Immuno-Oncology Functional Precision Medicine Assays by Trogocytosis

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ABSTRACT

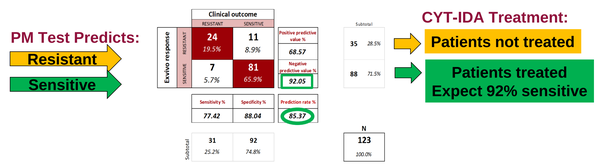
We pioneered in 2007 an **automated flow cytometry platform to evaluate drug activity in samples of hematological malignancies (HMs) patients**. Preserving the bone marrow Native Environment during compound incubation prevented artifacts induced by isolating leukocytes. We have validated this platform achieving a 93% correct prediction of sensitive patients achieving complete remission, in a cohort of 123 samples of 1st line AML all treated homogeneously with Cytarabine+Idarubicin (fig 1) We have now developed novel assays for Functional Precision Medicine (FPM) of immune oncology (IO) drugs presented here enabled by measuring trogocytosis, such as CAR-Ts, Bispecific antibodies (BiTEs), and immune check points (IChPs). The mechanisms of IO drugs leverage the capacity of immune cells such as T cells or NK cells to kill tumor cells. **We focus here on T cells and CAR-Ts, that kill by selective recognition of tumor cells.** A major challenge for FPM of immune oncology drugs is that T cell killing require T cell activation and proliferation, but this is normally achieved unselectively. The standard approach to T cell activation and proliferation is incubation with CD3 CD28 beads, which activate T cells indiscriminately. However, **only a % of these T activated cells would be able to recognize selectively and kill tumor cells**. For IChPs reactivating native tumor-selective T cells this would be a very small %. For CAR-Ts this % is not well characterized, but there is increasing recognition of the heterogeneity of CAR-T clones in the transfection process. There are cases of only 1 single CAR-T cell clone being responsible for tumor killing in patients, **suggesting a small % of these CAR-T may be responsible for clinical efficacy**. For BiTEs, they are hypothesized to kill tumor cells by bringing in proximity T cells with tumor cells, but we and others are finding that some of these T cells may be more efficacious than others. **We present a new approach to solve the problem of unselective activation of T cells for FPM of immune oncology drugs**. Quantitative assessment of the pharmacological activity of IO drugs in tumor samples **require a correct measurement of the stoichiometry between reactants**; i.e. activated T cells killing vs tumor cells. Tumor cells are identified by well know antibody markers. However, unselective activation of T cells by CD3 CD28 beads that cannot be distinguished from each other, without distinguishing which ones are doing the killing, prevents a correct stoichiometry. When T cells kill tumor cells by an immune synapse, they can take parts of the tumor cell membrane with them in a process called trogocytosis. This has been measured by staining the tumor cells selectively with a membrane cell tracker dye before mixing with the T cells, measuring the incorporation of this dye into the activated T cells. **Trogocytotic T cells would thus identify the killer T cells within a population**, in particular within an heterogenous population of T cells. **Trogocytosis may enable correct stoichiometry of killing T cells vs tumor cells to enable accurate FPM of IO drugs**. FPM assays have been developed for CAR-Ts measuring to dose response killing of tumor cells for CAR-Ts, using a CD19 CAR-T on ALL samples and a NKG2D CAR-T on AML samples. We have found a high interpatient variability of these activities, supporting the need for a FPM approach. Tumor killing started at 4 h incubation and was measured at different time points up to 24 hours. A small 1-3% of trogocytotic T cells have been consistently observed in these CAR-Ts at only 1 hour, when tumor killing was not observed. These trogocytotic T cells could be identified as either doblets representing a T cell attached to a tumor cell, as expected, or singlets being only a T cell that has been stained with the tumor cell membrane tracker dye. Different experimental conditions could shift the relative % of doblets vs singlets trogocytotic T cells. **To validate that these trogocytotic T cells were indeed the killing T cells, we FACS sorted both populations**; trogocytotic (CD5+CD25+Dye+) vs non-trogocytotic (CD5+CD25+Dye-) T cells. When these 2 populations were mixed with the same tumor sample and incubated over 24 hours, trogocytotic T cells were responsible for >95% of the killing of tumor cells relative to non-trogocytotic T cells. FPM assays have been developed on BiTEs for AML/MDS (CD3xCD123) and ALL/CLL/NHL (CD3xCD19). Although BiTEs are hypothesized to kill tumor cells by proximity assuming all T cells are equal, **we observed a 10% of trogocytotic T cells after 120 h incubation with the BiTE for some samples**. This suggested that **only a small fraction of activated T cells may be responsible for killing tumor cells**. In fact, mechanistically BiTEs are prodrugs, activating T cells that are the "active ingredient", and thus the correct FPM measurement is the potency of the activated T cells killing tumor cells. We divide the exact number of tumor cells killed during incubation by a BiTE relative to control no drug wells, with the exact number of activated T cells (CD5+CD25+) newly generated during the incubation, by flow cytometry at 72 h or 120 h. This provides a T Cell Killing Score that calculates how many tumor cells are killed, on average, by a single activated T cell. This parameter would represent the real activity of the BiTE as a prodrug, and varies greatly among patient samples from less than 1 to 100. If the number of trogocytotic activated T cells is used instead of all activated T cells, this T Cell Killing Score can change by a 10-fold, showing **the importance of using trogocytosis to measure BiTE activity in FPM assays**. FPM assays for Immune Check Point inhibitors (IChPs) developed rely on the prior assays for CAR-Ts and BiTEs. IChPs incubated alone with patient samples did not induce T cell activation and tumor cell killing. However, when IChPs are added to the CAR-T or BiTE assays described above can lead to enhanced T cell activation and/or enhanced tumor cell killing. These IChPs are rarely expressed in acute leukemias, and our assays focus on BiTEs that induce expression of IChPs during incubation. Our IChPs assays evaluate 2 conditions: first, identifying samples in which BiTE incubation results in a significant % of tumor cells left alive, i.e. immune resistant to the BiTE-activated T cells. In these cases we can evaluate adding 1 or 2 IChPs to the incubation to identify those that revert the resistance enabling killing of the resistant tumor cells. We can also evaluate the expression of these IChPs in both the control no BiTE wells vs the subpopulation of immune resistant tumor cells; IchPs overexpressed in resistant tumor cells could be responsible for the observed resistance. **Combining both criterions, that an IChPs reverses BiTE resistance and at the same is overexpressed in the BiTE resistant tumor cells, provide a FPM assay**. Sometimes there is a need to add more than 1 IChP.

