

Immunologic Monitoring

or

The many joys of immunologic assessments
in clinical trial patients

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Presenter Disclosure Information

Lisa H. Butterfield, Ph.D.

The following relationships exist related to this presentation:

<No Relationships to Disclose>

Table I. *Summary of some known immunological differences between mouse and human*
Of Mice and Not Men: Differences between Mouse and Human Immunology, Mestas and Hughes, JI 2004, 172: 2731

	Mouse	Human		
Hemotopoiesis in spleen	Active into adulthood	Ends before birth		
B7-H3 effects on T cells	Inhibits activation	Promotes activation		
Neutrophils in periph. blood	10–25%	50–70%		
Lymphocytes in periph. blood	75–90%	30–50%		
MUC1 on T cells	Absent	Present		
TLR2 expression on PBL	Low (induced on many cells including T cells)	Constitutive (but not on T cells)		
TLR3	Expressed on DC, Mac. Induced by LPS	Expressed by DC. No LPS induction		
TLR9	On all myeloid cells, plasmacytoid DC and B cells	Expressed only on B cells, plasmacytoid DC		
TLR10	Pseudogene	Widely expressed		
ICAM3	Absent	Present		
P-selectin promoter	Activated by TNF and LPS	Unresponsive to inflammation		
Leukocyte defensins	Absent	Present		
GlyCAM	Present	Absent		
MHC II expression on T cells	Absent	Present		
Macrophage NO	Induced by IFN- and LPS	Induced by IFN- / , IL-4⁺ anti-CD23		
CD4 on macrophages	Absent	Present		
Effect of IL-7R deficiency	Blocks T and B cell development	Only blocks T cell development		
ZAP70 deficiency	No CD4 ⁺ or CD8 ⁺ T cells	No CD8⁺ T but many nonfunctional CD4⁺		
CD1 genes	CD1d	CD1a,b,c,d		
NK inhibitory Rs for MHC 1	Ly49 family (except Ly49D and H)	KIR		
NKG2D ligands	H-60, Rae1	MIC A, MIC B, ULBP		
Th expression of IL-10	Th2	Th1 and Th2		
Fc RI	Absent	Present		
Fc RIIA, C	Absent	Present		
Ig classes	IgA, IgD, IgE, IgG1, IgG2a*, IgG2b, IgG3, IgM * absent in C57BL/6, /10, SJL and NOD mice, which have IgG2c	IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, IgM		

Question: what immune readout correlates best with disease prognosis and clinical outcome?

Goal: Identification of patients who should be enrolled in trials, who is going to respond

Issues of Variability

Real life example of MART-1 peptide-pulsed DC trial

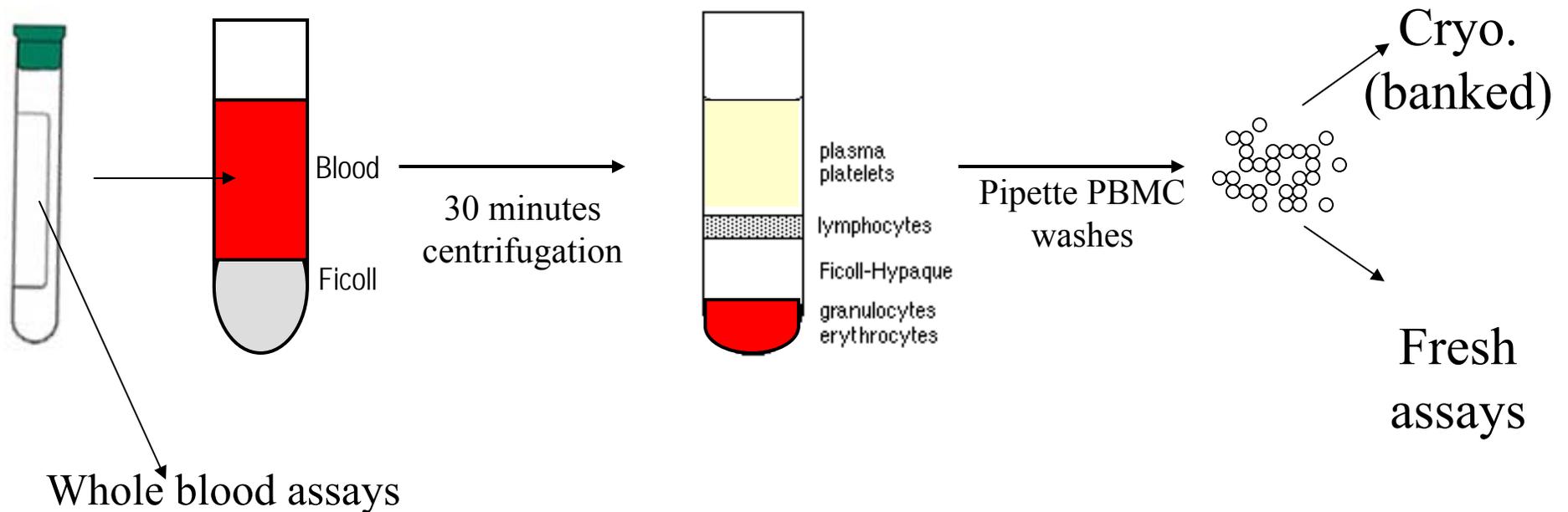
Survey of different assays, successes and limitations

Clinical trial analysis design issues

Standardization and Validation

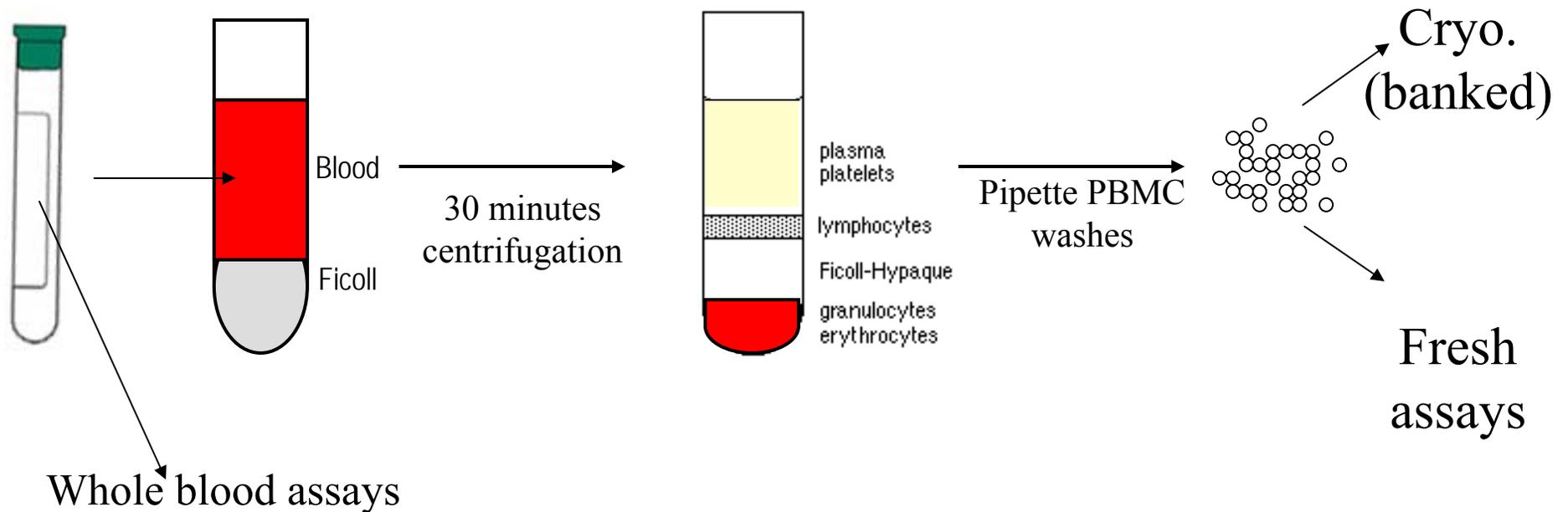
Peripheral Blood: easy to obtain at multiple time points

Separation of blood components on a Ficoll-Hypaque gradient.



Limitations: for solid tumors, may not reflect what occurs at the tumor, and for leukemia/lymphoma, may be all tumor/blasts.

Peripheral Blood



Variability: *Hemolysed? Anti-coagulant in tube (heparin/EDTA)?
Time/temperature since blood draw? Volume in tube?*

Healthy Donor Absolute Counts and Percentages

CD3 % positive	CD3 cells/μL	CD3/CD4 % positive	CD3/CD4 cells/μL	CD3/CD8 % positive	CD3/CD8 cells/μL	CD3 % positive	CD3 cells/μL	CD16+CD56 % positive	CD16+CD56 cells/μL	CD19 % positive	CD19 cells/μL
90	1918	63	1345	26	562	90	2088	6	104	4	100
81	1077	49	644	29	388	82	1081	6	76	12	163
71	1505	62	1311	9	192	70	1542	9	206	20	446
86	2324	59	1576	27	729	85	2512	5	143	10	288
82	1442	50	887	27	478	82	1428	9	151	9	159
79	1301	50	824	31	511	80	1291	9	143	11	181
77	1307	45	772	31	527	77	1309	7	114	16	269
81	986	58	706	23	279	81	992	6	68	14	165
90	2178	67	1618	23	548	90	2189	3	76	7	171
78	2027	45	1175	29	766	77	1886	5	114	18	438
76	1155	63	965	13	193	78	1183	8	127	14	214
77	1372	48	842	30	527	76	1400	10	180	14	251
75	1017	56	763	16	211	76	1065	9	120	15	216
83	1916	62	1420	20	458	83	1929	4	98	13	301
67	1498	47	1049	21	457	67	1520	16	373	16	372
83	2143	62	1609	19	501	82	2257	7	198	11	301
82	1300	39	624	43	687	82	1407	11	183	8	129
76	1222	54	865	21	337	77	1290	11	185	12	200
73	1097	54	808	17	264	74	1174	16	248	11	168
86	1784	63	1321	21	432	85	1814	4	90	11	236

CD3+: 986-2,512 cells/ul (2.5x)

CD3+/CD4+: 39-67% (1.7x)

CD3+/CD8+: 9-43% (4.7x)

CD19: 4-20% (5.0x)

CD16+/CD56+: 3-16% (5.3x)

Cancer Patients: Cellular Product Production

DC production from leukapheresis

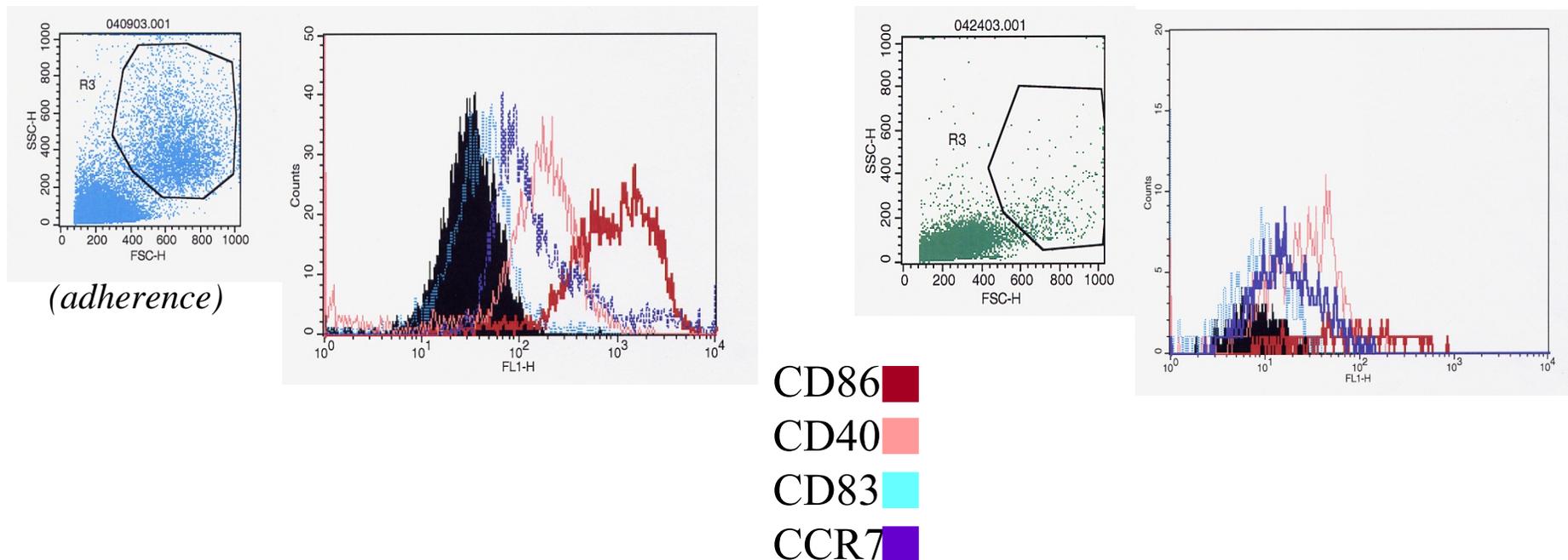
CPL: 03-156	Pheresis	Initial Cell Count	Initial Monocytes Count (%)	Total Recovered Cells from Elutra	Elutriated Cells (Monocytes) Total (F4 or F5)	CD14+	# Aastrom Inoculated	Recovery (%)
08-149-000001	142ml	1.6x10 ¹⁰	24.7	7.54x10 ⁹	4.4x10 ⁹	94%	2.55x10 ⁹	22%
07-339-000004	144ml	7.0x10 ⁹	14.0	2.64x10 ⁹	1.76x10 ⁹	75%	1.76x10 ⁹	35%
08-086-000010	164ml	1.9x10 ¹⁰	12.2	1.19x10 ¹⁰	4.52x10 ⁹	95%	2.55x10 ⁹	45%
CPL: 05-063							# Plated/ in Flasks	Recovery (%)
08-115-000014	78ml	3.4x10 ⁹	25.0	2.1x10 ⁹	1.8x10 ⁹	97%	3.90x10 ⁸	22%
08-338-000015	80ml	4.12x10 ⁹	20.4	8.7x10 ⁸	4.7x10 ⁸	86%	3.9x10 ⁸	43%
08-094-000019	70ml	5.48x10 ⁹	17.0	2.1x10 ⁹	1.14x10 ⁹	90%	1.32x10 ⁸	2%

Long or short leukapheresis?

