Presenter Disclosure Information

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The following relationships exist related to this presentation:

CTL's Founder, Owner and CEO

Challenges of T cell monitoring:

- A. What to measure?
- B. How to measure it?
- C. How to measure it precisely, reproducibly?

A. What to measure (that has not been measured sufficiently in the past possibly explaining when T cell data and clinical outcomes did not match up)?

- Extended antigen-specificity (epitope spreading)
- Affinity/avidity (antigen-dose)
- Unanue Type A/B peptide configuration
- Killing vs. cytokine
- Effector classes beyond IFN-γ (e.g.,Th17, etc)
- Polyfunctional T cells
- Suppression, etc…

B. How to measure it…

- Ex vivo vs. after in vitro culture
- Sensitivity: frequencies mostly << 1/10,000
- Efficient cell utilization (and re-utilization)
- Robustness of assay
- High throughput suitability

→ **ELISPOT**

C. How to measure it accurately and reproducibly

- we are dealing with perishable, live cell material that is sensitive to shipping, handling and culture conditions
- complex assays – many steps and reagents involved
- green thumb requirement?

accurate, reproducible, and comparable measurements are a basic requirement of science.

….. CTL's story of successful standardization

Milestone 1 towards standardization:
Functionally loss-free freezing of PBMC

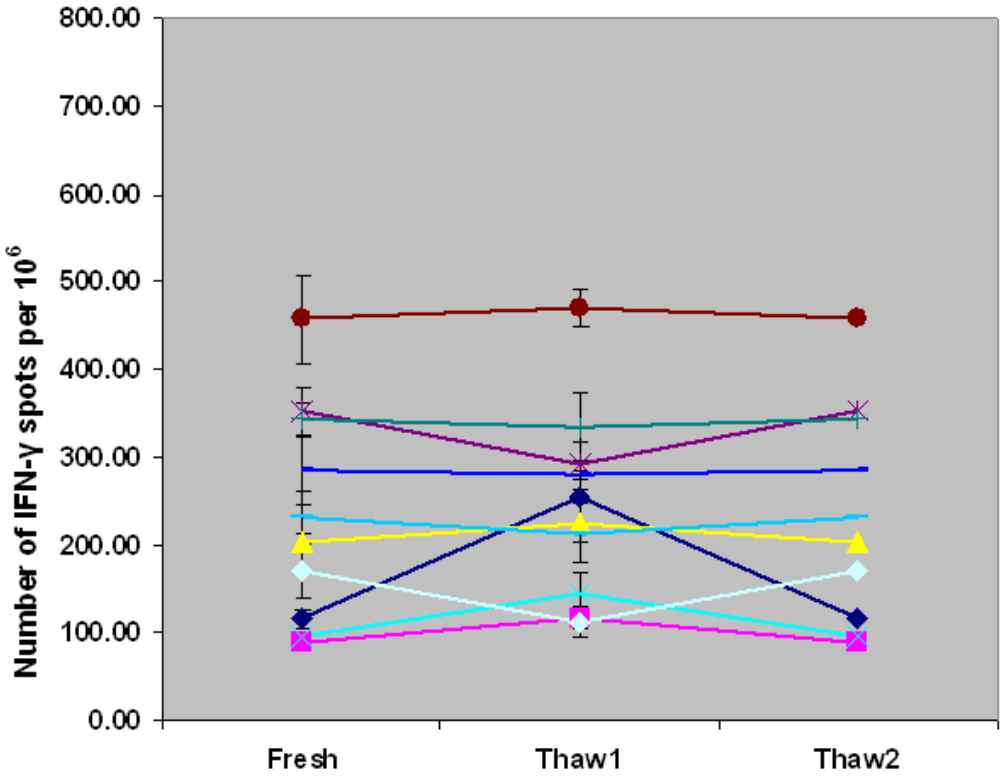
In 2003 CTL scientists introduced protocols for functionally loss-free freezing of PBMC:

Kreher et al. : CD4+ and CD8+ cells in cryopreserved human PBMC maintain full functionality in cytokine ELISPOT assays. J. Immunol. Methods, 278:79-93, 2003

(IFN-γ, IL-2, IL-4 and IL-5 were measured)

1a. Following this protocol, we found that high and low frequency, high and low avidity CD4 and CD8 T cells are preserved, functionally loss free. No “resting” needed after thawing.

Ex vivo/ Ex cryo



NIAID Contr. HHSN266200400098I, ELISPOT Qualification, Vaccinia

1a. The role of temperature:

Blood should be shipped, stored, and processed all times at room temperature, not chilled. For freezing, the cells and the DMSO-containing cryomedium must be at room temperature (not ice-cold) when mixed. When thawing, bring cells rapidly to 37 °C.

