Immunological Monitoring of Cancer Vaccine Clinical Trials:

Why do we need a centralized lab?

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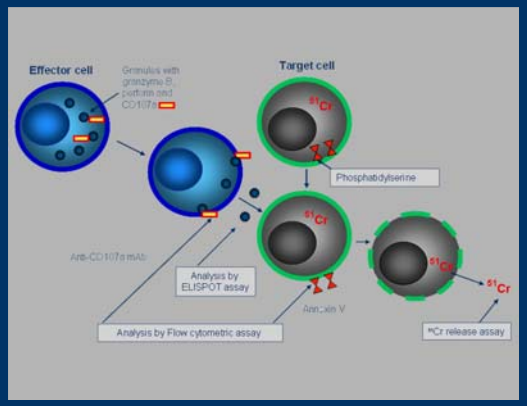

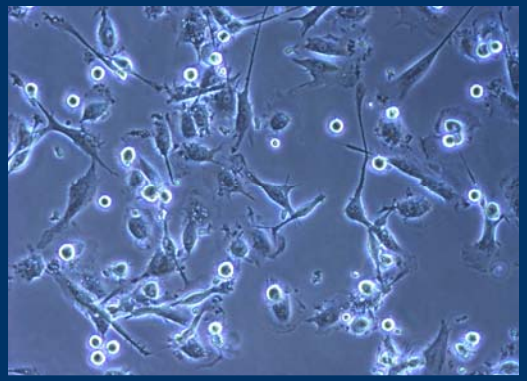

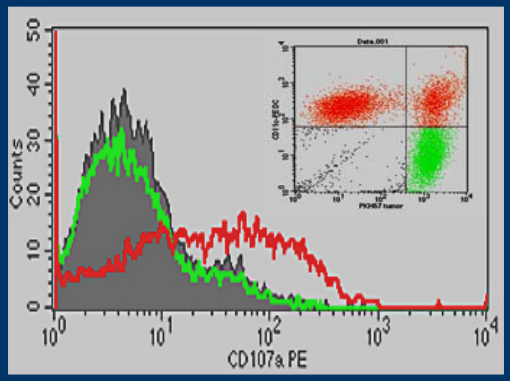

The Laboratory of Cell Mediated Immunity (LCMI) is part of the Applied & Developmental Research Support Program, SAIC-Frederick, Inc., located on the NCI-Frederick campus, Frederick, MD

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Clinical Monitoring

- Sample login and processing
- Cell Culture
- Magnetic bead cell enrichment
- EBV Transformation
- Nucleic acid extraction and quantification

Flow Cytometry

- Immunophenotyping
- Cell Cycle Analysis
- Bioassays – antibody binding and titration assays
- Viability and apoptosis

Lymphokine Testing

- ELISA assays for cytokines, soluble receptors
- Multiplex cytokine assays

Cytokine bioassays
Proliferation assay and recertification testing

Clinical monitoring

- Peptide ELISPOT assay
- Whole protein ELISPOT assay
- Tumor ELISPOT assay
- Granzyme B ELISPOT assay
- Cytokine induction assay
- Proliferation assay
- CTL induction and cytotoxicity assay

Basic research support

- Murine IFN-γ, IL-2, IL-5, IL-17, CM-CSF, MCP-1 and Granzyme B ELISPOT assays
- Non-human primates IFN-γ ELISPOT

Assay development, optimization and validation

To date, seven immunological assays have been approved for clinical use by the NCI Vaccine Working Group Steering Committee (Dr. Jay Berzofsky, Chair):

Peptide ELISPOT assay (*reactivity to HLA.A2-binding 9-10 mer peptides*)

Whole protein ELISPOT assay *(reactivity to whole proteins or peptides >10 mer with or without DC)*

Tumor cell ELISPOT assay

Granzyme B ELISPOT assay

Cytokine induction assay

Proliferation assay

Cytotoxic T-lymphocyte (CTL) induction and ^{51}Cr release assay

All assays are available to NIH researchers and can be optimized for individual research needs.

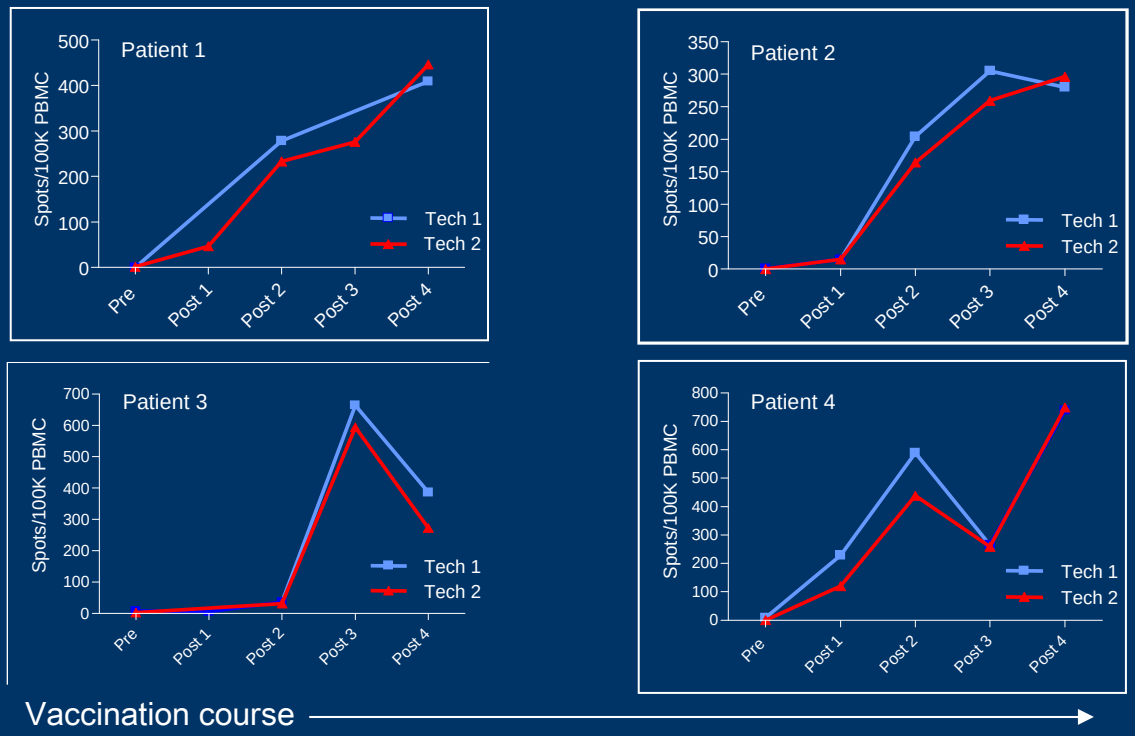
In addition to immunoassays for human samples, the LCMI has also adapted and optimized:

IFN-γ, IL-2, IL-5, IL-17, GM-CSF, MCP-1 and Granzyme B ELISPOT assays and Flow Cytometric Cytotoxicity assay for murine studies

IFN-γ ELISPOT assay for non-human primates

LCMI is CLIA Certified

Clinical applications of IFN-γ Peptide ELISPOT assay. Assay reproducibility

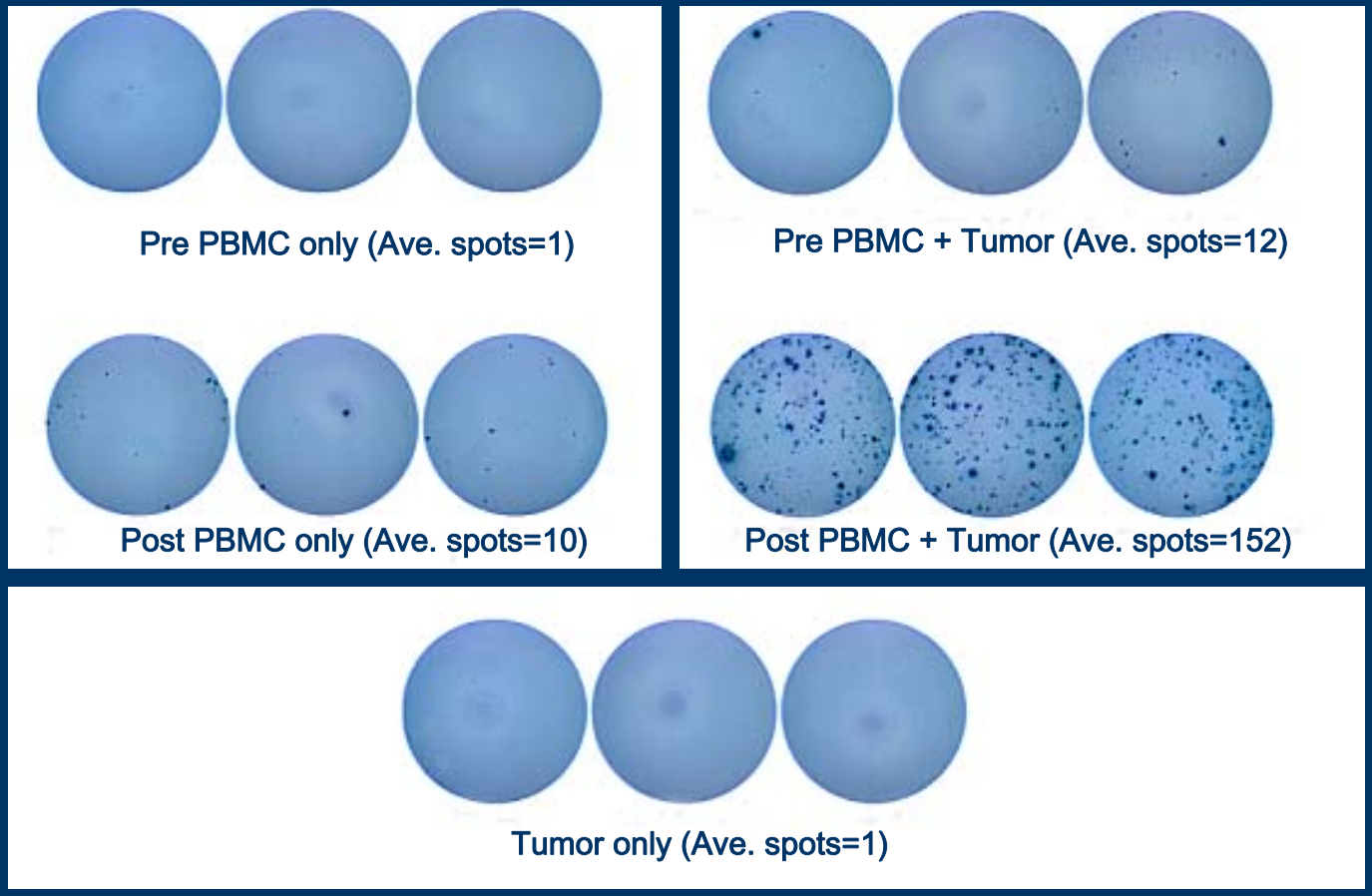


Dr. Steven Rosenberg's (NCI) melanoma patients vaccinated with gp100:209M. Blinded PBMC samples were tested by two technicians on different dates. Response to gp100:209 shown in four representative patients.

Autologous Tumor IFN-γ ELISPOT

IFN-γ secretion in ELISPOT assay by PBMC of follicular lymphoma patients vaccinated with Id, stimulated in vitro with CD40L activated autologous follicular lymphoma cells

Malyguine et al., J Transl Med. 2004 Mar 29;2(1):9.