%Monocytes: 12-25%; %CD14 from elutriated “monocyte fraction”: 75-97%

%DC from monos.: 2-45% ; *more variation*: monocytes from plastic adherence

Patient Autologous DC Vaccine Cells



Example from an immunotherapy vaccine study. Some patients were able to expand large numbers of DC bearing cell surface markers CD40, CD83, CD86 and CCR7, but not all. **These 2 patients did not receive the same vaccine.**

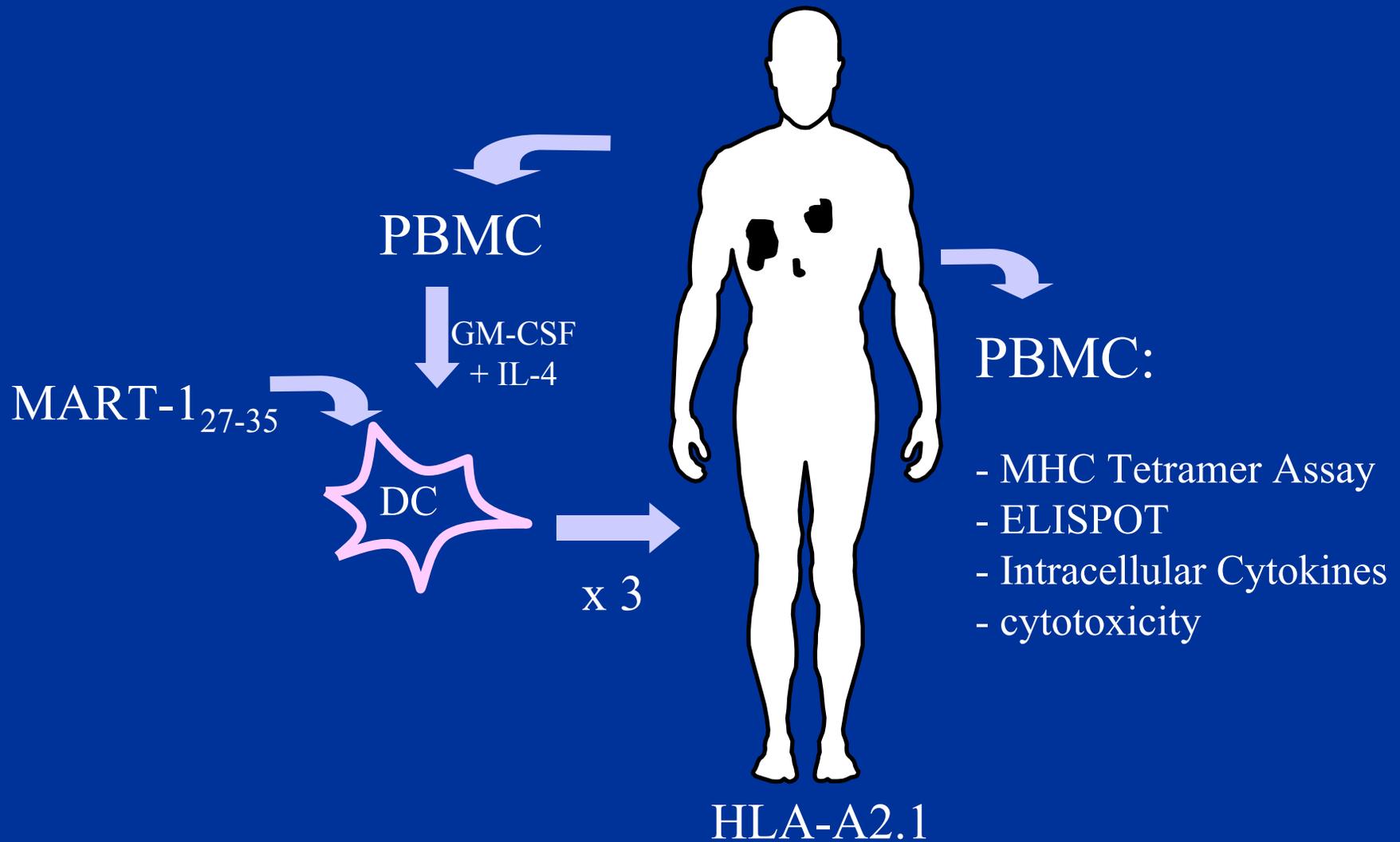
Important data in dot plots and histograms often not presented in published papers

Butterfield, CCR 2006

Real life (historical) example:

Immunotherapy Clinical Trial Monitoring
(circa 1999-2000)

MART-1₂₇₋₃₅ Peptide-pulsed DC Clinical Trial Design



CLINICAL TRIAL DESIGN

DESIGN: Phase I/II dose-escalation clinical trial

TREATMENT GROUPS:	<u>Group</u>	<u>No.</u>	<u>DC Dose</u>	<u>Route</u>
	A	3	10^5	i.d.
	B	3	10^5	i.v.
	C	3	10^6	i.d.
	D	3	10^6	i.v.
	E	3	10^7	i.d.
	F	3	10^7	i.v.
	G	12	$>10^7$	i.d.

TREATMENT SCHEDULE

<u>Day:</u>	<u>Events:</u>
-14 to -7	Leukapheresis for PBMC and serum collection
0	MART-1 peptide pulsed DC vaccination (1st)
14	Blood draw for immunological tests MART-1 peptide pulsed DC vaccination (2nd)
28	Blood draw for immunological tests MART-1 peptide pulsed DC vaccination (3rd)
35	Blood draw for immunological tests (wk 5)
56	Blood draw for immunological tests (wk 8)
112	Blood draw for immunological tests (wk 16)

Clinical Outcome

- 10 Patients Measurable Disease:
 - Complete Response 1 (i.d. 10^7 DC)
 - Disease Stabilization 2 (i.d. 10^5 and i.d. 10^6 DC)
 - Progression 7

Clinical Immunology Assays

1. MHC Tetramer
2. IFN γ ELISPOT Cytokine Frequency
3. Intracellular Cytokine Staining (IFN γ /IL-4)
4. Cytotoxic T Lymphocyte Assay

Immunological Assay Summary

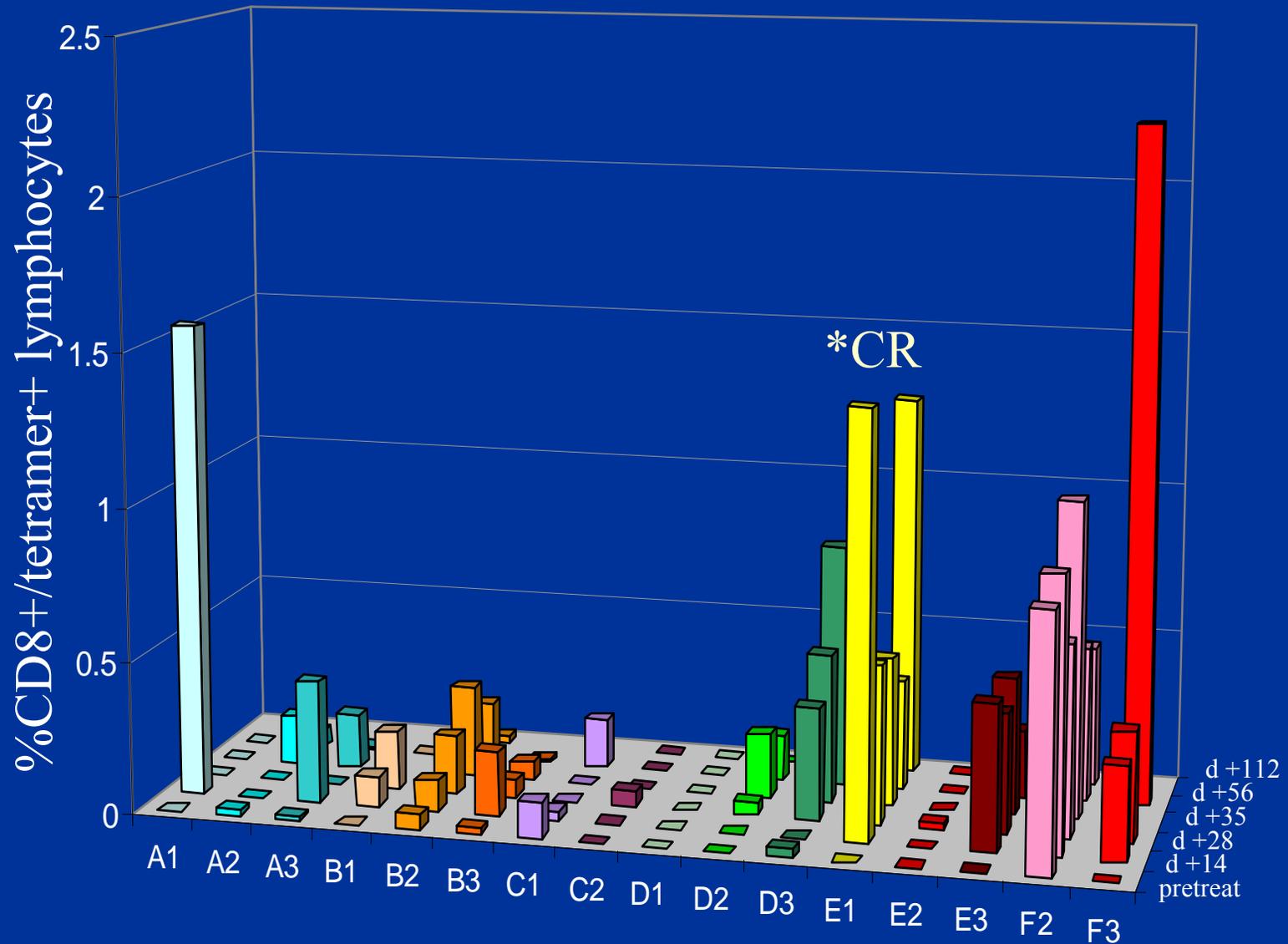
MHC Tetramer: Flow cytometry-based assay to detect MART-1₂₇₋₃₅ specific T cells.

Cytokine synthesis: ELISPOT: Functional assay requiring O/N *in vitro* culture, very sensitive and quantitative.
Could detect CD8 and CD4 responses.

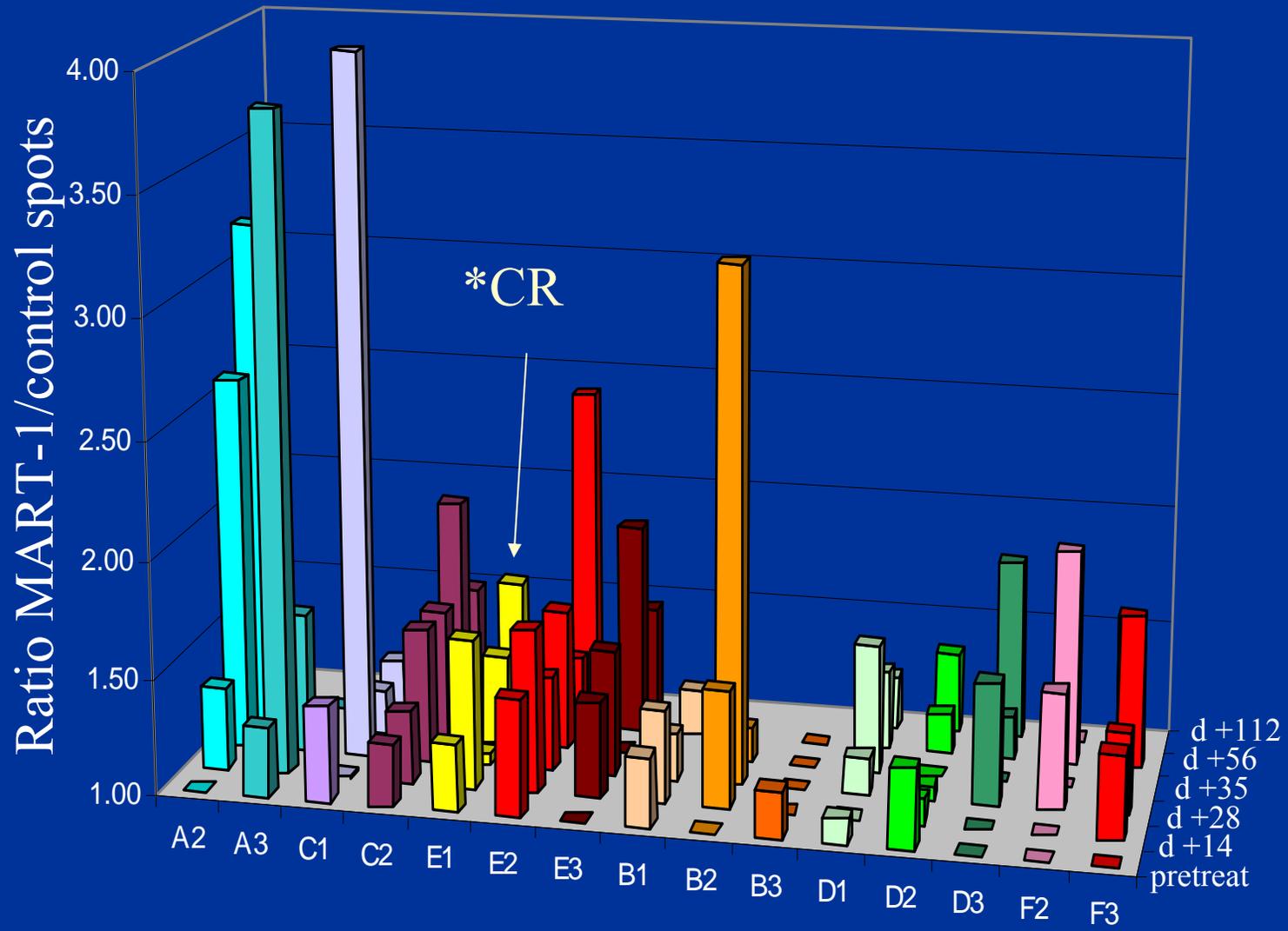
Intracellular Cytokines: Flow cytometry-based functional assay that allows for cell surface phenotyping of responding cells.

Cytotoxicity: Functional assay for direct tumor cell killing, requires weekly *in vitro* restimulations.