In conclusion, trogocytosis may enable measuring the subset of activated T cells being responsible for killing tumor cells, enabling appropriate stoichiometry between killing T cells and tumor cells required for accurate FPM assays for these IO drugs

PM TEST *EX VIVO* CORRELATION ON AML PATIENTS

PM Test Predicts Clinical Complete Response with 92% Accuracy 1st Line AML Treatment CYT+IDA N=123

Observational clinical trial, patients treated independently of PM Test, correlating PM Test with patient clinical outcome afterwards.
Clinical Response Complete Response=Sensitive, Others=Resistant



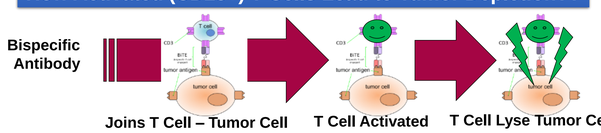
92% Prediction sensitive patients significantly higher than 74.8% 1st line response rate

When we say "sensitive" we'll be right 92% for CR *"Unprecedented"*
PM Test results seem better than new targeted therapies

Figure 1. PharmaFlow PM AML Test validation achieves 92% correct prediction of sensitive patients with CR. This test can provide more than 90% response rates for drugs as CDx under clinical trial and use, impacting in ROI.

BI-SPECIFIC ANTIBODIES

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



Cytotoxic T Cell Is the real drug, bispecific Ab functions as a prodrug

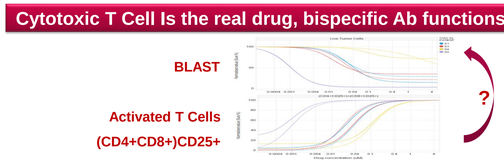
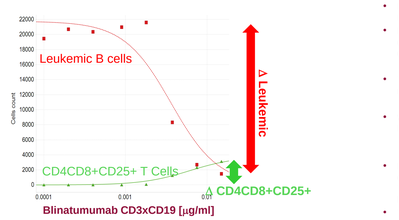


Figure 2. Mechanism of action of BiTEs. BiTEs act through the formation of an immunologic synapse between T-cells (CD3) and a tumor-associated surface antigen (TAA) producing T-cell activation and serial lysis of tumor cells. However, the correct FPM measurement is the potency of the activated cytotoxic T cells killing tumor cells. BiTEs can be considered as prodrugs being the activated T cells the "active ingredient".