Pre- and post-vaccine PBMC (1 $\times 10^5$ cells/well) were co-cultured with patient specific tumor cells (2×10^5 cells/well) in the IFN-γ ELISPOT assay for 48 hr. Tumor cells were activated with sCD40L and IL-4 for 3 days prior to experimental setup. Wells containing PBMC only and tumor cells only served as a control. Samples were tested blinded. Images are from the plate scan generated by the CTL Analyzer and are a representative experiment from *nine* with similar results. Comparable results were observed with mantle cell lymphoma patients. *Dr. Larry Kwak patients.*

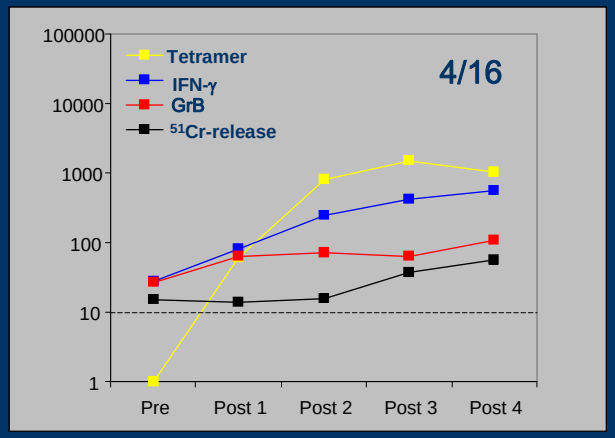
Granzyme B ELISPOT assay

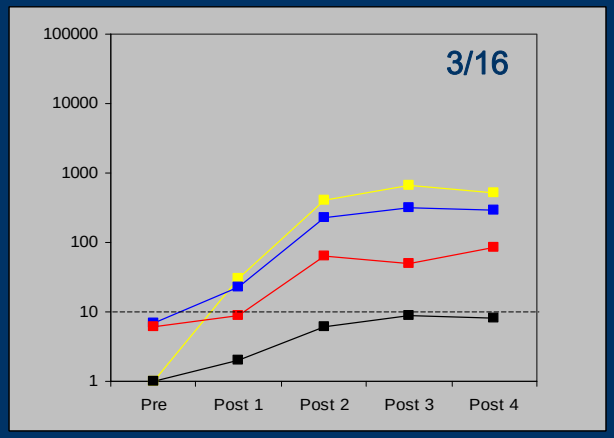
GrB is the most abundant granzyme present in cytolytic granules of CTL and natural killer (NK) cells, and is a key mediator of target cell death. Therefore, the release of GrB may be used to measure NK and CTL cytotoxicity. Our findings demonstrate that the GrB ELISPOT assay is a superior alternative to the ^{51}Cr-release assay. Moreover, unlike the IFN-γ ELISPOT assay which is a surrogate marker of killing, the GrB ELISPOT directly measures cytotoxic cell activity.

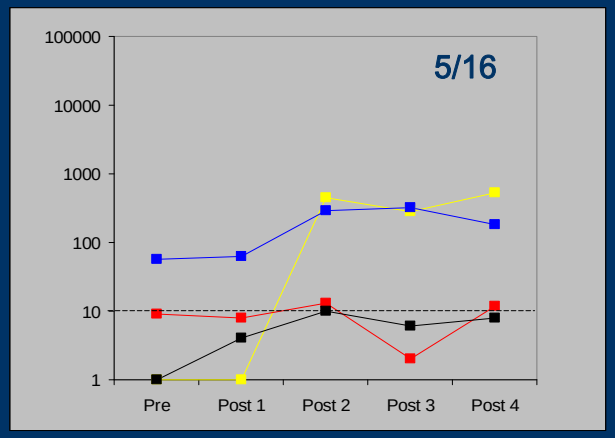
Granzyme B and IFN-γ release in the ELISPOT assays by PBMC from vaccinated melanoma patients, cytotoxicity in the ^{51}Cr release assay and tetramer data. Collaboration with Dr. S. Rosenberg, NCI.

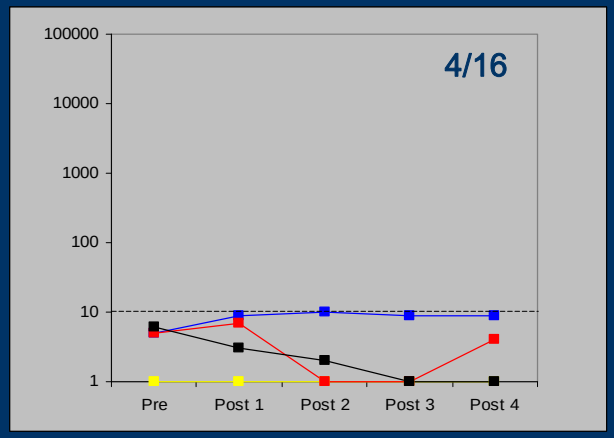
Shafer-Weaver KA et al, *J.Immunother* 29(3):328-335, 2006

All melanoma patients were HLA-A*0201 positive, and received peptide immunization (gp100:209-217 [210M], in the Surgery Branch, NCI. PBMC utilized in this study were obtained before and at three weeks after each vaccination. PBMC were tested against C1R.A2 antigen-presenting cells pulsed with g280 (gp100:280-288 peptide, control) or g209 (gp100:209-217 peptide). Tetramer data are from Surgery Branch and shown as the number of tetramer positive cells per 10^4 CD8^{bright} T-cell. IFN-γ and GrB ELISPOT values are average number of IFN-γ or GrB secreting cells per 10^5 effector cells. The ^{51}Cr-release assay data are presented as percent specific lysis at E:T ratio of 50:1. The data presented are a representative patient for each pattern of response category.