MHC Tetramer Analysis

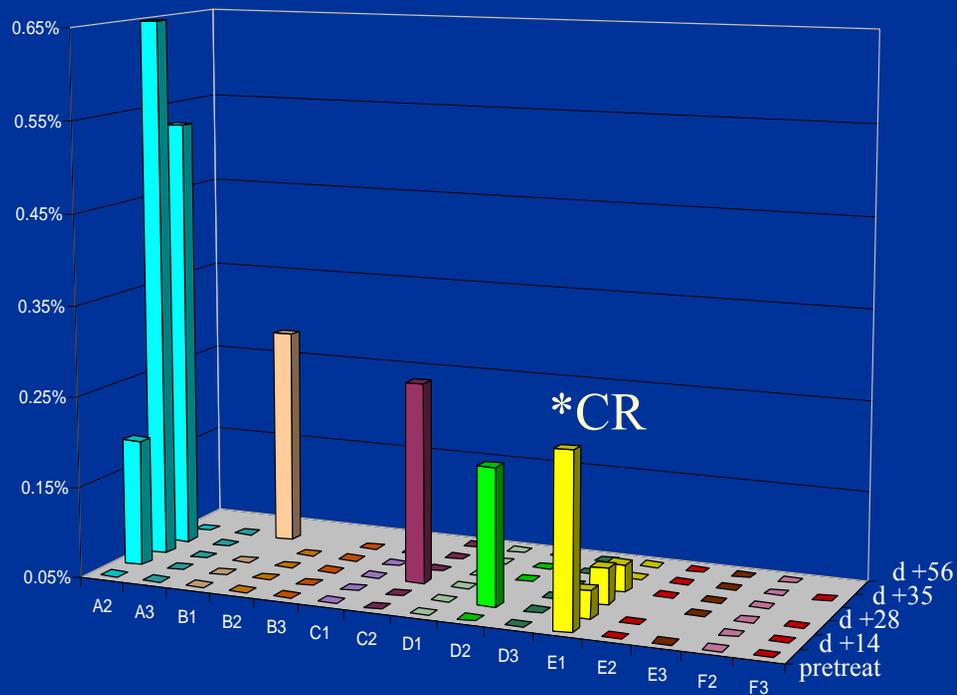


IFN γ ELISPOT Analysis

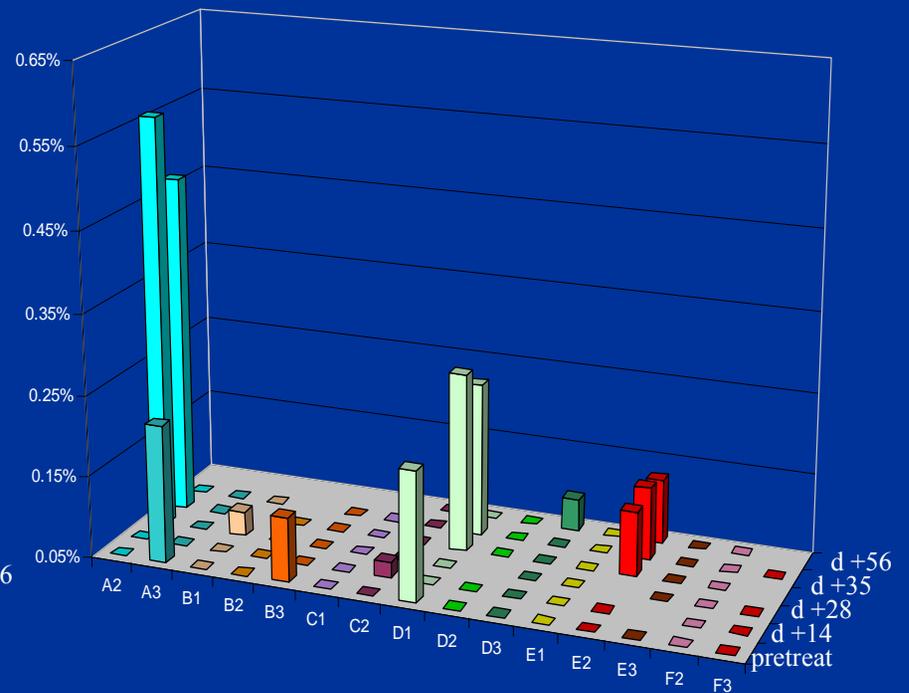


Intracellular Cytokine Analysis

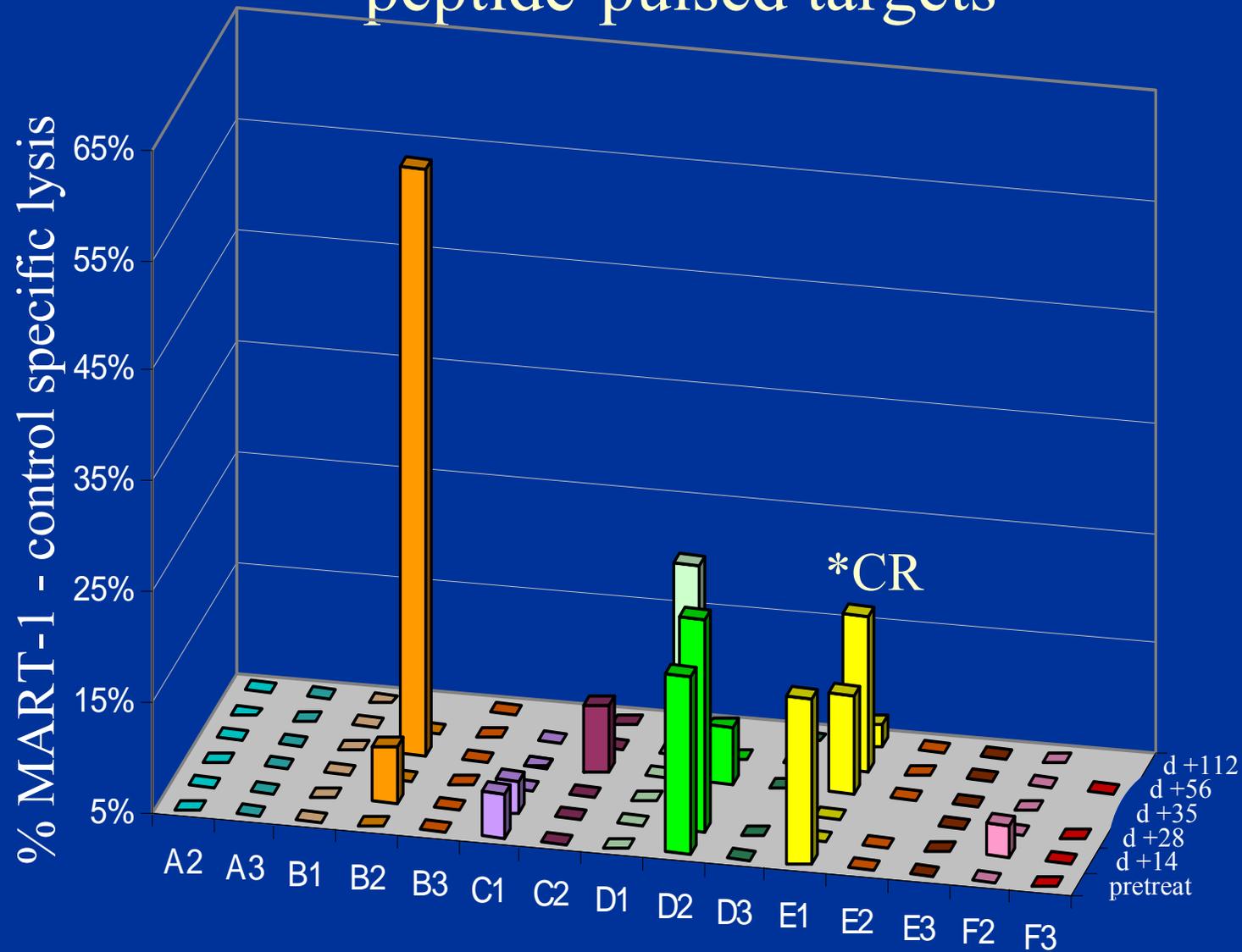
CD3+ MART-1 specific IFN γ + cells



CD3+ MART-1 specific IL-4+ cells



Cytotoxicity Analysis: peptide-pulsed targets



Patient E1 (now 10 years NED)

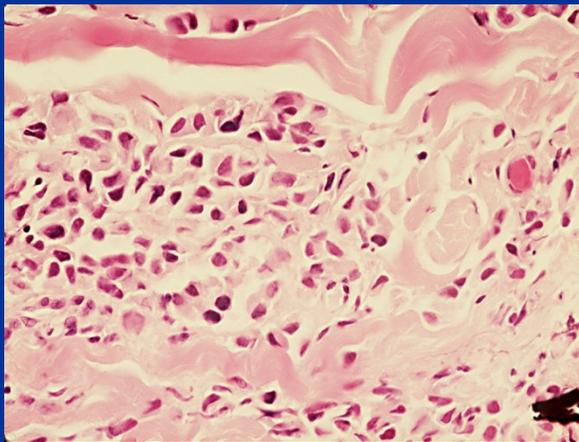
Pretreatment



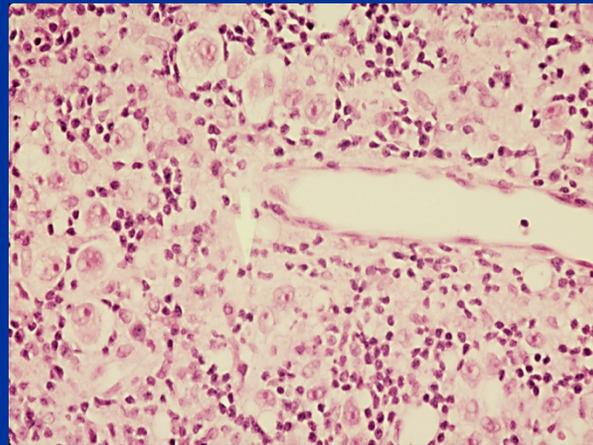
+ 56 days



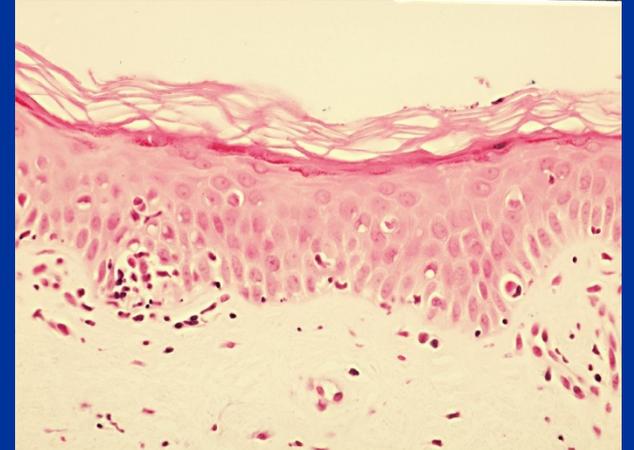
+ 130 days



Melanoma Tumor



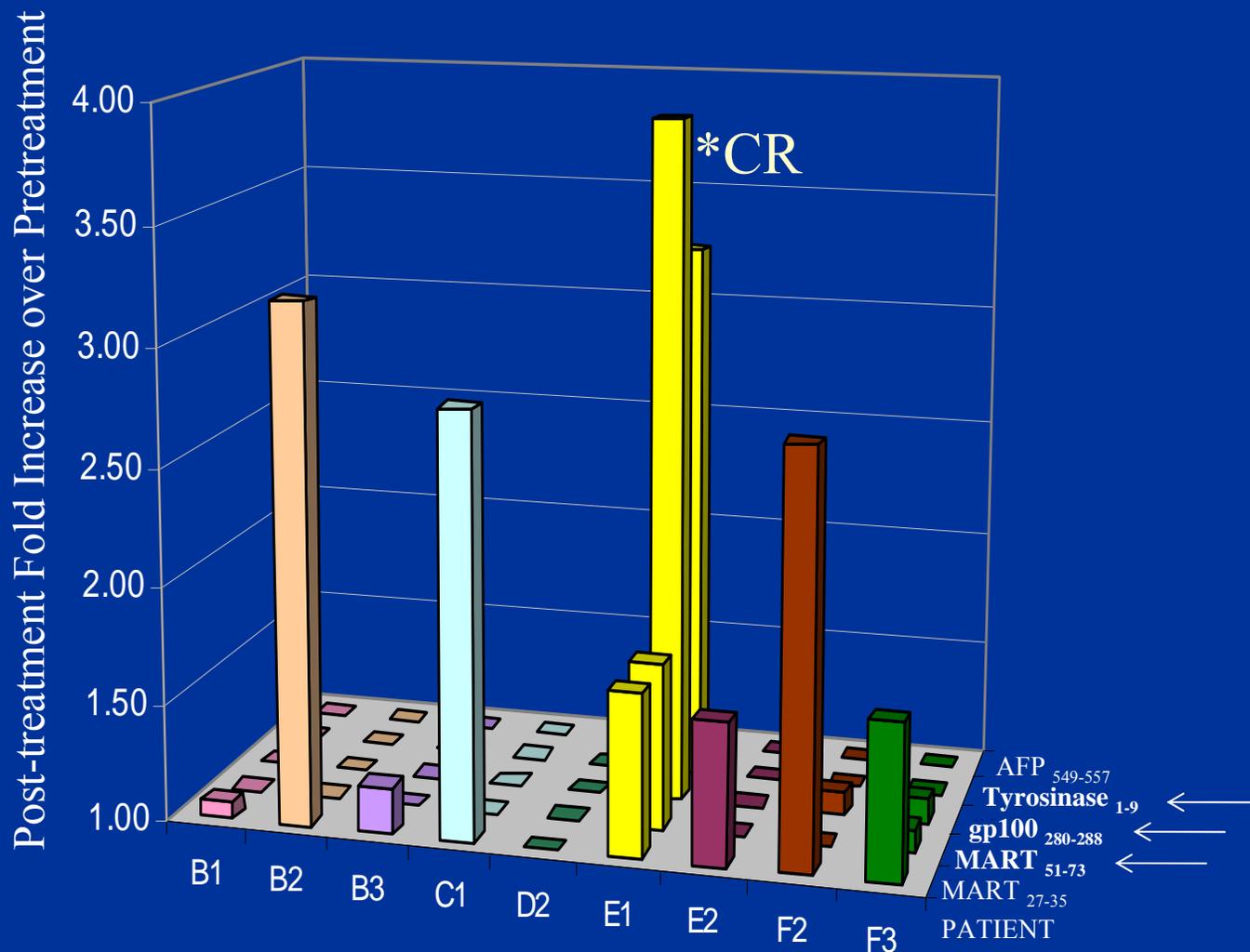
Lymphocytic Infiltrate



Absence of Melanoma

Patient E1: Determinant Spreading

IFN γ ELISPOT



Summary

- (1) The MART-1- Dendritic Cell vaccine is safe and immunogenic.
- (2) MART-1-specific T cell responses are detected even at the lowest DC vaccine dose.
- (3) Intradermal vaccination may be superior to intravenous administration.
- (4) In many patients the increase in circulating antigen-reactive cells is transient.
- (5) Complete clinical responses occurred in patients who developed T cell responses to additional class I and class II melanoma determinants.

