant patient
2. **Effective E:T Ratio equivalent standard EC50**
   - Can be validated measuring dose responses with FACS sorted activated T Cells
   - High Effective E:T Ratios predicts sensitive patients
3. **Emax**
   - Emax near 100% required for a sensitive patient
4. **Kinetics of response**

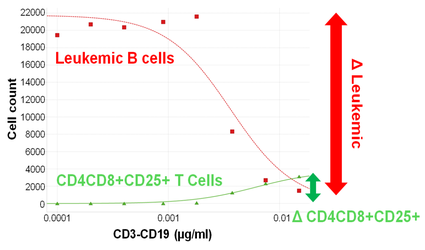
The integration of all these parameters quantifies the BsAb activity selecting cases with higher possibility of BsAb response.

RESULTS

Simple Version Immune-Tumor Response How Activated (CD25+) T Cells Lead to Tumor Depletion?



Activated T cells are the real drug: Effective E:T Ratios



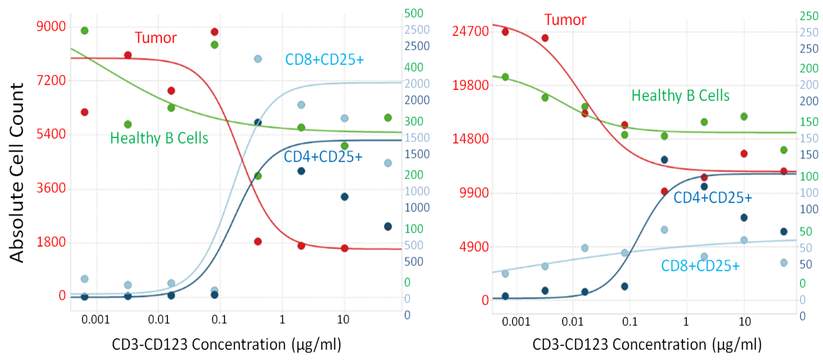
Blinatumomab CD3xCD19 [ng/ml]

	T-Cells	CD19+	Basal E:T
Cells # at baseline	1101	33166	1:30.1

	T- Cells CD4CD8+CD25+	Live Tumor Cells	Effective E:T Ratio
Cell # max dose	3799	21761	1:5.7

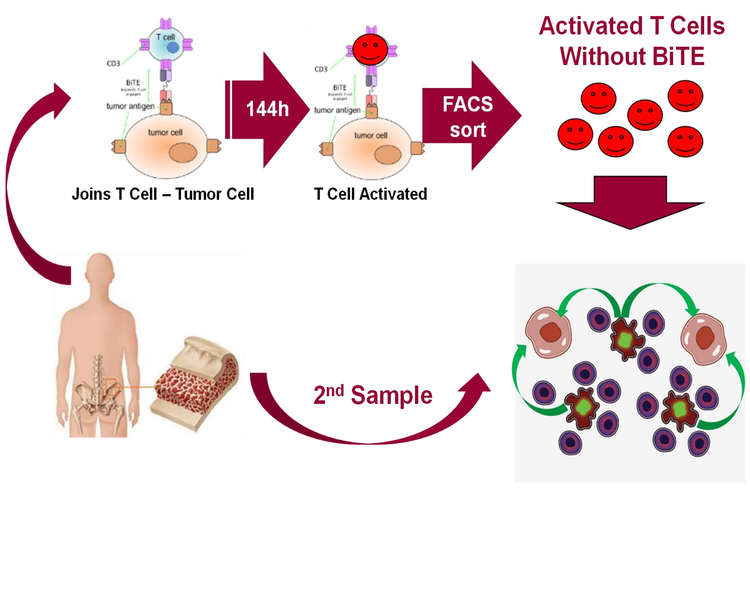
- Basal E:T ratios measure basal tumor vs total T cells
- Bispecific antibody induces cytotoxic CD4CD8+CD25+ T cells not present at basal
  - **Δ CD4CD8+CD25+**
- These cytotoxic T cells kill a number of leukemic cells
  - **Δ Leukemic**
- We define an Effective E:T Ratio as the ratio between
  Δ CD4CD8+CD25+ : Δ Leukemic
- Measures how many cancer cells are killed by each cytotoxic T Cell, i.e. the T Cell cancer-killing activity
- Effective E:T Ratios are different than Basal E:T ratios and may represent a better measurement of bispecific antibody activity

If Activated BM T Cells are TSA They Should Kill Selectively Tumor Cells and Not Healthy Cells