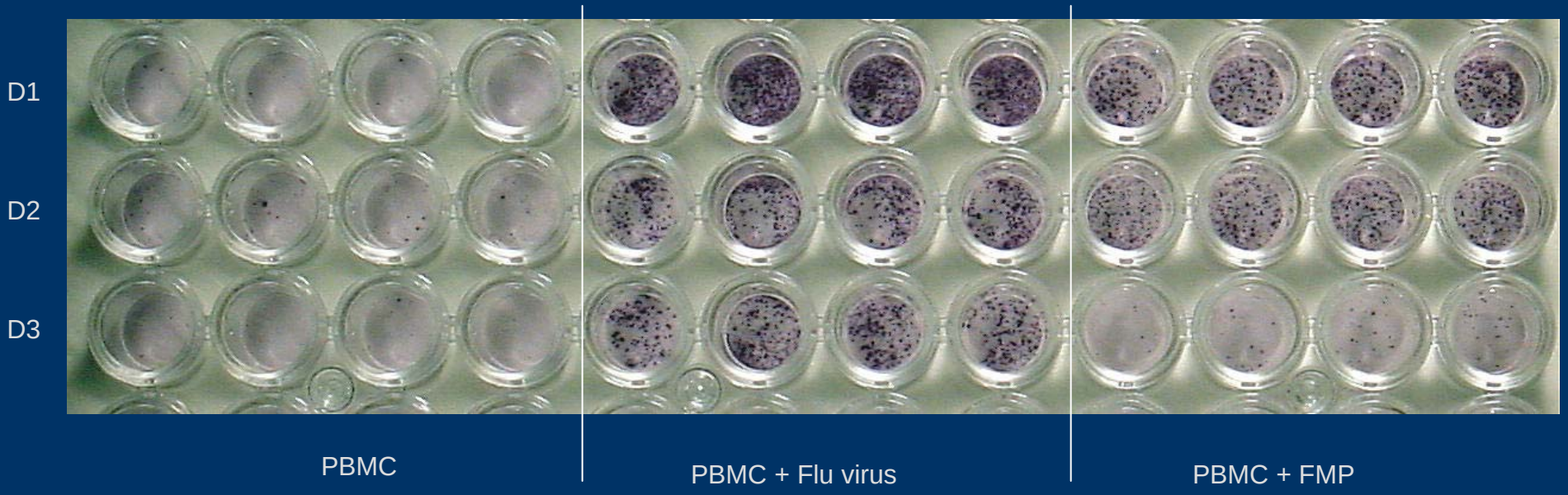






The Phi Coefficient demonstrated that the IFN-γ and tetramer assays perfectly correlated (Phi = 1.00, $p < 0.0001$). The GrB ELISPOT assay was significantly associated with all three of the other assays (p values of 0.015, 0.015, and 0.0059 with tetramer, IFN-γ ELISPOT, and ^{51}Cr-release assays, respectively). The Phi Coefficient was 0.595 for GrB with the tetramer and IFN-γ ELISPOT assays ($p = 0.015$, respectively) and 0.655 ($p = 0.0059$) with the ^{51}Cr-release assay. ^{51}Cr-release assay significantly correlated only with the GrB ELISPOT ($p = 0.0059$).

Whole protein IFN-γ ELISPOT assay



Flu virus* and FMP response. 24 hr incubation. $3x10^5$ PBMC/well. Representative experiment

*Influenza A Hong Kong/H3N2/8/68 virus

DC-based ELISPOT assays

PBMC

EFFECTORS (PBMC)

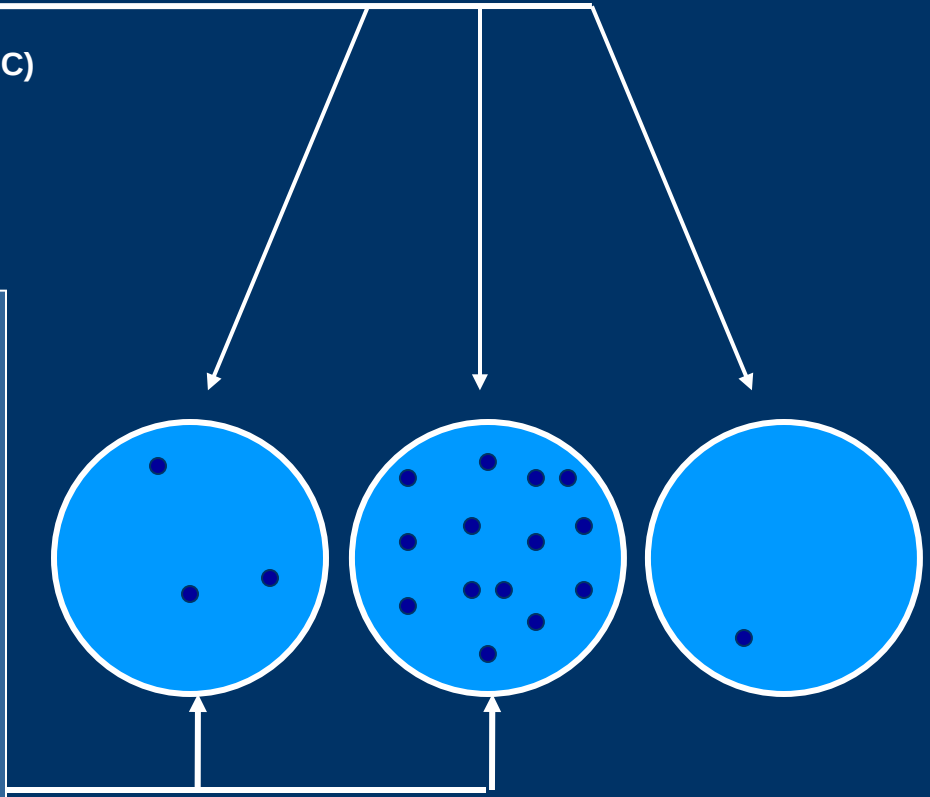
- PBMCs were thawed, rested, washed and placed into dendritic cell media on plastic (DCM, RPMI with 10% FBS & supplements) for 2 hours at 37^0C.