New Measurement of Activity for Bispecific Antibodies
T-Cell Killing Potency for Blinatumumab on CLL



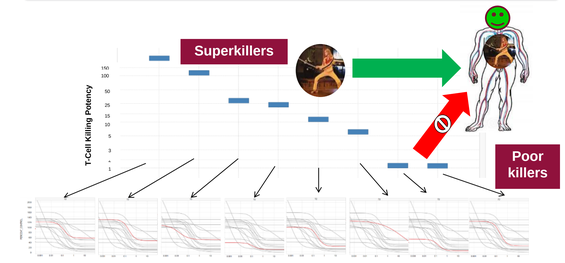
- Basal E:T ratios measure basal tumor vs total T cells
- Bispecific antibody induces cytotoxic CD4CD8+CD25+ T cells not present at basal
  - Δ CD4CD8+CD25+
- These cytotoxic T cells kill a number of leukemic cells
  - **Δ Leukemic**
- We define a T-Cell Killing Potency as the ratio between
  Δ CD4CD8+CD25+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

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

	T- Cells CD4CD8+CD25+	Live Tumor Cells	T-Cell Killing Score
Cell # max dose	3799	21761	1:5.7

Figure 3. Novel measurement of BiTEs activity based on a T-cell killing score that consider both blast and T-cells. The T-cell killing potency are the effective E:T ratios that may represent a better measurement of BiTE activity. Activated T cells are the real drug.

T-Cell Killing Potency Becomes PharmaFlow PM Test



T-Cell Killing Potency Does Not Correlate with Standard Tumor Depletion Dose Responses

New Method T-Cell Killing Potency Identifies Superkiller T Cells as PM Test
Different patients have T Cells with very different Killing Potencies

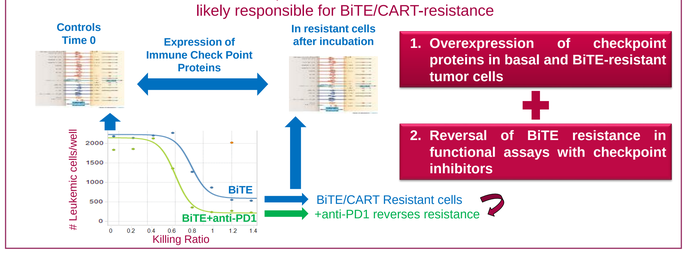
Figure 4. Representative example of the T Cells killing potency (y-axis) ratio from 8 AML samples (x-axis). This method clearly stratify patients with high (left side) vs low T-cell killing activity (right side).

BiTEs & IMMUNE CHECK POINTS

PharmaFlow PM: Immune Check Point Inhibitor Test (I)

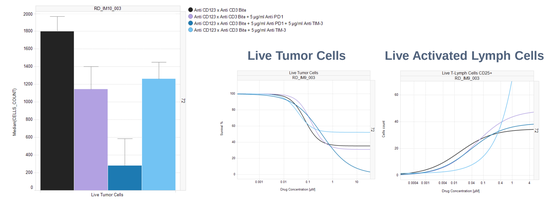
Novel PM Test To Combine BiTEs with Immune Check Point Inhibitors

Immune Check Point Proteins overexpressed in BiTE/CART-resistant tumor cells vs controls → likely responsible for BiTE/CART-resistance



1. **Overexpression of checkpoint proteins in basal and BiTE-resistant tumor cells**
2. **Reversal of BiTE resistance in functional assays with checkpoint inhibitors**

PharmaFlow PM Immune Check Point Inhibitor Test (II)

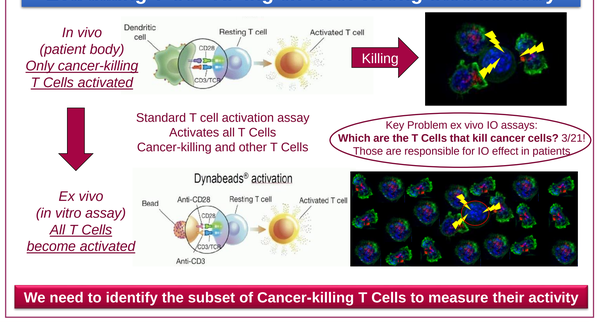


- AML Sample was incubated with CD3xCD123 BiTE, leaving resistant leukemic cells.
- IChPIs PD1 or TIM3 alone could not reverse the resistance, but their combination did.

Figure 6. PM Test to predict IChPs combinations with a BiTE. for AML. Left; expression levels of IChPs in BiTE treated resistant tumor cells, and adding PD1, TIM3, or both IChPs. Middle; dose response curves of BiTE and combinations with these IChPs. Right; dose response curves of BiTE-activated T cells (CD25+ CD5+). Sample treated with CD3xCD123 BiTE requires PD1 + TIM3.

TROGOCYTOSIS & BiTES

Evaluating T Cell Killing ex vivo: Wrong Stochiometry

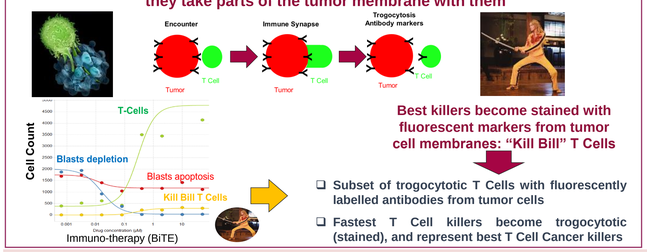


We need to identify the subset of Cancer-killing T Cells to measure their activity

Figure 7. Evaluation of T-Cell killing ex vivo. Incubation with CD3 & CD28 beads is the standard approach to T-Cell activation and proliferation which activate T-cells indiscriminately, but only a few of them could be able to recognize selectively and kill tumor cells.