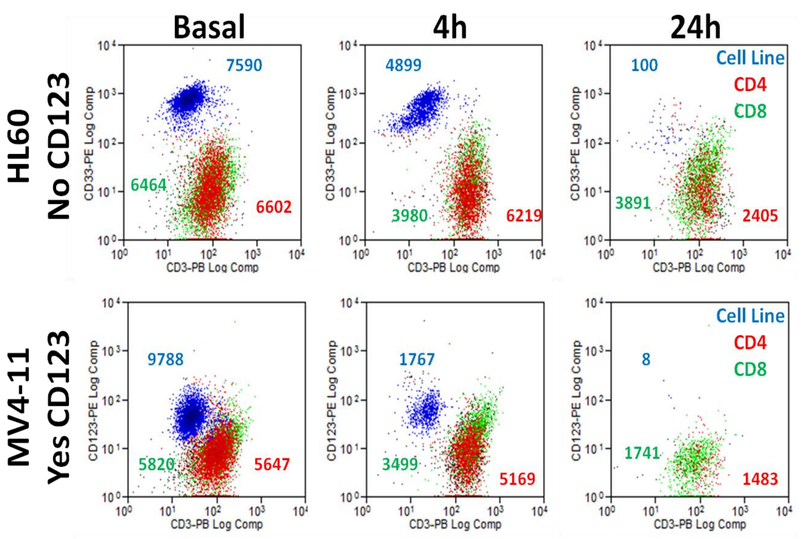


Activated proliferating T Cells kill tumor cells but not healthy B Cells within the same bone marrow sample

Activated Cytotoxic T Cell Kills Blasts Through a CD123 Independent MOA

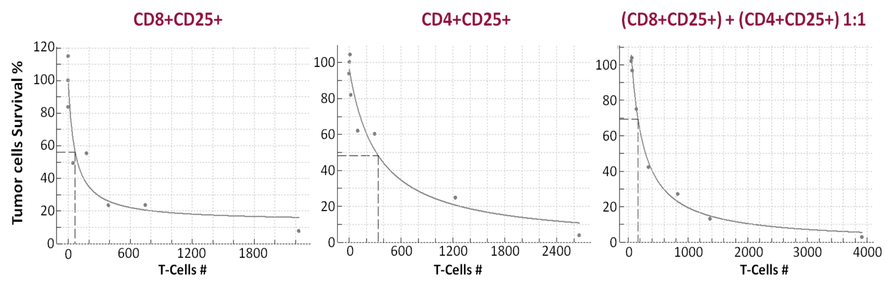


Can They Kill AML Cells Lines w/o CD123 Expression? YES



FACS sorted activated cells kill in a CD123 independent MOA

Measuring Dose Responses of Sorted Activated T Cells Without Bispecific Antibody



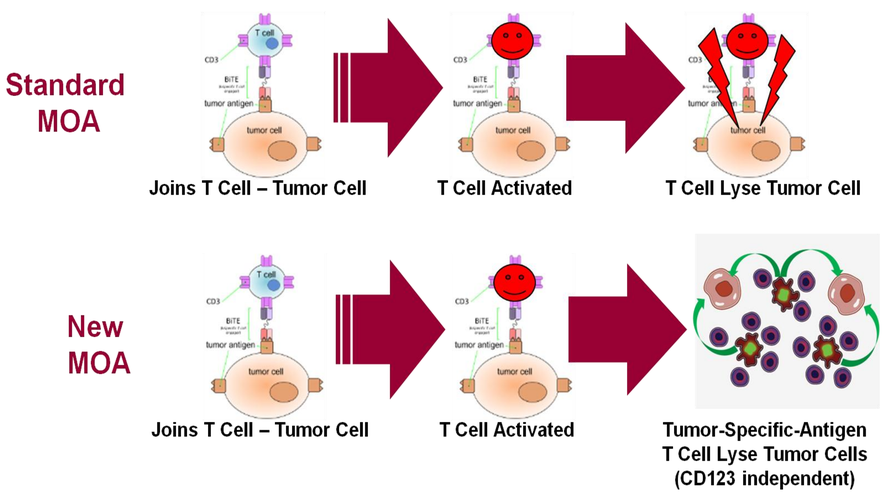
Percentage of tumor cells survival estimated relative to plate control with no-drug
Intercept dashed line corresponds to EC50 value

	EC50 (T-Cells#)	E0 (% Survival)	Emax (% Survival)	AUC
CD8+CD25+	67	84.8	13.7	29392.1
CD4+CD25+	336	96.5	0.0	44769.0
(CD4+CD25+)&(CD8+CD25+) [1:1]	164	138.4	0.0	44499.9

Both CD8 & CD4 activated T Cells kill tumor cells
CD8+ 5x more potent than CD4+
Effective E:T Ratios with CD4 & CD8 activated T Cells

CONCLUSIONS

Standard MOA: BsAbs Promote Direct Tumor Lysis by Proximity
New MOA: BsAbs may activate Tumor-Specific-Antigen T Cells



High Effective E:T Ratios (e.g. 25) samples may activate TSA-T Cells
Low Effective E:T Ratios (e.g. 1-5) may kill only by low potency proximity

- **Our findings are consistent with a model where, in addition to the standard MOA inducing tumor cells lysis by proximity, BsAbs can highly enrich cytotoxic clonal T-cell subsets with TSA and induce strong activation and proliferation of T-cells capable of killing tumor cells in an effective and selective manner.**
- **The PharmaFlow platform selects different *in vitro* T-cytotoxicity effects across patients identifying best patient candidates for adoptive antitumor immunotherapy with BsAbs with the integration of Effective E:T ratios and pharmacological parameters (EC_{50} & Emax): quantitative pharmacology of BsAbs in patient samples.**
- **New design of multi-specific antibodies from our new MOA are empowered by our screening of hundreds constructs ex vivo.**
- **CDx opportunity may increase substantially the clinical outcomes (ISTs).**