- After 2 hours, the suspended cells were removed by washing with PBS.

- DCM containing 100 IU/ml GM-CSF and 50 ng/ml IL4 were added to the adhered cells and cultured for 7 days.

- Cells were fed at day 3 or 4 with ½ volume of fresh media containing GM-CSF & IL4.

- On Day 7, cells were harvested and pulsed with appropriate antigen before plating into the assay.

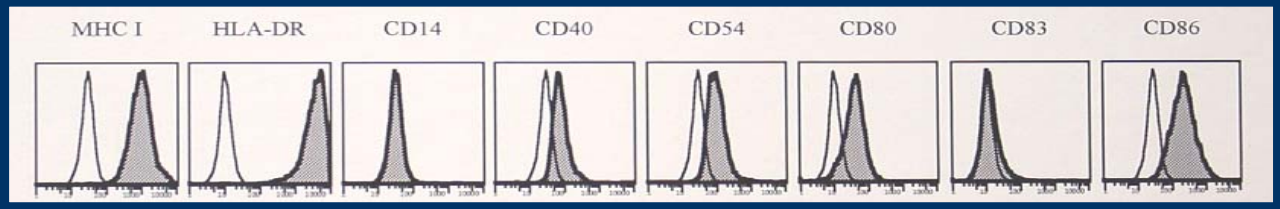


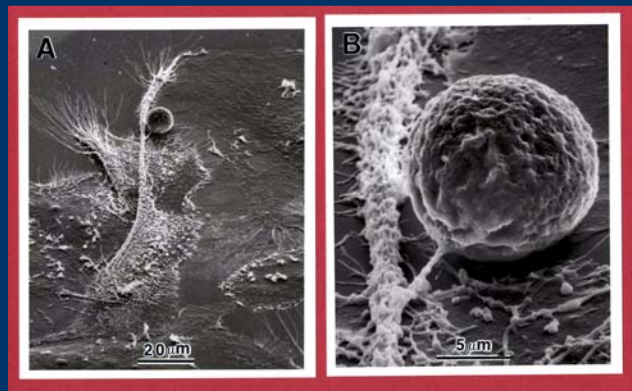
Non-pulsed APC (DC)

Pulsed APC (DC)

No APC

Phenotype of immature dendritic cells used in DC-ELISPOT assay





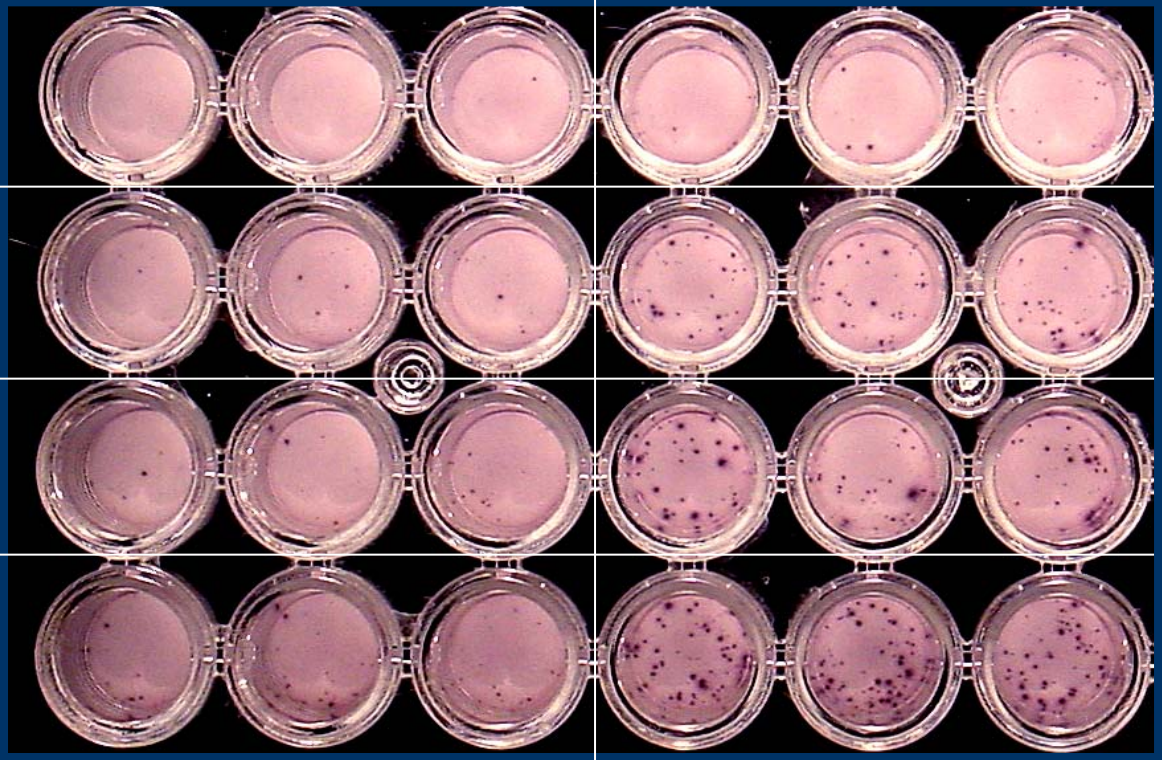
Effect of anti-CD28 mAb and DC on IFN-γ production by PMBC from KLH immunized patient in the ELISPOT assay