NEXT : capitalize on immune monitoring observations: promote determinant spreading to multiple antigens, CD4+ and CD8+ T cells

Which vaccine has superior efficacy and should be moved forward?

Vaccine A: IFN γ /CD8 $^+$ ELISPOT of 20 spots/10e5

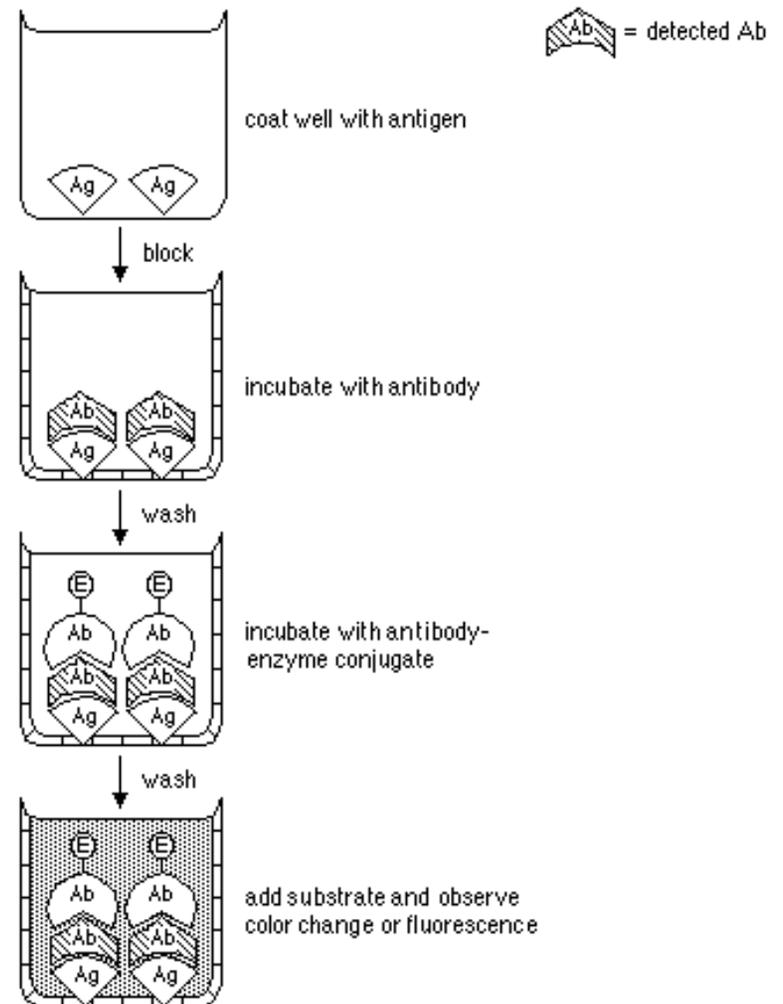
Vaccine B: IFN γ /CD8 $^+$ ELISPOT of 200 spots/10e5?

Can you tell from the publications what is assay variation, patient-patient variation vs. real biological effect?

Survey of Assays

Enzyme-Linked ImmunoSorbent Assay (ELISA)

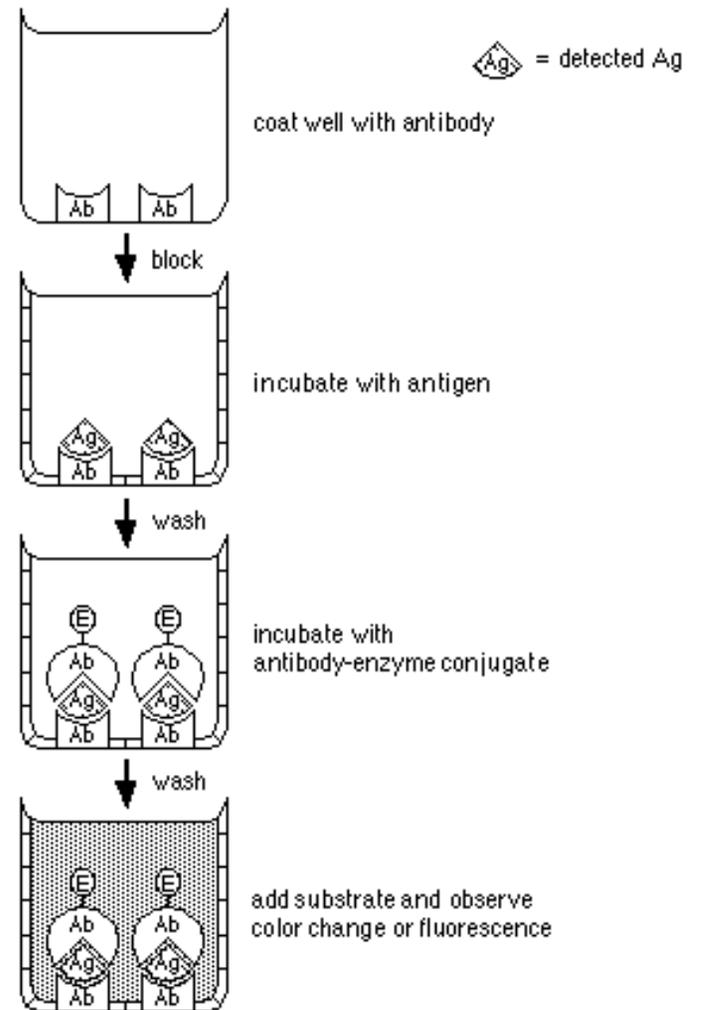
Indirect ELISA to detect specific **antibodies**.



Ag = antigen; Ab = antibody; E = enzyme.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Antibody-sandwich ELISA to detect **antigen**.



Luminex multiplex Cytokine Analysis

This 30-plex kit provides a series of combined reagents for the simultaneous measurement of human:

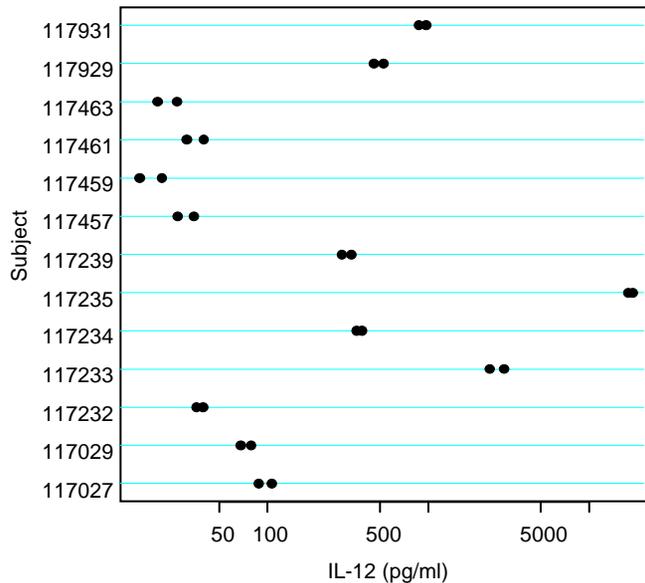
IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF- α , IFN- α , IFN- γ , GM-CSF, MIP-1 α , MIP-1 β , IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF

in serum, plasma, or tissue culture supernatant.

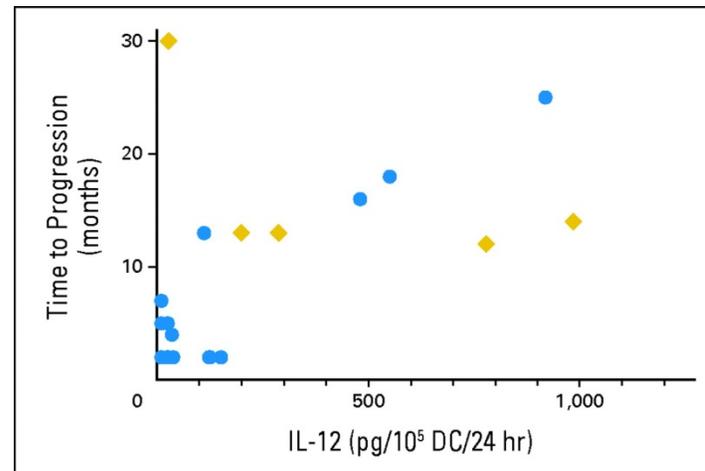
Sample size: 50 ul

Assay Standardization and Validation IL-12p70

candidate potency assay for DC vaccines



Reproducibility of the IL-12p70 production assay. Two series of samples obtained from DC generated/matured under various conditions were tested at multiple times in the same or different assays (*Butterfield, JIT 2008*).



IL-12p70 production levels positively correlated with time to progression. $P = .0255$ is based on Cox regression followed by likelihood-ratio test. Circles ● indicate patients who have already experienced disease progression; diamonds ◆ represent patients who have not experienced recurrence to date.

IL-12p70 (and IL-10) data for healthy donor controls is under biostatistical analysis for reproducibility, etc., in preparation for analysis of additional trials, and patient vaccines.

(*Okada, H., JCO 2010*)

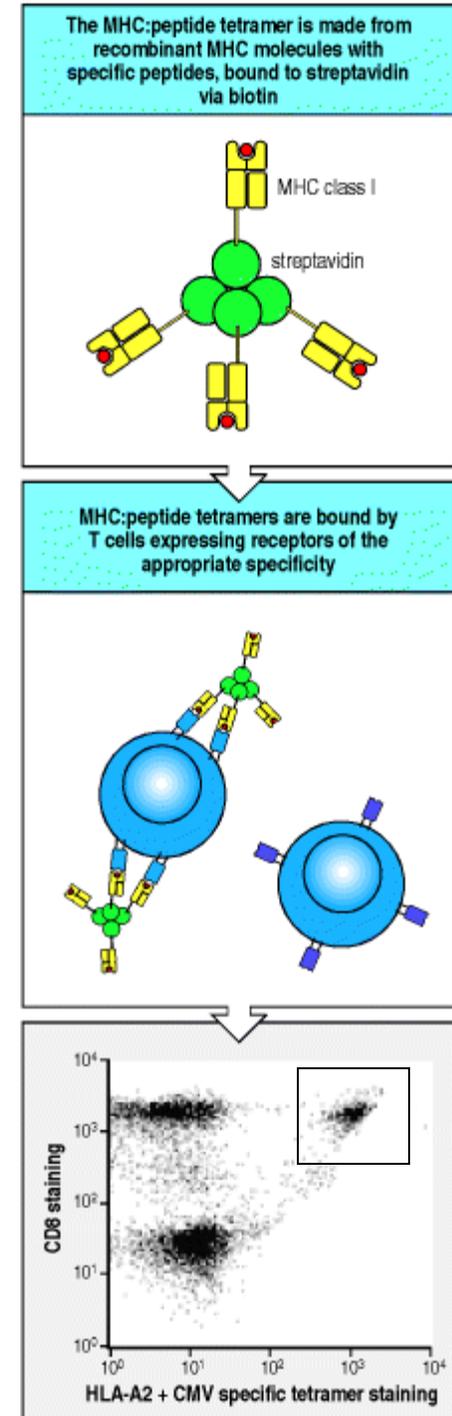
MHC:peptide tetramers (or pentamers, decamers...) are able to stain peptide-specific T cells

MHC:peptide tetramers are formed from recombinant refolded MHC:peptide complexes containing a single defined peptide epitope. The MHC molecules can be chemically derivatized to Streptavidin via biotin, which creates a tetramer of MHC:peptide complexes.

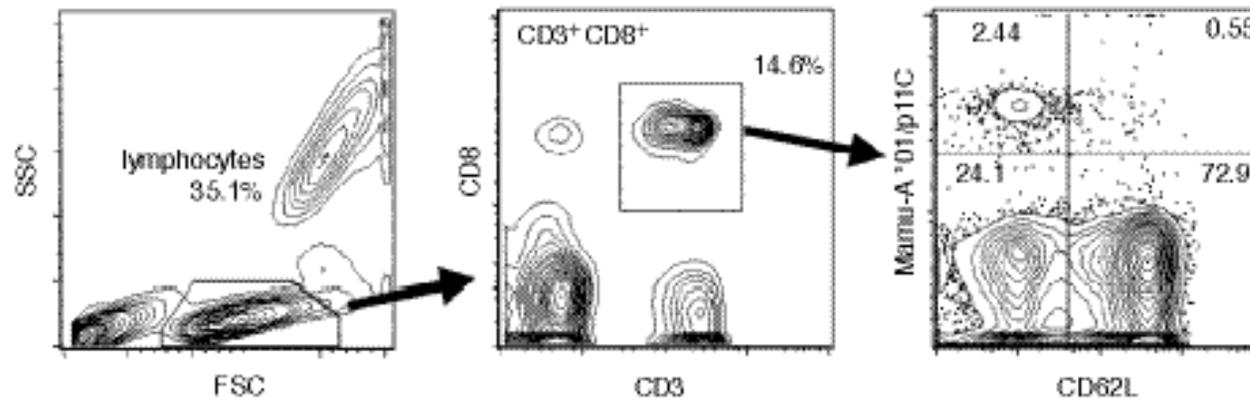
While the affinity between the T-cell receptor and its MHC:peptide ligand alone is too low to bind stably to a T cell, the tetramer, by being able to make a more avid interaction with multiple MHC:peptide complexes binding simultaneously, is able to bind to T cells.