Key Problem of ex-vivo IO Assays
Which are the T cells that kill cancer cells?

When T Cells kill tumor cells through an immune synapse, they take parts of the tumor membrane with them



Best killers become stained with fluorescent markers from tumor cell membranes: "Kill Bill" T Cells

- **Subset of trogocytotic T Cells with fluorescently labelled antibodies from tumor cells**
- **Fastest T Cell killers become trogocytotic (stained), and represent best T Cell Cancer killers**

Figure 8. Trogocytosis evaluation. Tumor cell were stained with a membrane cell dye before mixing with T-cells and then the incorporation of this dye to the activated T-cells were measured. These trogocytotic cells were sorted and incubated with another AML patient sample evaluating the killer capacity. Trogocytosis may enable correct stoichiometry of killing T cells vs tumor cells to perform accurate FPM of IO drugs.

CARTs & TROGOCYTOSIS

PM Test CAR-T: NKG2D on 6 AML Samples

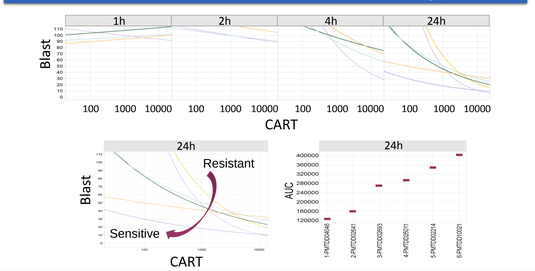


Figure 9. FPM assays for CART-NKG2D CART in AML patient samples. Figure top, time dependent kinetic effects of the tumor-killing activity of CART-NKG2D on AML samples. Down left, overlap dose response curves at 24h showing the direction towards sensitive vs resistant samples. Down right, quantitative ranking of activity of the Area Under the Curve (AUC) calculated for each sample. High interpatient variability was observed between the samples assayed, supporting the need for a FPM approach

Trogocytosis CAR-T CD19 ALL: Doblets CART-Blast

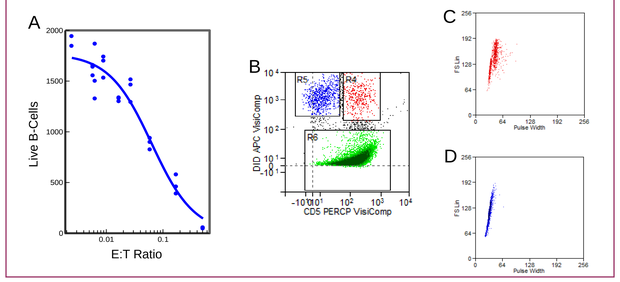


Figure 10. Activity and trogocytosis of CART-CD19 on a B-ALL sample. (A) Dose response curve on a B-ALL sample with CART cells. (B) Red region shows the trogocytotic CART cells with CD5+++ and DID dye. (C) Forwards scatter vs Pulse identifies most trogocytotic CART cells as doblets (right shifted cell population) than singlets (left shifted cell population). (D) Singlets in leukemic control.

FACS Sort NKG2D AML CAR-T
Trogocytotic vs not Trogocytotic

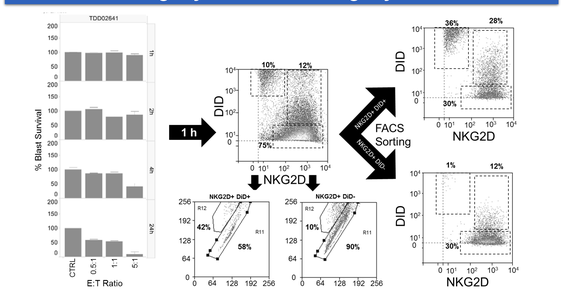


Figure 11. FACS sorting of trogocytotic CART-NKG2D cells on an AML sample

NKG2D AML CAR-T
Trogocytotic Clones Kill More than Non-Trogocytotic

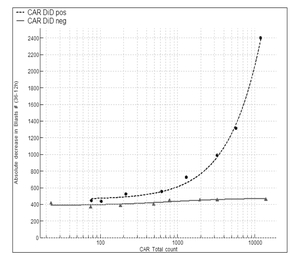


Figure 12. Enhanced tumor-killing activity of trogocytotic (DID+, dotted line) vs non-trogocytotic (DID-, continuous line), shown as the absolute decrease of leukemic blasts between 12 to 36 h incubation, relative to the number of CART-NKG2D T cells