A NOVEL *IN VITRO* METHOD TO QUANTIFY THE PHARMACOLOGICAL ACTIVITY OF BISPECIFIC ANTIBODIES IN HEMATOLOGICAL SAMPLES

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ABSTRACT

Background: The PharmaFlow automated flow platform has achieved 85% clinical correlation with AML samples with its novel Native Environment assay. Recently, novel Bi-specific antibodies (BsAbs) or analogous constructions acting through the formation of an immunologic synapse between T-cells (CD3) and a tumor-associated surface antigen (TAA) have been used as immunotherapy leading to T-cell activation and serial lysis of tumor cells.

Aims: The aim of the present study is develop and *in vitro* assay with multiples variables to better quantify the activity of bispecific antibodies and reflect the interpatient variability.

Material & Methods: For this purpose, different fresh whole Bone Marrow (BM) or Peripheral Blood (PB) were tested with their corresponding BsAbs at 8 different concentrations in different time points (24h-144h). In this sense, we tested 31 AML BM samples (5 paired BM and PB) with the CD123XCD3 (Creative Biolabs) and 7 CLL and 3 B-ALL samples with Blinatumumab (Amgen). When appropriate, basal quantification of TAA was performed by flow cytometry (FCM). The PharmaFlow platform efficiently count by FCM how many tumor cells are killed by every activated T-cells, here called effective E:T ratio. For each sample, 8-colour FCM staining was performed to simultaneously analyze the leukemic population, activated CD4 and CD8 T-cells and the residual normal cells. EC50 or Emax was calculated to evaluate potency or efficacy. Kinetics of activity was measured repeating the dose response curves in 3 different days.

Results: Most of the samples present both T-cell activation (CD25+) and an effective lysis of tumor cells after BsAbs exposure in a time and dose dependent manner, even starting with low basal E:T ratios (<1:100). For AML, basal quantification of CD123 by FCM density does not reflect a correlation with the in vitro response. By contrast, differences in T-cell cytotoxicity or leukemic immunoresistance were observed between samples in terms of EC50 or Emax, even more marked between CLL samples. The integration of effective E:T ratios, EC50, Emax, and kinetics allow us to generate an in vitro response model and select those samples with higher T-cell cytotoxicity after the different BsAbs exposure. Interestingly, many of the samples for all the BsAbs leave a significant proportion of live cells, even at the higher BsAb concentrations or with a remarkable expansion of activated T-cells that suggest the use of immunecheckpoint to unblock this immnunoresistant status.

Conclusion: We have developed an automated flow cytometry assay for bispecific antibodies screening that keep intact both basal effector to target (E:T) ratios and Native environment using whole blood or bone marrow samples. In this context, the PharmaFlow platform selects different in vitro T-cytotoxicity effects across patients identifying best patient candidates for adoptive antitumor immunotherapy with BsAbs. The integration of Effective E:T ratios and pharmacological parameters better predict the in vitro response of BsAbs. Because of the high capacity of the PharmaFlow platform, additional antibodies constructions alone or in combinations with immunomodulatory agents could be tested to identify the better agents or immunotherapeutics combinations in hematological diseases.