PBMC 7

PBMC+ αCD28 20±6

PBMC+ DC 15±3

PBMC + DC+ αCD28 34±9



PBMC 15±5

PBMC+ αCD28 47±2

PBMC+ DC 42±5

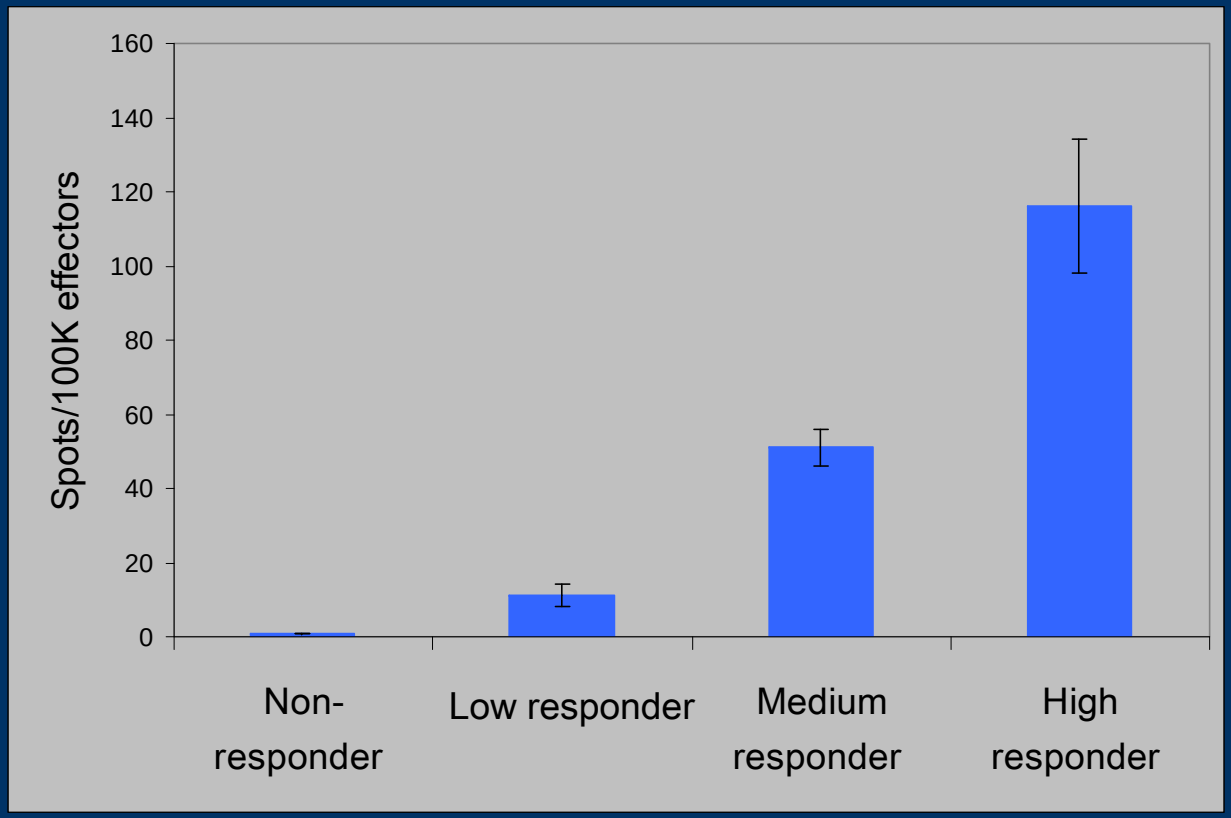
PBMC+ DC + αCD28 84±2

No KLH **+ KLH**

24 hr incubation. $3x10^5$ PBMC/well. PVDF membranes. Number of spots per well ± SD shown. One representative experiment.

Dr. Larry Kwak patients

CLIA Competency testing



PBMC from donors with different levels of response to CMV peptide were tested blinded on different dates by different technicians in peptide IFN-γ ELISPOT assay. Averages ± SD from 4-9 assays shown. Representative group of donors.

Infrastructure support

1. CLIA certification
    a. Daily instrument and laboratory monitoring/quality control
    b. Daily Z1 quality control
    c. Weekly Z1 cleaning
    d. Monthly Z1 maintenance
    e. Proficiency/competency testing for CLIA ES assay(2 validated ES per year)
    f. Instrument logging and trouble shooting
    h. CLIA certification for proliferation assay
        i. compiling and analysis of normal donors
        ii. Statistical consultation for setting normal parameters
        iii. Literature/information searches for setting standards for proliferation assays.
        iv. Setting up a standard order for monthly delivery of fresh 3H-thyminde
        v. Creating and modifying an SOP to CLIA format/standards

2. Assay reagent quality control (side-by side lot testing)
    a. Human AB serum screen – proliferation, IFN-γ ES, IL-5 ES, GrB ES, Cytokine production
    b. FBS serum screen – DC cultures, IFN-γ ES, IL-5 ES, GrB ES, Cr-release
    c. ELISPOT plate lots testing /trouble shooting
    d. Antigen/mitogen testing – use in ES, proliferation assays,
    e. DC reagents – cytokines and maturation reagents

3. Screening and monitoring normal donors to use as assay controls
    a. Flow cytometry for HLA typing
    b. Initial testing in ES and proliferation assays
    c. Compiling and analysis of repeated measures for individual donors for setting quality control parameters
    d. Cost for normal blood draws, isolation of PBMC, and freezing/storage of cells.
    e. Inventory control of normal donors

4. General laboratory maintenance
   a. Changing of LN2 tank and ordering new tanks – 1 tank/10days
   b. Ordering of reagents
   c. Reagent inventory
   d. Incubators/water baths (water levels)
   e. Logging and filing of paperwork
   f. General laboratory clean up
   g. Generation/updating laboratory SOP
   h. Preparation of IBC protocols for new antigens/stimulants

5. Customer service (non-yellow task)
   a. Addressing emails for potential yellow task requests
   b. Phone conversations for potential yellow task requests
   c. Pricing of potential yellow tasks
   d. Designing assays for potential yellow tasks
   e. Providing expertise to potential yellow task customers or the greater NIH community, ie, 2 meetings with two scientists from Ira Pastan lab to help them get their ELISPOT up & running

6. Research and development to enhance customer service
   a. Literature searches for new reagents and new or improved assays
   b. Obtaining free samples/reagents from vendors
   c. Testing new antigens in biological assays
   d. Optimizing or improving current assays
   e. Development and testing of new biological assays.