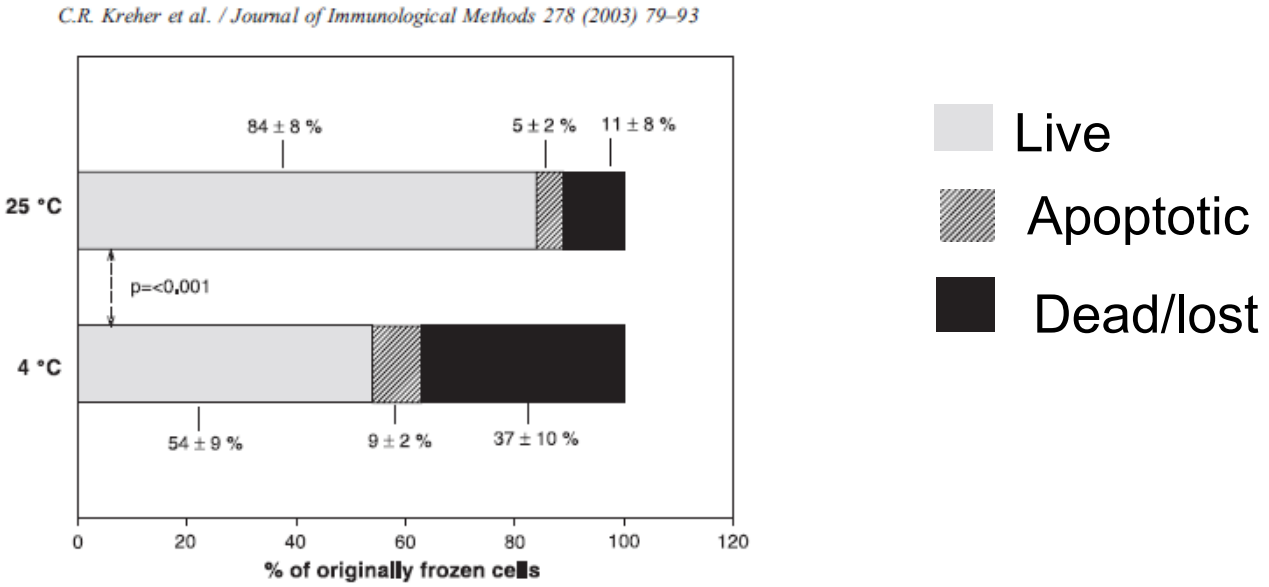


Fig. 1. Increased recovery of cell numbers and functionality when DMSO containing freezing medium is added at room temperature.

Milestone 2 towards standardization:

Reference PBMC

- Since 2005, CTL offers a reference PBMC library, presently consisting of over 70 PBMC donors to chose from, each
  - High resolution HLA-typed (Class I and Class II)
  - Immune-characterized for
    - 20 antigens, recognized by CD8 and CD4 cells
    - IFN-γ, IL-2, IL-4, IL-5, IL-17, Granzyme B
    - High and low frequency T cell responses
    - High and low avidity T cell responses

- Essentially unlimited availability of identical cell material: between 500 and 1,000 aliquots of 10 million PBMC/vial from each donor available from each draw.

- ***Reference PBMC are essential for assay comparisons, qualification / validation, and harmonization across institutions***

Milestone 3 towards standardization:
Serum Free Media

- In 2005, CTL introduced serum free media for all steps of PBMC processing and testing
  - Freezing (CTL-Cryo™)
  - Thawing (CTL-Anti-Aggregate ™)
  - Washing (CTL-Wash ™)
  - Testing (CTL-Test ™)

We have found no serum that performs significantly better, but many sera that perform (much) worse.

Milestone 4 towards standardization:
Objective, automated analysis

- In 2009, CTL introduced the ImmunoSpot® Version 5 Software with which subjective user judgments can be avoided for ELISPOT counting by:
  - automated spot recognition (SmartCount™)
  - Automated gating (SmartGate™)
- Starting Series 6, each reader can perform identically.

Automated, user judgment-independent data analysis sets our new ELISPOT platform way ahead of flow cytometry.

Milestone 5 towards standardization:

ELISPOT assay qualification, validation, and high throughput testing.

- Since 2007, CTL has qualified/validated 19 ELISPOT assays (single and double color, various analytes) for the NIH and major pharmaceutical clients, assays for:

- human
  - mice
  - macaques
  - pigs

- High throughput: CTL's GLP lab can run up to 200 PBMC samples a day.

ELISPOT assays can be highly reproducible within a laboratory that uses highly trained personnel and strictly adheres to SOP,

but,

how reproducible are ELISPOT results among different laboratories without involving such resources (most academic labs)? How much training and infrastructure is needed?