METHODS

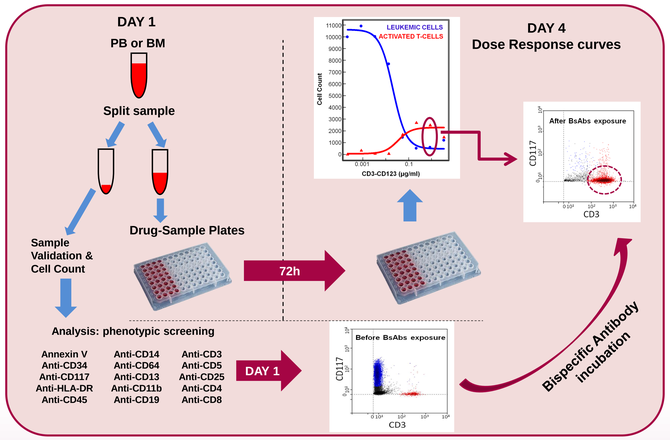


Figure 1. Screening set-up and Workflow

RESULTS

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

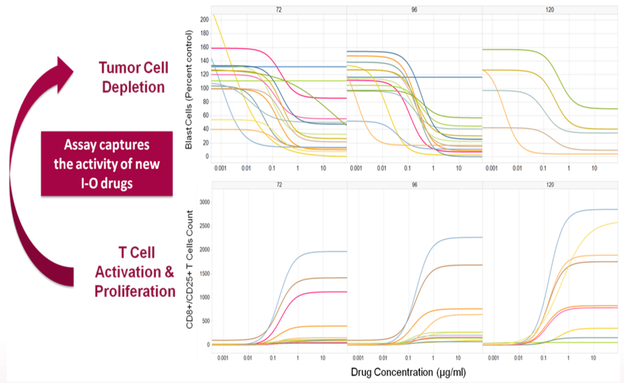


Figure 2. Dose response curves to assess the CD3-CD123 bispecific antibody activity at different time points (72-96-120h) in AML samples. Upper panel displays leukemic cell depletion curves. The survival index (y-axis) ranges from 100% to 0% displaying the leukemic cell depletion after exposure to dose 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.

RESULTS

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

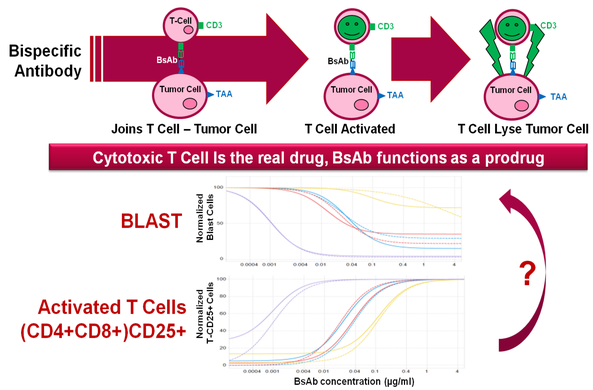
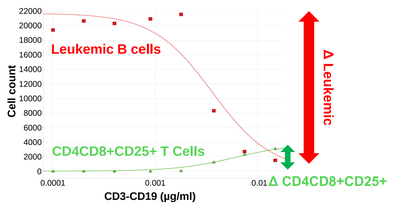


Figure 3

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



- 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

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

Figure 4

New Method Effective E:T Ratios Captures I-O Activity Bispecific Abs

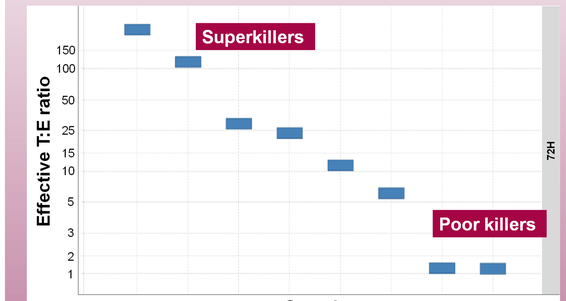


Figure 5: Representative example of the Effective T:E (y-axis) ratio from 8 AML samples (x-axis) . This method clearly stratify patients with high (left side) vs low T-cell killing activity (right side).

Quantitative Pharmacology for Bispecific Antibodies Activity In Patient Samples

1. **EC50 tumor depletion (same T Cell proliferation)**
   - Very similar across most samples
   - 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 predicted sensitive patients
3. **Emax**
   - Emax near 100% required for a sensitive patient
   - High % resistant tumor cells suggest combination e.g. PD1
4. **Kinetics of response**