7. Training
   a. Yearly biosafety training
   b. Yearly radiation safety training
   c. Yearly ethics training
   d. Yearly time charging training
   e. Yearly SAIC corporate training
   f. New software/software up grades (e.g. CTL analyzer software, flow cytometry software)
   g. Monthly CSP seminars
   h. Seminars, meetings, lectures, research festivals
   i. Posters and article preparation

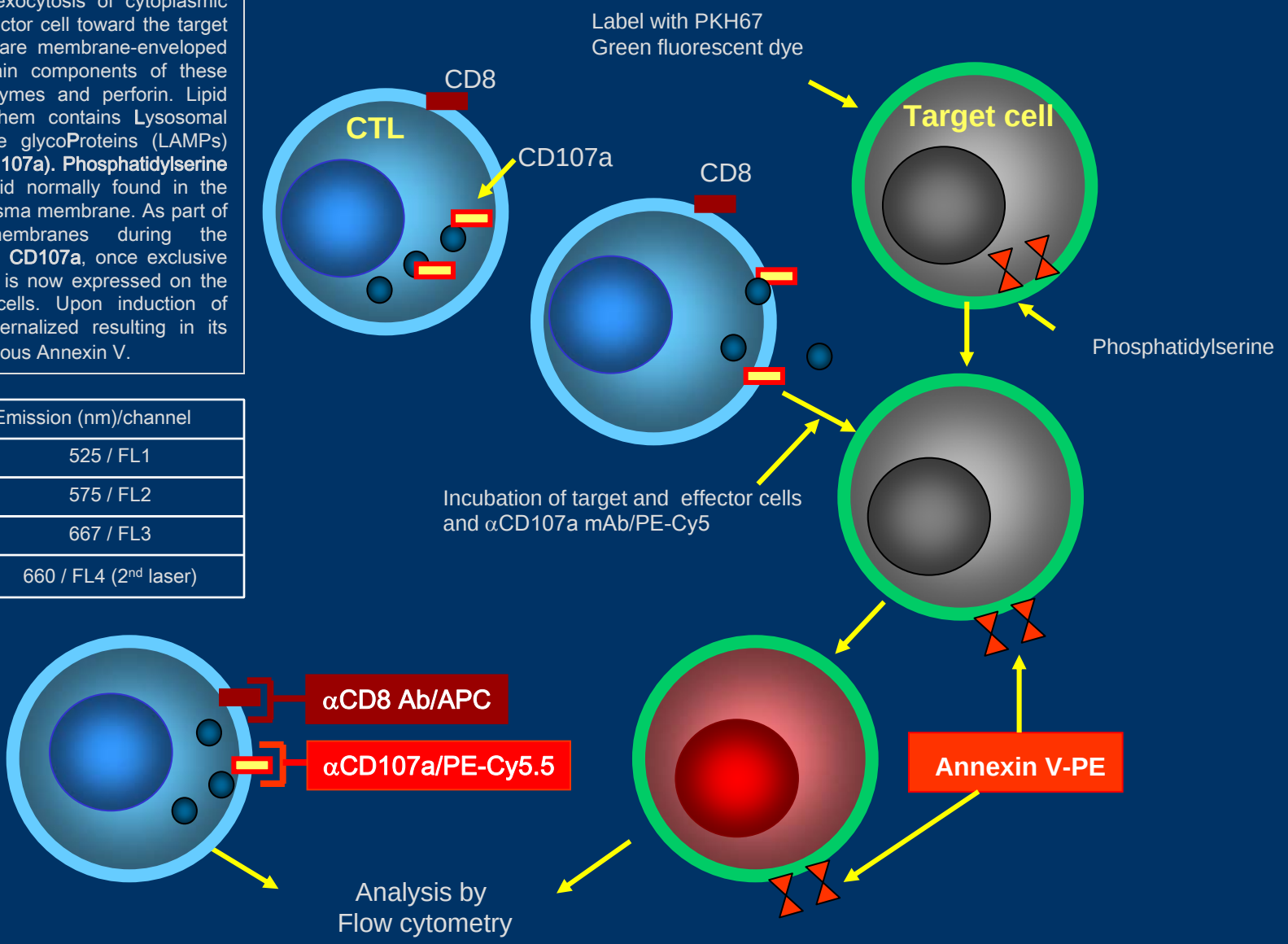
8. Administration
   a. Preparation of semi-annual and annual reports
   b. Preparation of budgets and monthly reviews
   c. Updating M&S costs by reagent & vendor
   d. Yearly reviews (2-3 hours/person for 4-5 people)