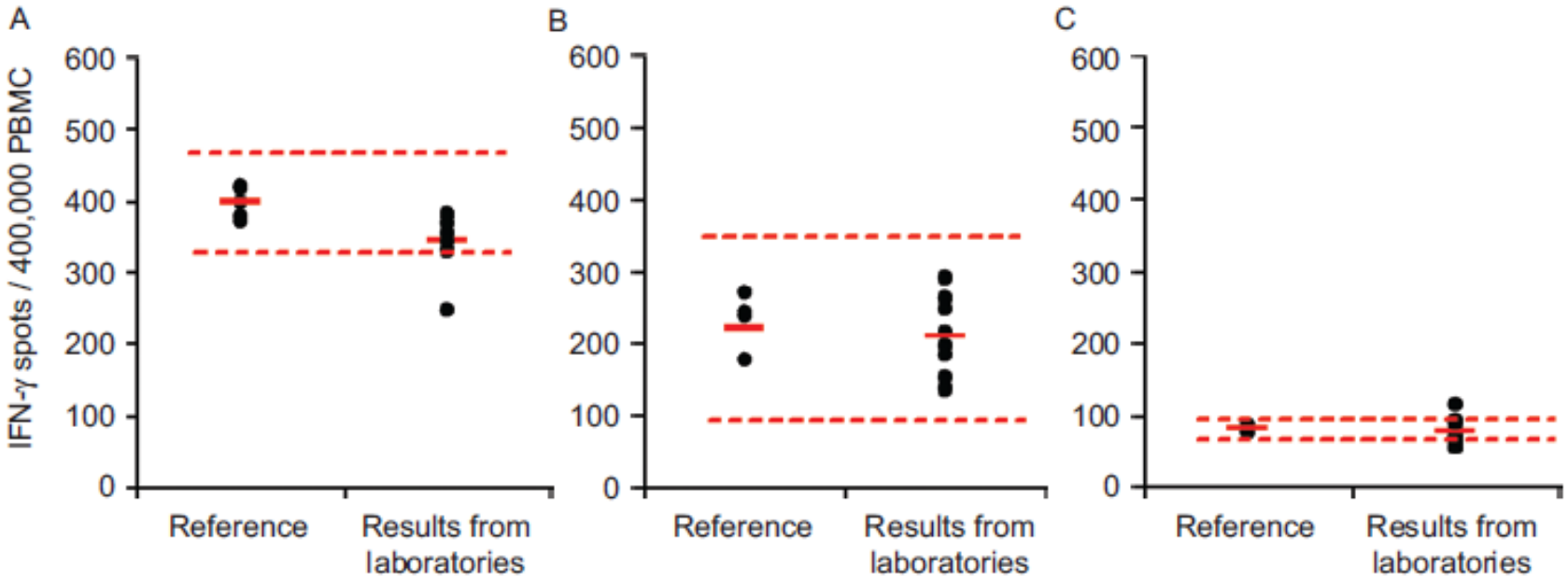
Milestone 6 towards standardization:

Demonstration that a unified platform suffices for obtaining highly reproducible ELISPOT data across institutions – GLP and training are not necessary.

Zhang, et al.: ELISPOT assays provide highly reproducible results among different laboratories for T-cell immune monitoring – even in hands of ELISPOT-inexperienced investigators. J. Immunotoxicology, 2009, 6:227-34

Our claim can be readily reproduced: all reagents, protocols are available!

Inter-lab reproducibility for the 3 Reference PBMC (Zhang et al.)



- 100% of labs detected the response – no false negative or false positive
- Mean of the lab's results was within 3 SD of the reference value
- All measurements were within 42% of each other

Implication 1: essentials for reproducible ELISPOT data

- Not essential:
  - GLP infrastructure
  - Extensive training of personnel
  - Expertise/“green thumb” of investigator

    (Good news: those are major hurdles and many times unaffordable for the majority of the scientific community)

- Essential: same reagents, protocols
  - PBMC (thawing protocols, cell counting)
  - Media (for thawing and testing)
  - Antigen, concentration
  - Test kit (with test protocol)
  - Analysis instrument/software

    (Good news: all the above are easily accessible “materials and methods” that can be readily shared among institutions.

Implication 2: reference standards can be generated for T cell monitoring (and are already available from CTL)

Reference standards are “yard sticks” that are essential for any measurement/comparison. Without a reference standard

- how can it be tested whether a change in protocol is indeed an innovation that improves assay performance if there is nothing to compare it to?
- how can one compare results generated in different experiments or labs without introducing standardized, precise means of comparison?
- how can proficiency of a lab/investigator be established?

Questions?