A discrete population of tetramer positive CD8⁺ cells, comprising some 5% of the total CD8⁺ cells, can clearly be seen.

Can be combined with additional fluorescent antibodies to identify phenotype/function of the T cells

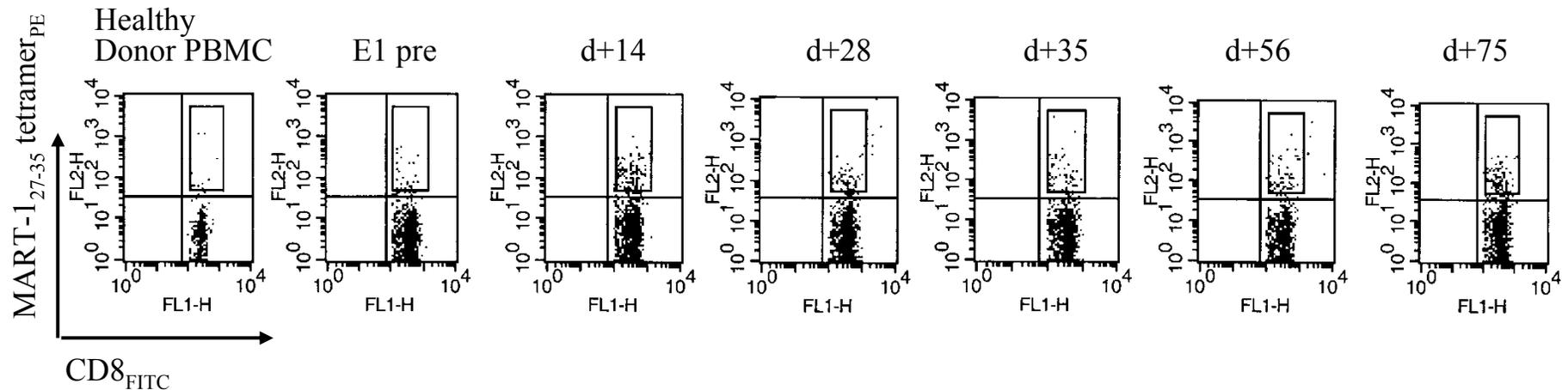


MHC-Peptide Tetramers to Visualize Antigen-Specific T Cells



Data shows full gating strategy
Clear, distinct tetramer+ (or multimer+) population

Example of melanoma “self” tumor associated antigen



Data does not show full gating strategy:

How clean was CD3/CD8 gating?

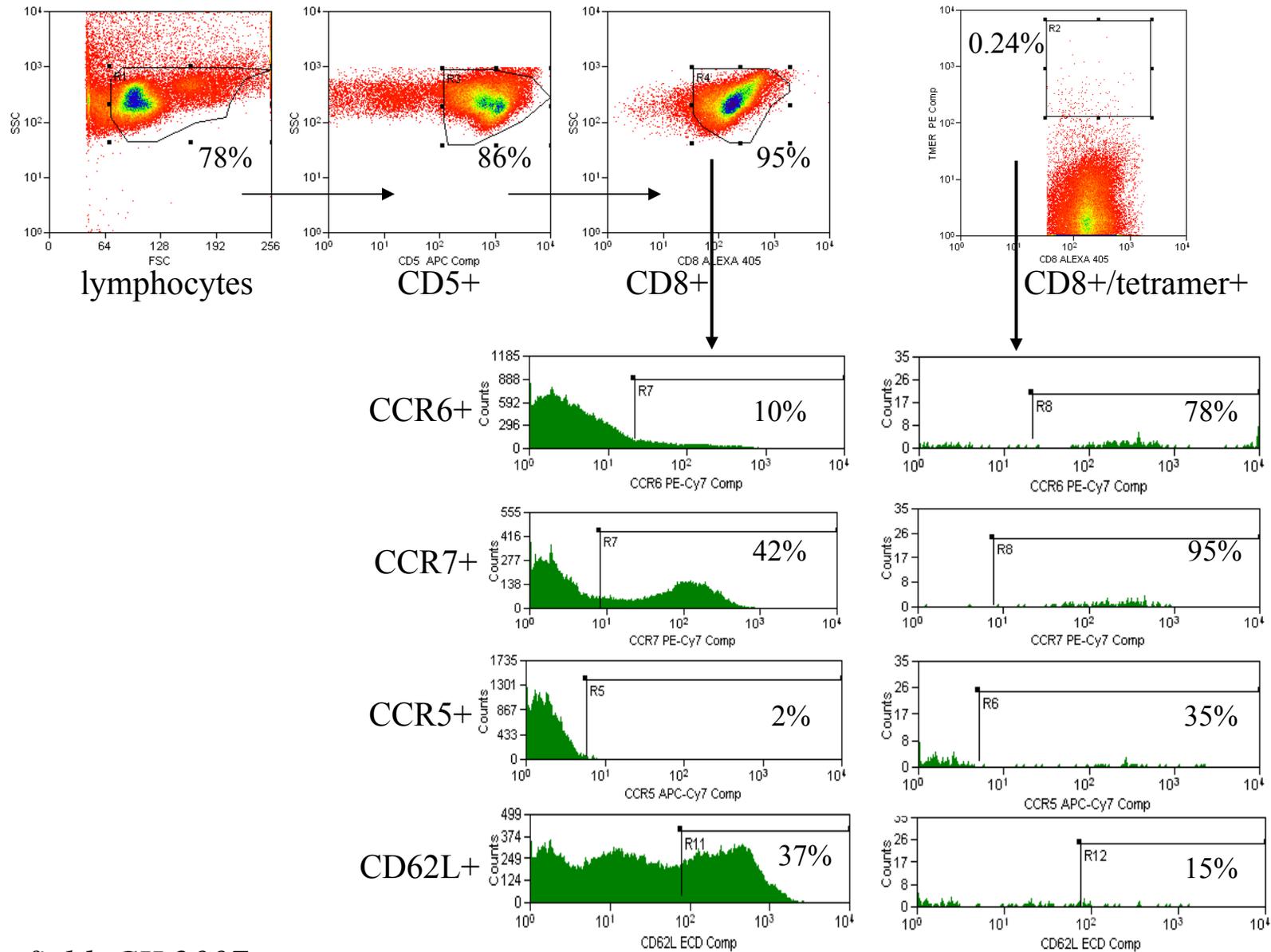
How clean were lymphocytes?

How sticky were multimers or were “dump” channels/
non-CD8-lineage markers also used?

Where is the distinct, separate population?

How standardized was the set up for the flow cytometer?

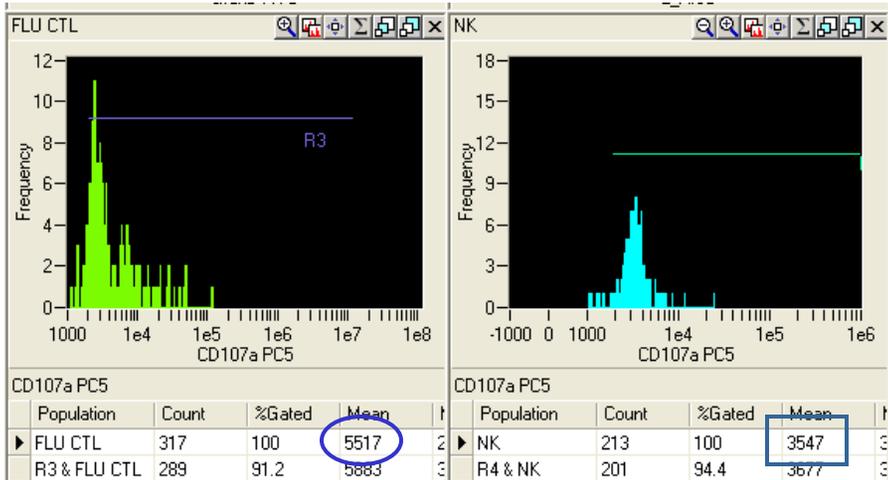
Patient B10 AFP₁₃₇ Tetramer Phenotype Analysis Strategy



Granzyme B and CD107a Profiles—CTL and NK Cells (Amnis)

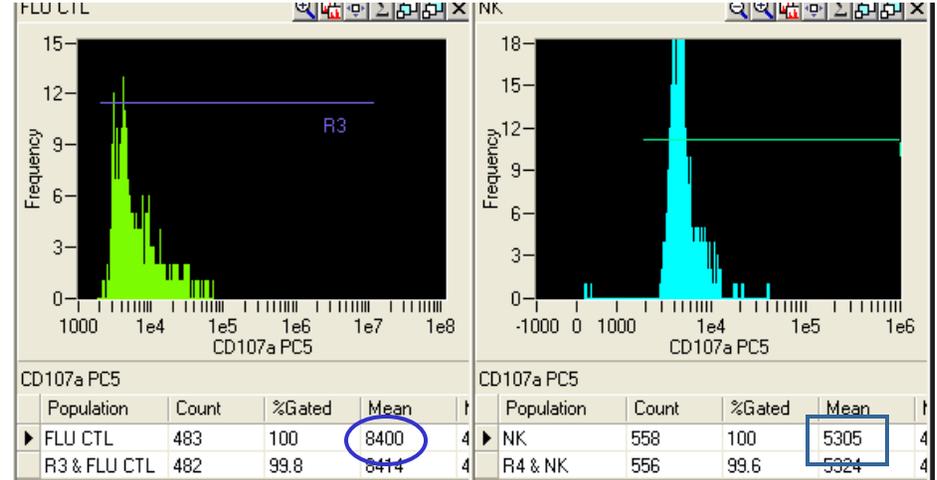
CTL + no treatment

CTL + OKT3/IL-2

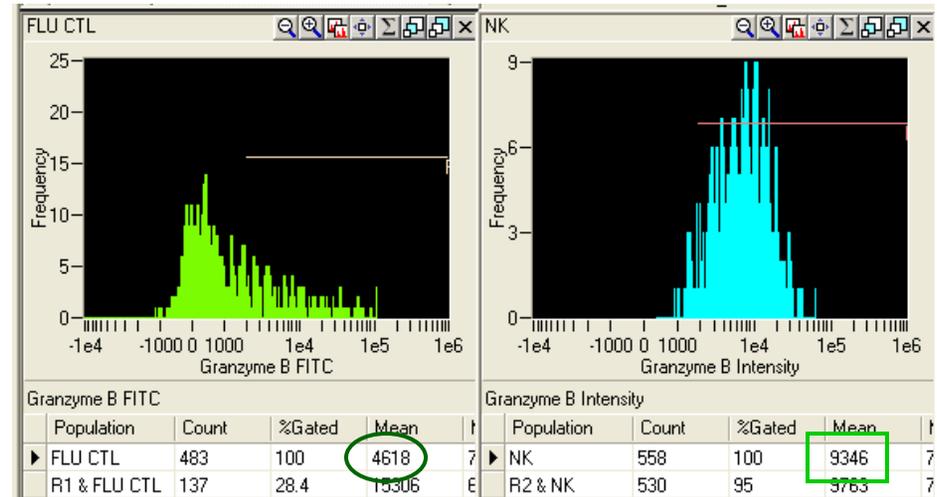
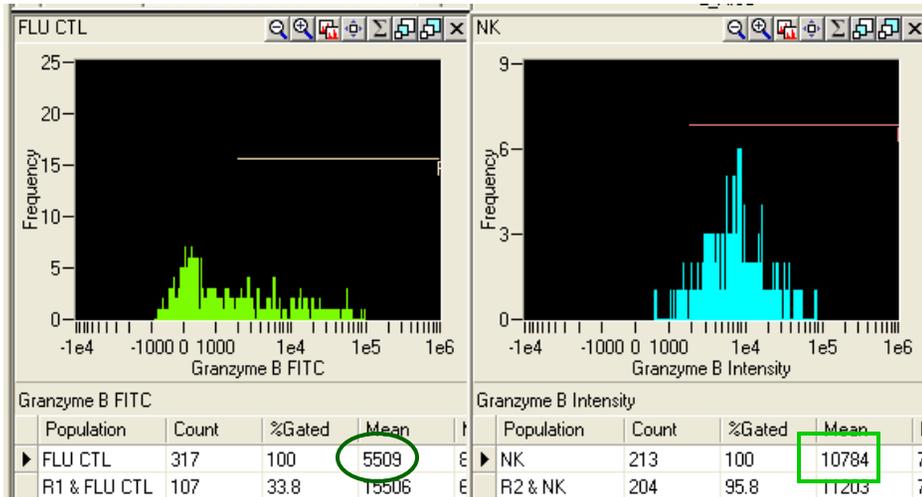


CTL

NK



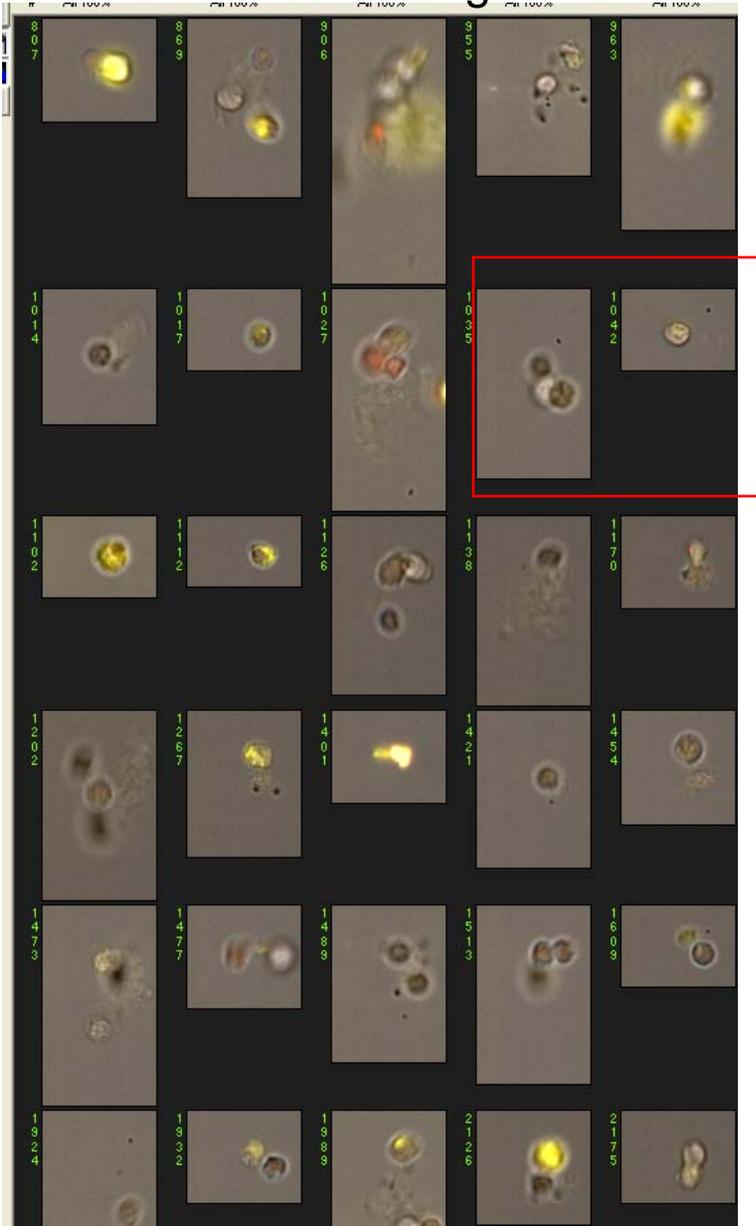
Increased CD107a in Flu CTL and NK cells