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

Figure 6

Effective E:T ratios on the AML samples

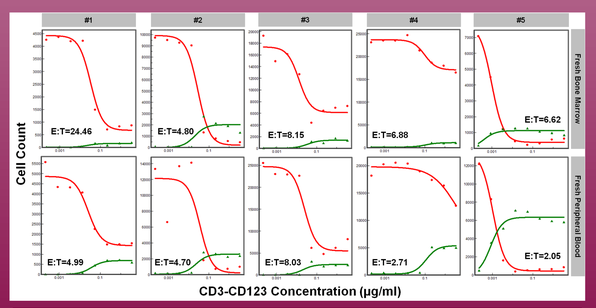


Figure 7: Effective E:T ratios on AML samples (N=5) after the CD3-CD123 exposure (72h). Upper panel correspond to the BM and bottom panel to the PB compartment. Red Line correspond to the Leukemic cells and green line to the activated (CD25+) T-cells (CD4+ or CD8+). BM T-cells are better killers in 3/5 samples (#1, #4 and #5) consistent with BM immunosuppressed Tumor Infiltrated Lymphocytes (TILs).

Effective E:T ratios on the CLL samples

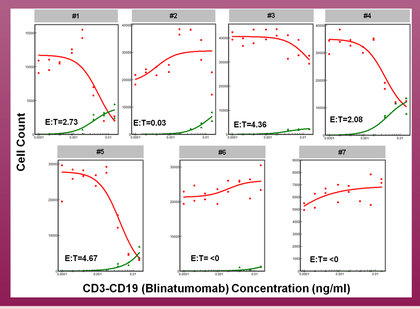


Figure 8: Effective E:T ratios on CLL samples (N=7) after the CD3-CD19 exposure (120h). Red Line correspond to the leukemic cells and green line to the activated (CD25+) T-cells (CD4+ or CD8+). Effective E:T ratios, AUC values, EC_{50} and E_{max} reflect both T-cell efficacy and B-cell immunoresistance identifying patients likely to receive blinatumomab therapy.

Effective E:T ratios on the ALL samples

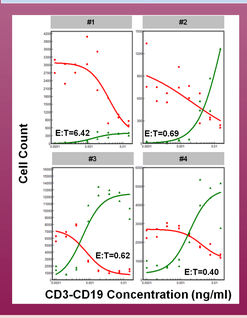


Figure 9: Effective E:T ratios on ALL samples (N=4) after the CD3-CD19 exposure (72h). Red Line correspond to the Leukemic cells and green line to the activated (CD25+) T-cells (CD4+ or CD8+). Samples #1 and #2 correspond to paired BM and PB patient sample, being BM T-cells (#1) better killers than PB (#2).

PD-1 increases T-cell activity

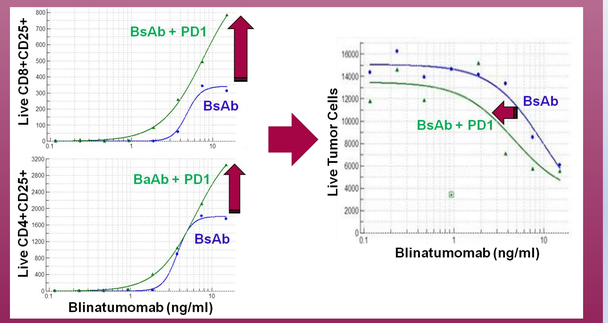


Figure 10: Absolute cell counts in a CLL sample for activated T-cells (left panels) and Live tumor cells (right panel) incubated with Blinatumomab in presence (green lines) or absence (blue lines) of the immunomodulatory inhibitor checkpoint PD1. Results show increased numbers of both activated T-cells (CD4 and CD8) in presence of PD1, enhancing the overall tumor killing.

CONCLUSION

- We report a novel proprietary *ex vivo* automated flow cytometry assay for I-O drugs keeping intact both basal effector to target (E:T) ratios and native environment using whole BM or PB.
- 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.
- Our findings are consistent with a model in which BsAb can enrich highly cytotoxic clonal T-subsets with Tumor-Specific Antigen in some patients.
- This assay enable evaluate multiple combinations with immunomodulators (PD1, CTLA-4, TIM-3, LAG-3) or BsAbs candidates for hematological diseases.
- Clinical trials should not exclude patient for low expression of TargetxCD3.
- 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).