Flow Cytometric Cytotoxicity Assay

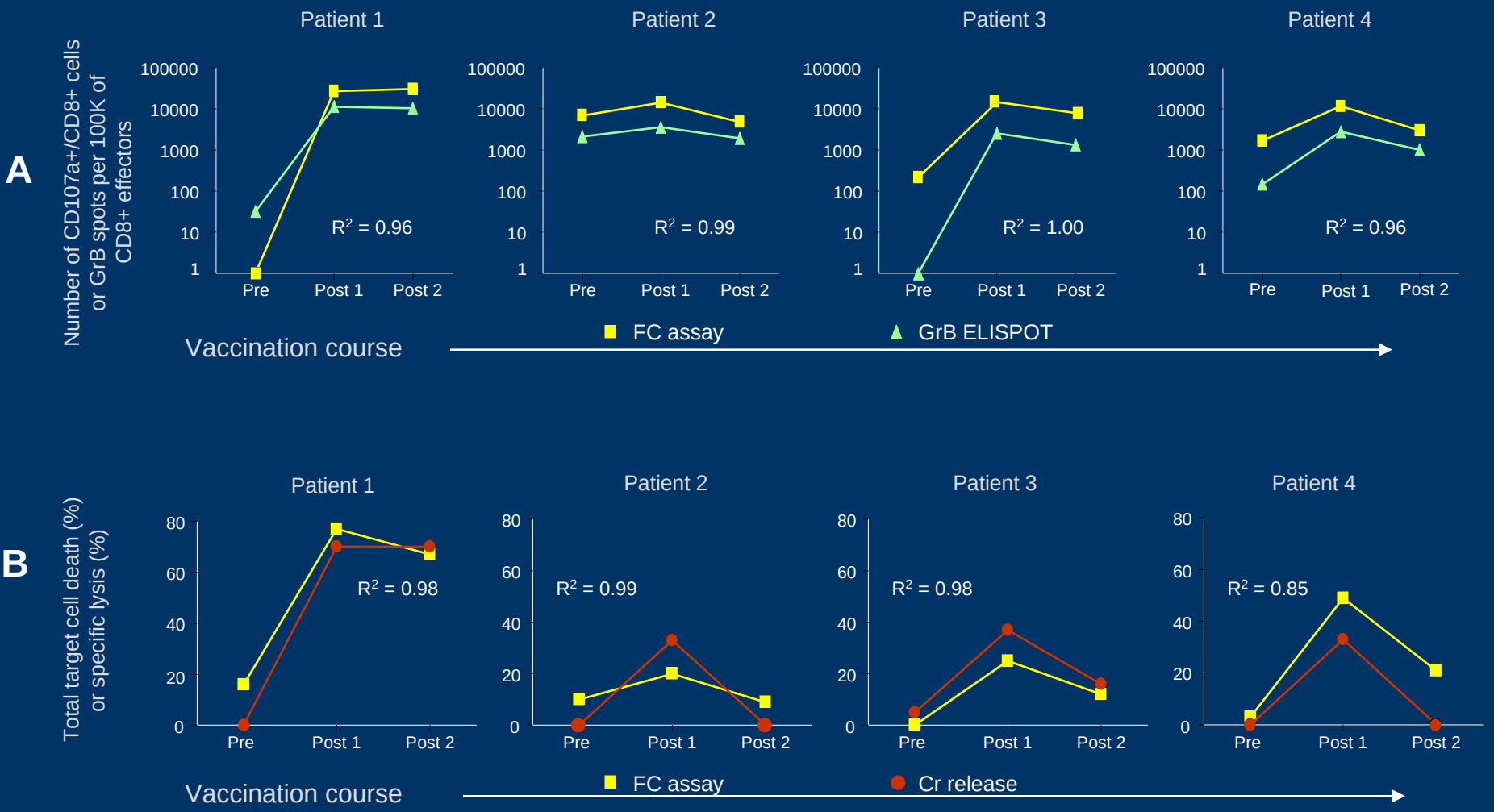
Zaritskaya L et al., J Immunotherapy 32(2): 186-194, 2009.

One of the major mechanisms of cell-mediated cytotoxicity involves exocytosis of cytoplasmic granules from the effector cell toward the target cell. These granules are membrane-enveloped lysosomes. The main components of these lysosomes are granzymes and perforin. Lipid bilayer surrounding them contains **L**ysosomal **A**ssociated **M**embrane glyco**P**roteins (LAMPs) including LAMP-1 (**CD107a**). **Phosphatidylserine (PS)** is a phospholipid normally found in the inner leaflet of the plasma membrane. As part of the fusion of membranes during the degranulation process **CD107a**, once exclusive to granule membrane is now expressed on the surface of effector cells. Upon induction of apoptosis, **PS** is externalized resulting in its accessibility to exogenous Annexin V.

Fluorochrome	Emission (nm)/channel
PKH 67	525 / FL1
R-PE	575 / FL2
PE-Cy5	667 / FL3
APC	660 / FL4 (2nd laser)



Correlation between results obtained in the FC assay and the GrB ELISPOT or standard ^{51}Cr-release assay using patient CTL



PBMC were obtained before (pre) and at three weeks after each vaccination (post 1 and post 2). The g209-specific CTL were generated as described above. CTLs were tested for activity against g209 peptide-pulsed C1R.A2 target cells in the FC assay, Granzyme B (GrB) ELISPOT, and standard ^{51}Cr-release assays.

A, degranulation (CD107a expression) of CD8+ effector cells measured in the FC assay compared to GrB secretion measured in the ELISPOT assay. Data is presented as either the number of CD8+ cells expressing CD107a (FC assay - triangles) or GrB spots per 1×10^5 CD8+ effector cells (ELISPOT - squares) incubated with g209-pulsed C1R.A2. Data for non-pulsed C1R.A2 was subtracted as background.

B, induction of target cell death (Annexin V binding) measured in the FC assay compared to specific lysis of g209-pulsed minus non-pulsed C1R.A2 in the ^{51}Cr release assay. Data is expressed as either the percentage of target cell death (squares) or the percentage of specific lysis at what E:T ratio (triangles). Correlation between the results from the assays was calculated using Pearson Correlation Coefficient (R2). Data for 4 representative patients of 7 tested is shown.

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