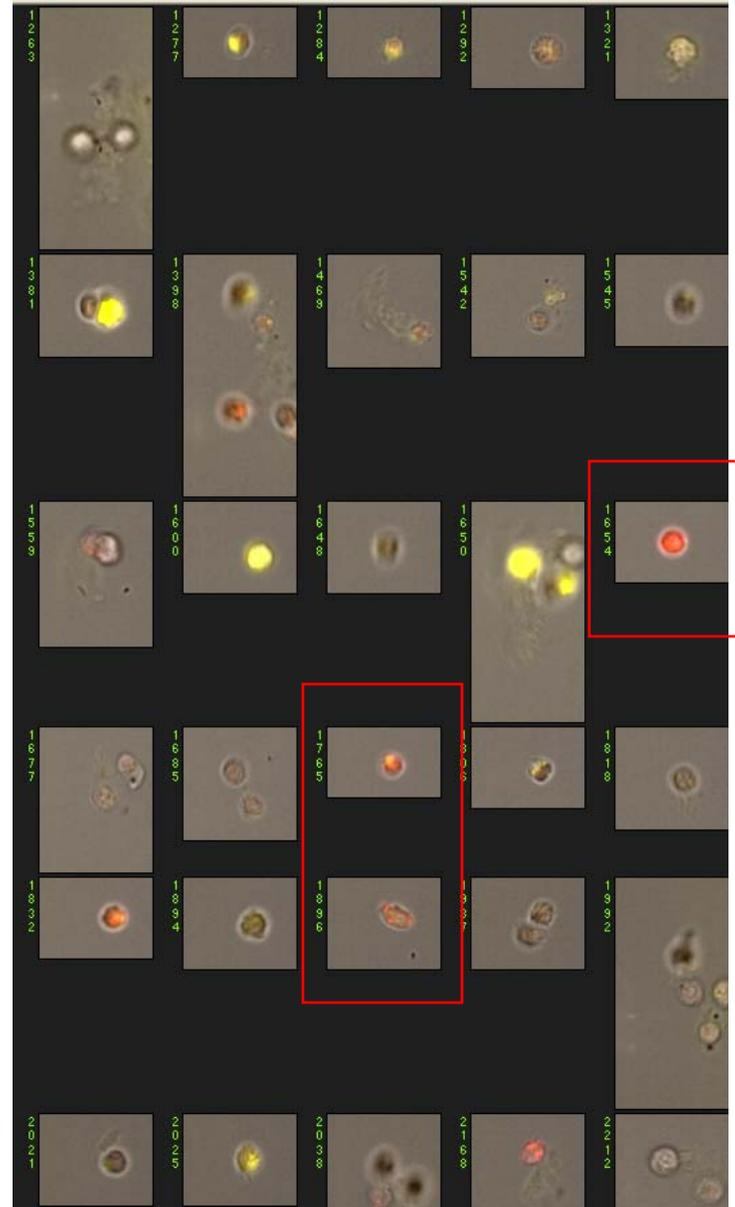
Decreased granzyme B in Flu CTL and NK cells

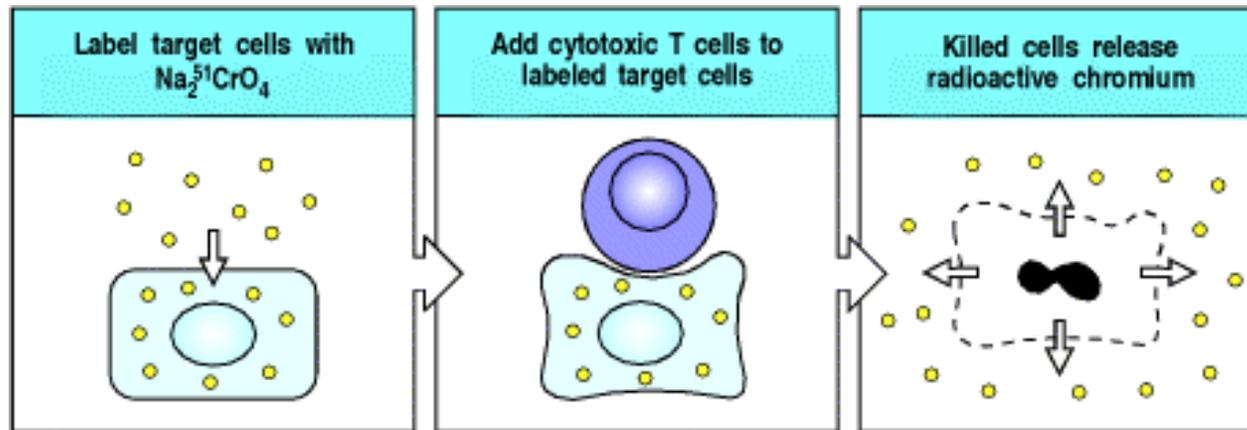
CTL (CD5+/CD8+) Granzyme B+/CD107a+ Cell Images

CTL + nothing



Increased Red = CD107a
CTL + OKT3/IL-2





Cytotoxic T-cell activity is often assessed by chromium release from labeled target cells. Target cells are labeled with radioactive chromium as $\text{Na}_2^{51}\text{CrO}_4$, washed to remove excess radioactivity and exposed to cytotoxic T cells. Cell destruction is measured by the release of radioactive chromium into the medium, detectable within 4-6 hours of mixing target cells with T cells.

Can also test after 24 hours to test for immediate killing (4-6 hours) by perforin/granzyme mechanism plus slower killing (24 hours) by $\text{TNF}\alpha$.

E:T ratios go as high as 100:1

Often requires in vitro stimulation to detect

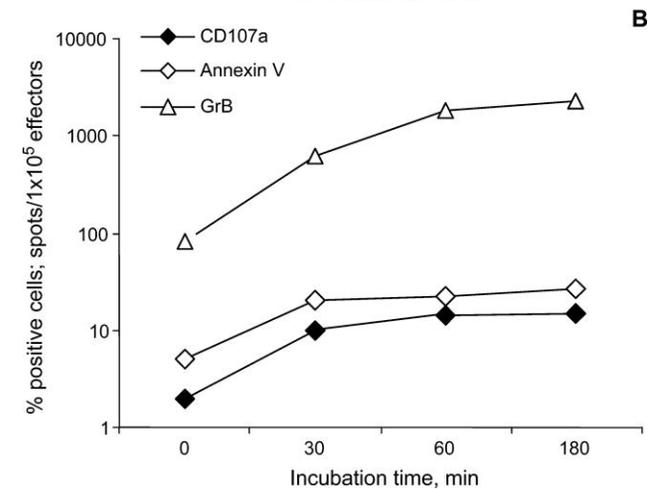
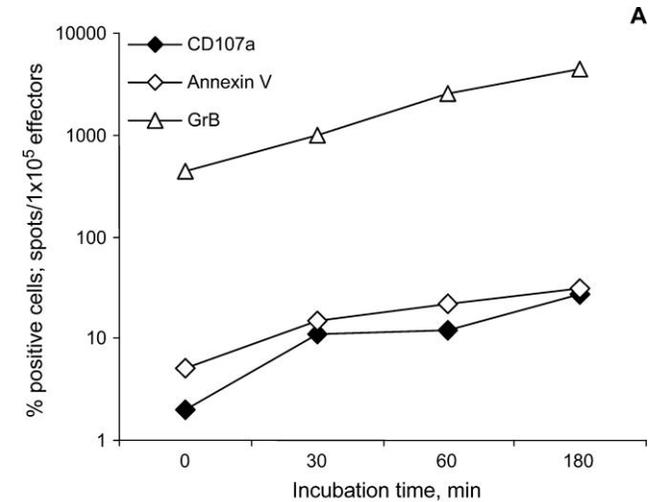
Variably affected by cryopreservation

A Novel Flow Cytometric Assay for Evaluating Cell-Mediated Cytotoxicity

Burkett, Mark W; Shafer-Weaver, Kimberly A; Strobl, Susan; Baseler, Michael; Malyguine, Anatoli

Correlation of CD107a expression by anti-peptide CTLs with annexin V binding by specific targets as measured in the flow cytometric assay and granzyme B release in the ELISPOT assay.

CD107a surface expression also correlates with Granzyme B release detected by ELISPOT.

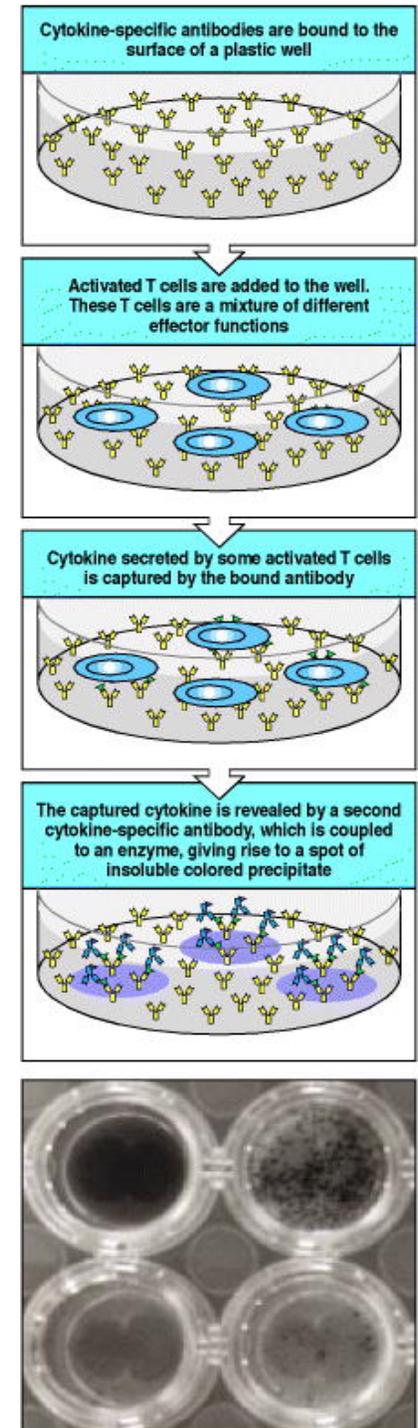


The frequency of cytokine-secreting T cells can be determined by the ELISPOT assay. The ELISPOT assay is a variant of the ELISA assay in which antibodies bound to a plastic surface are used to capture cytokines secreted by individual T cells.

If a T cell is secreting the appropriate cytokine, it will be captured by the antibody molecules on the plate surrounding the T cell. T cells are removed, and the presence of the specific cytokine is detected using an enzyme-labeled second antibody specific for the same cytokine.

Each T cell that originally secreted cytokine gives rise to a single spot.

Results: PHA >> CMV > melanoma antigen > negative control.



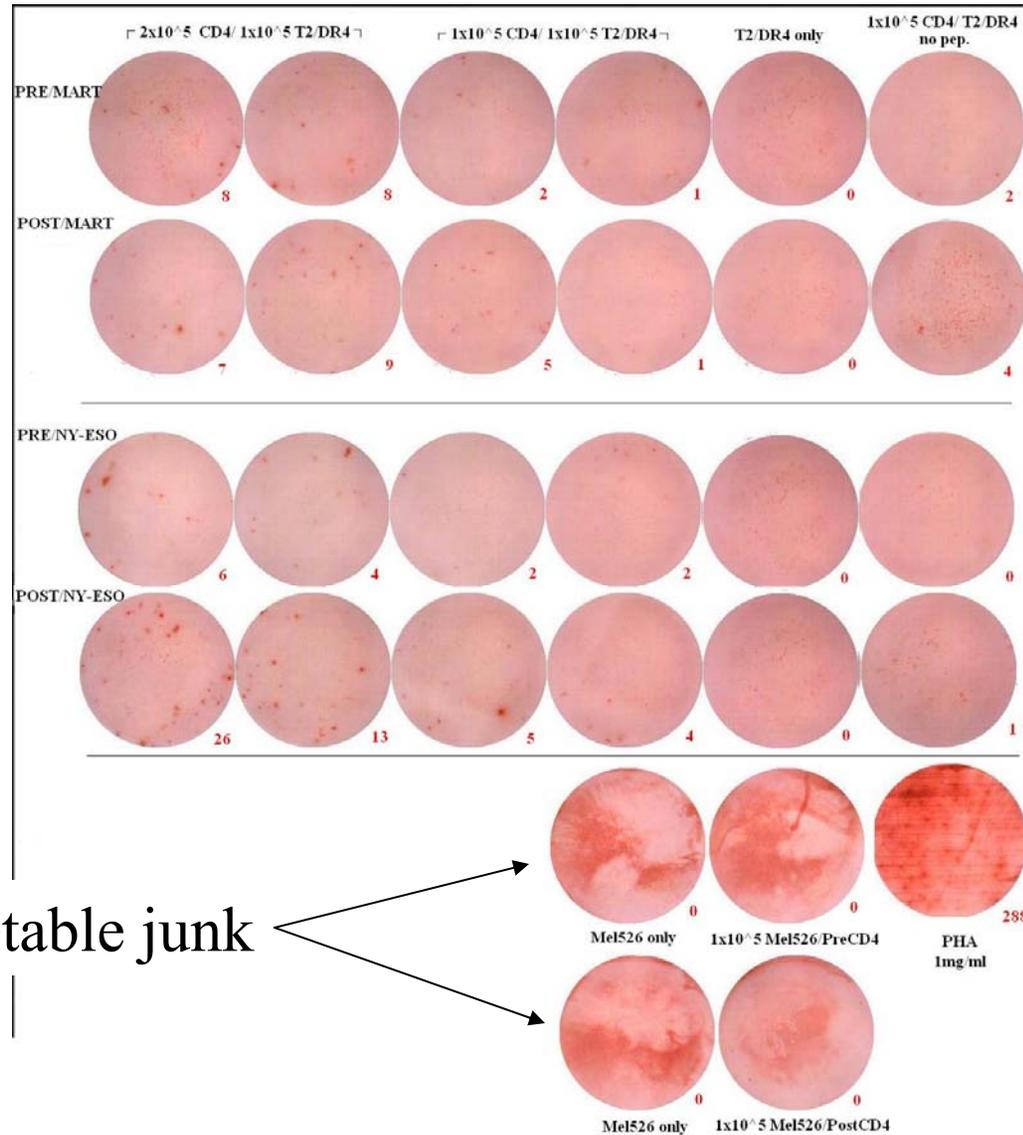
IFN γ ELISPOT

Melanoma Antigen Peptides, CD4+ T cells

Same frequency
pre vs. post

Increased frequency
Post vaccine

Un-interpretable junk

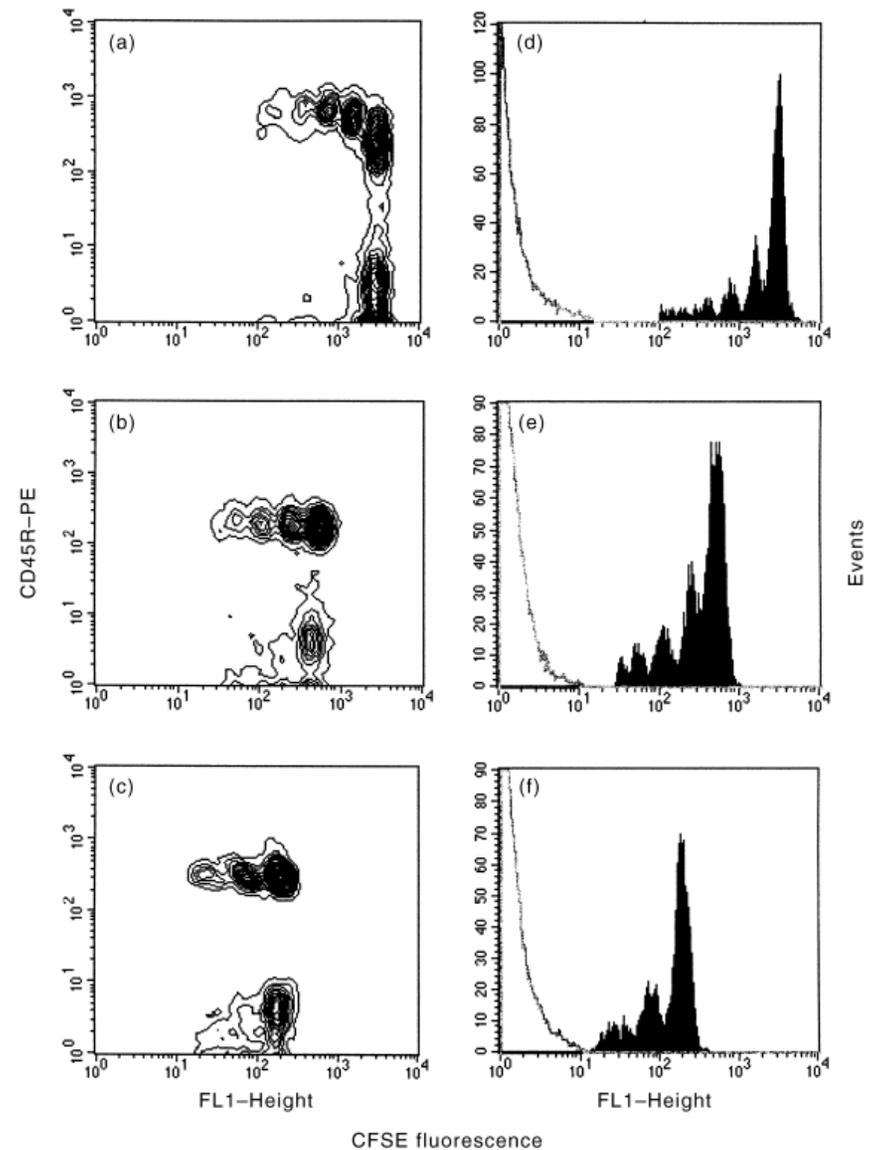


CFSE Proliferation

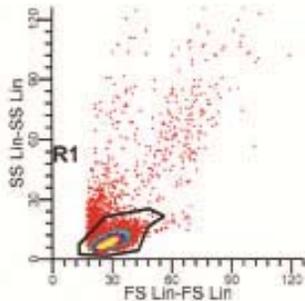
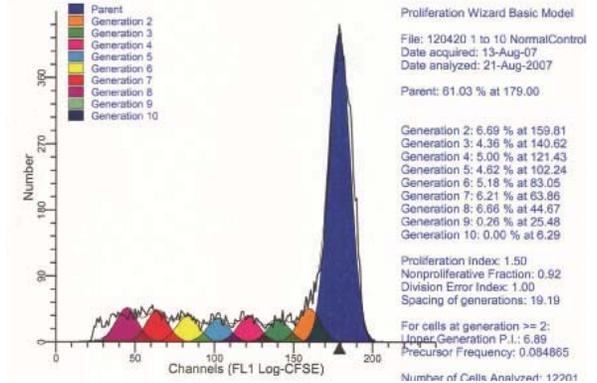
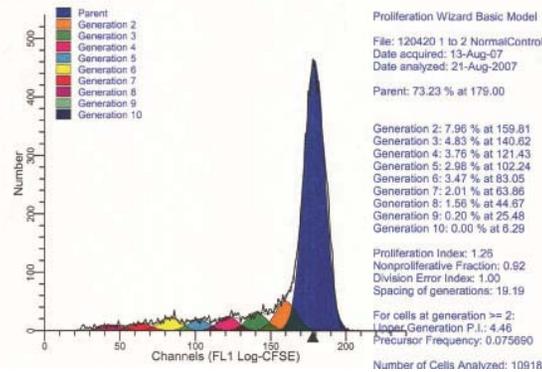
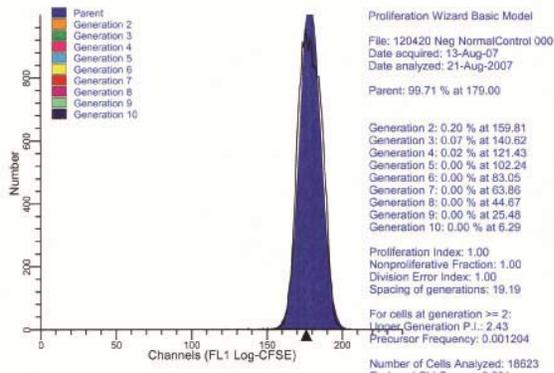
Most techniques for assessing cell division can only quantify overall proliferation (^3H thymidine incorporation).

This is a cell division analysis procedure based on the quantitative serial halving of the membrane permeant, stably incorporating fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE), that covalently attaches to free amines of cytoplasmic proteins.

The technique can be used both *in vitro* and *in vivo*, allowing eight to 10 successive divisions to be resolved by flow cytometry.

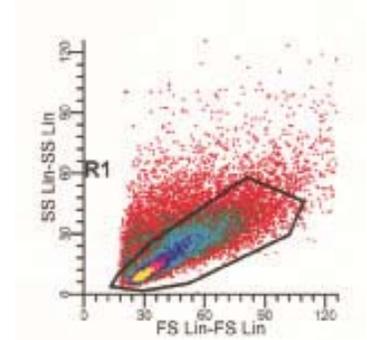


Functional Treg/Proliferation : CFSE dilution assay

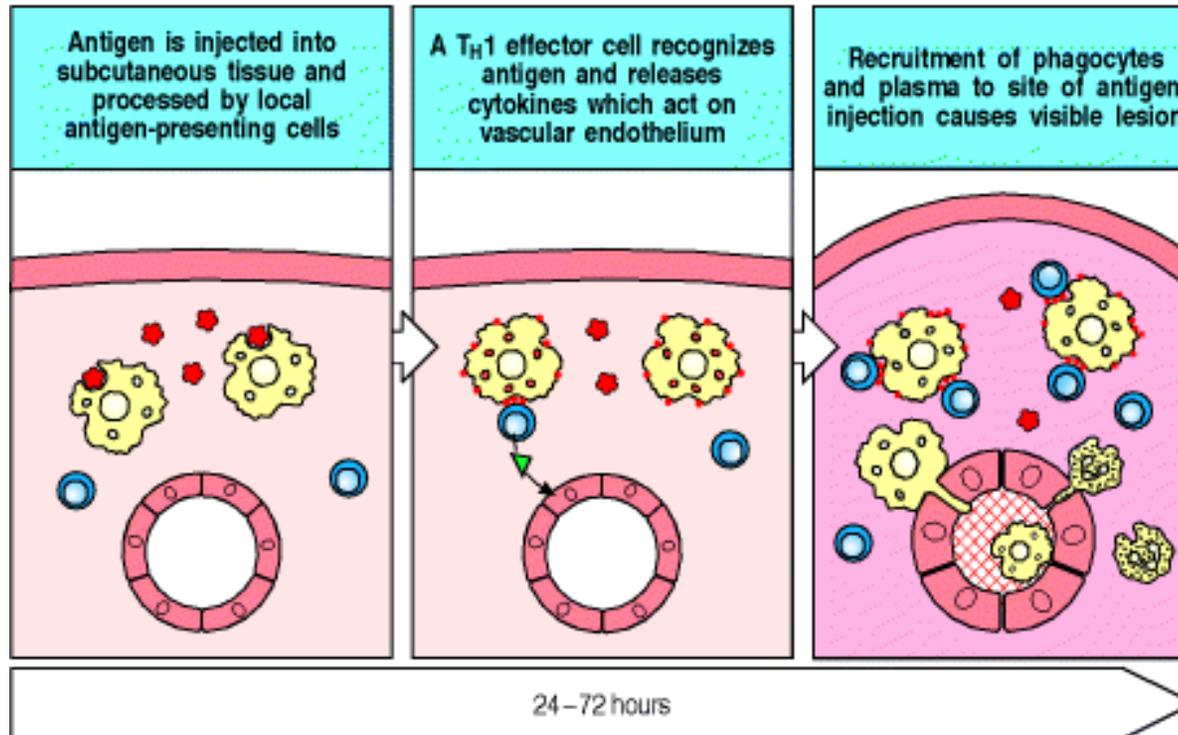


PBMC are stimulated (controls include PHA(+) and no stimulation(-)) and followed over 3-5 days for proliferation.

Initially untreated cells have not divided (100% in “parent” (blue) peak), and later, the cells have “blasted” in size, and only 61% remain undivided in the parent peak.



Delayed Type Hypersensitivity Reaction



The stages of a delayed-type hypersensitivity reaction. involves uptake, processing, and presentation of the antigen by local antigen-presenting cells.

T_H1 cells that were primed by a previous exposure to the antigen migrate into the site of injection and become activated. Because these specific cells are rare, and because there is little inflammation to attract cells into the site, it can take several hours for a T cell of the correct specificity to arrive.

These cells release mediators that activate local endothelial cells, recruiting an inflammatory cell infiltrate dominated by macrophages and causing the accumulation of fluid and protein. At this point, the lesion becomes apparent.

Analyses of delayed-type hypersensitivity (DTH) biopsies in situ.

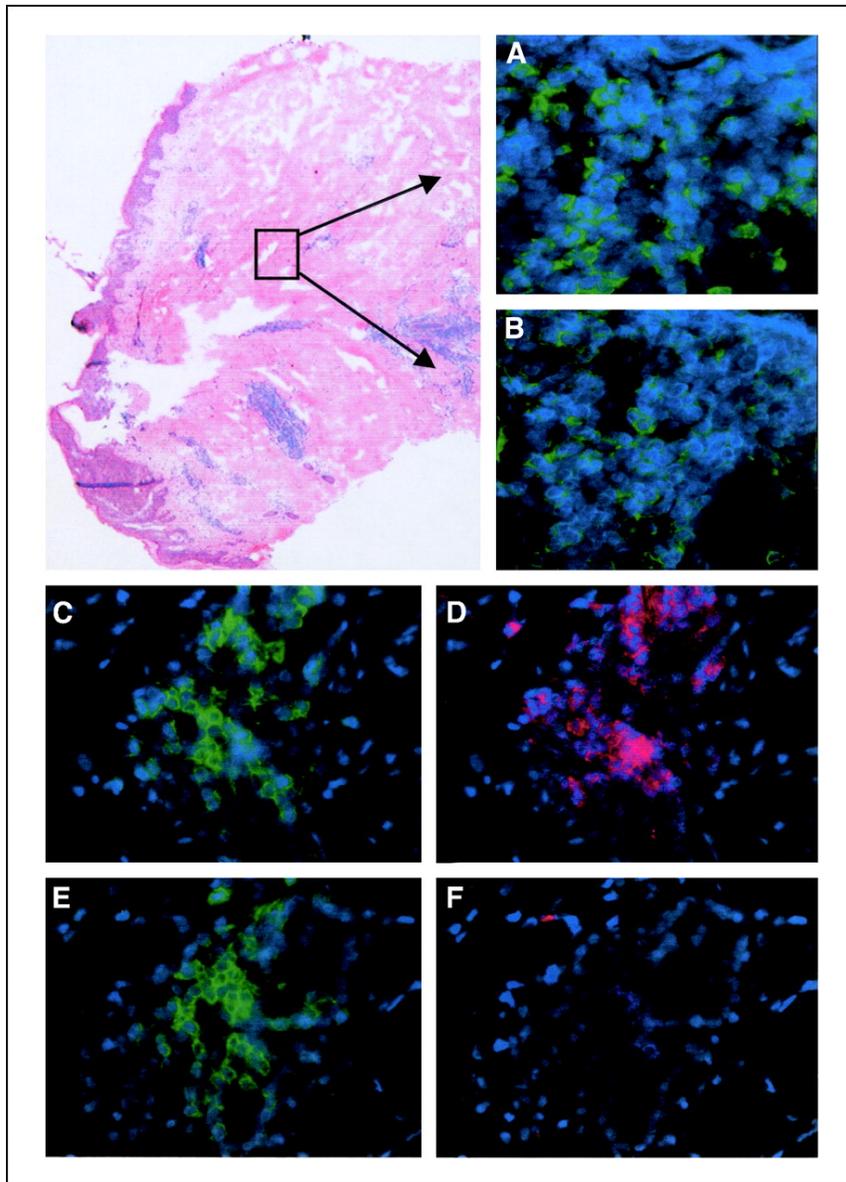


Fig 2. Analyses of delayed-type hypersensitivity (DTH) biopsies in situ. Staining of the DTH sections of a vaccinated patient revealed infiltrating clusters of (A) CD8 and (B) CD4 T cells. In situ tetramer staining on cryosections of biopsies: sections were stained with CD8 in green (C and E) in combination with tetramer tyrosinase (D) or gp100:280 (F) in red.

de Vries I J M et al. JCO 2005;23:5779-5787

JOURNAL OF CLINICAL ONCOLOGY

DTH -infiltrated lymphocytes

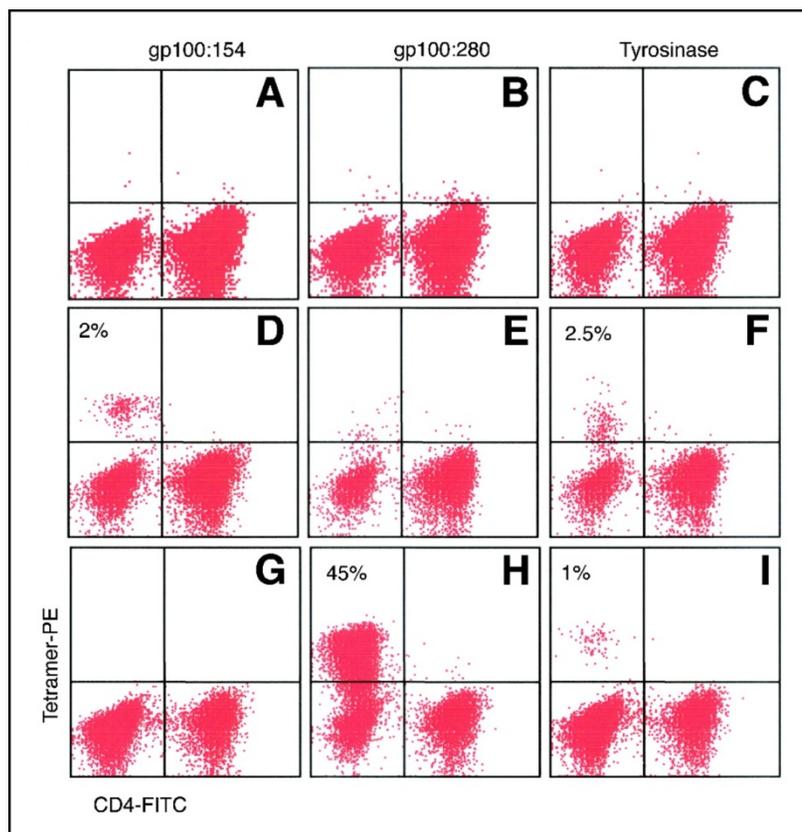


Fig 3. In delayed-type hypersensitivity (DTH) -infiltrated lymphocytes (DIL), dendritic cells (DC) + keyhole limpet hemocyanin (KLH), no tetramer-positive T cells were observed (A to C). In the same patient positivity for all three tetramers was observed in DTH: DC + three peptides (D to F). In DIL from DTH, DC + KLH + three peptides, positivity for two tetramers were observed (G to I).

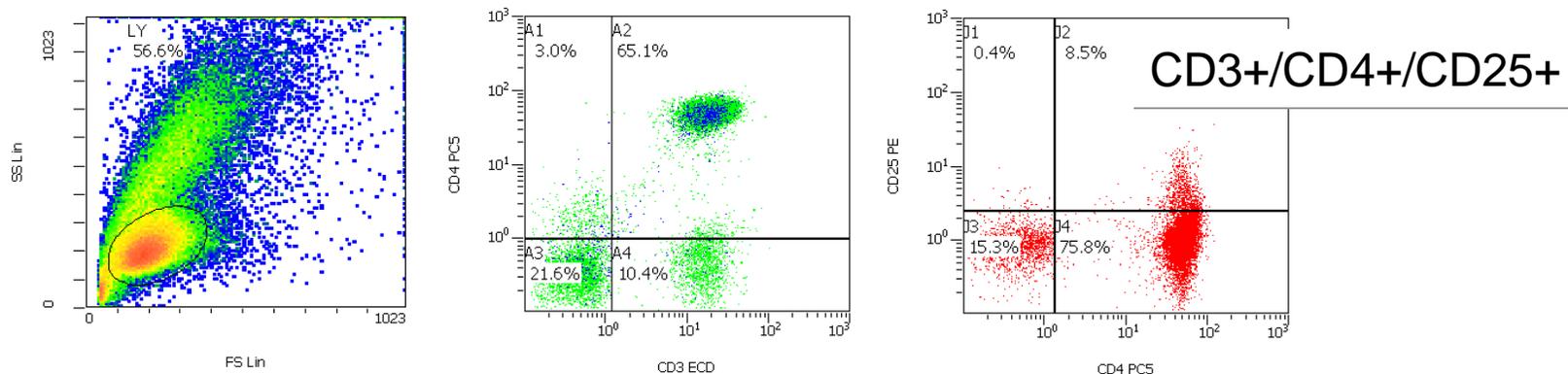
After intradermal administration of a DTH challenge with gp100- and tyrosinase peptide-loaded DC, *essentially all patients showed a positive induration*. In clinically responding patients, T cells specific for the antigen preferentially accumulated in the DTH site, as visualized by in situ tetramer staining. Furthermore, significant numbers of functional gp100 and tyrosinase tetramer-positive T cells could be isolated from these DTH biopsies, in accordance with the applied antigen in the DTH challenge. **We observed a direct correlation between the presence of DC vaccine-related T cells in the DTH biopsies of stage IV melanoma patients and a positive clinical outcome ($P = .0012$).**

What else?

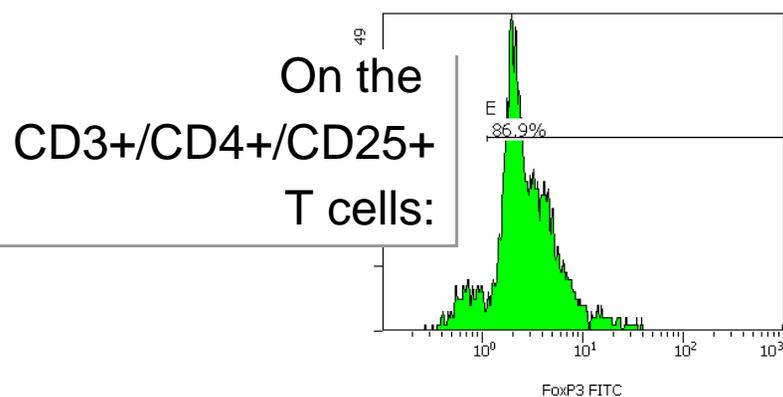
- Immune suppression
(Treg frequency, Treg function; MDSC frequency)
- Serum Antibody response (ELISA, SEREX)
- Systemic cytokines (serum/plasma Luminex)
- Tumor infiltrating lymphocytes
immunohistochemistry/fluorescence in tissue
single cells: multimer, cytokine production, killing
- Draining lymph node
immunohistochemistry/fluorescence in tissue
single cells: multimer, cytokine production, killing
- Molecular Assays
DNA polymorphisms, RNA expression, epigenetics,
signaling

Treg: flow cytometry phenotype

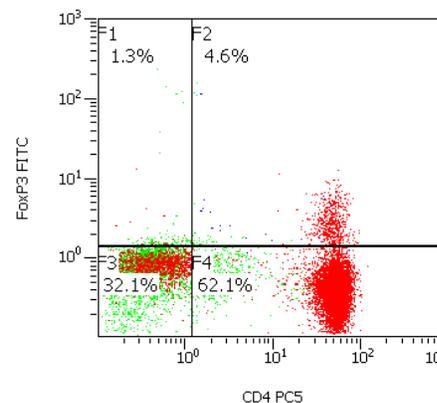
Ingated] 113669 10-261 IRX IC2 2006-03-06 002.LMD : FS Lin/SS L[LY] 113669 10-261 IRX IC2 2006-03-06 002.LMD : FL3 Log/FL4 Log[I] 113669 10-261 IRX IC2 2006-03-06 002.LMD : FL4 Log/FL2 Log - /



(F1)[J2] 113669 10-261 IRX IC2 2006-03-06 002.LMD : FL1 Log - AD[LY] 113669 10-261 IRX IC2 2006-03-06 002.LMD : FL4 Log/FL1 Log -



On the
CD3+/CD4+/CD25+
T cells:



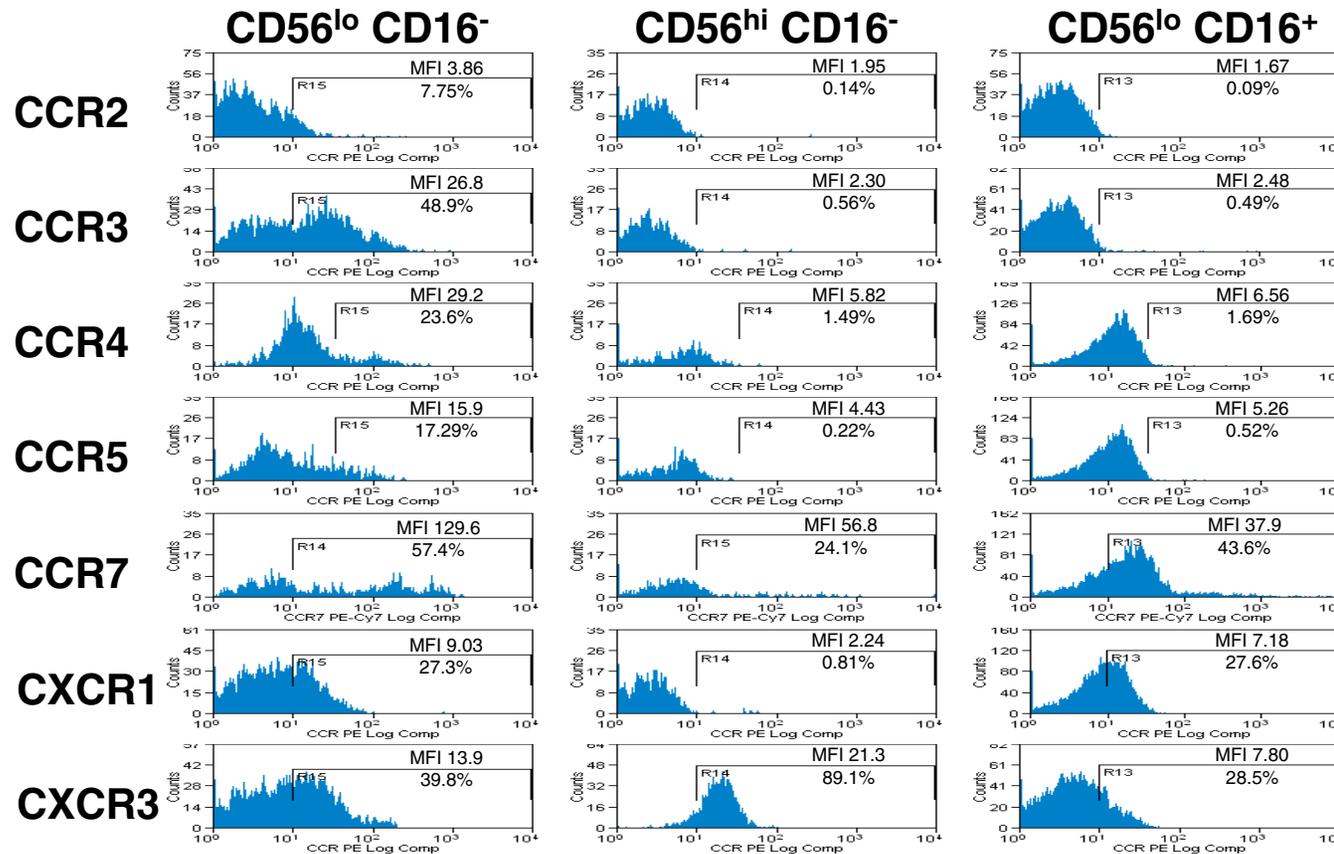
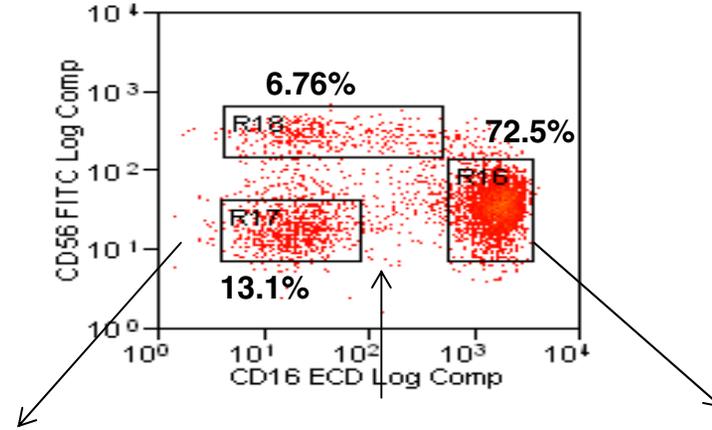
Total FoxP3 by CD4+: histogram and dot plot

Also: CD127, CD39, CTLA-4...

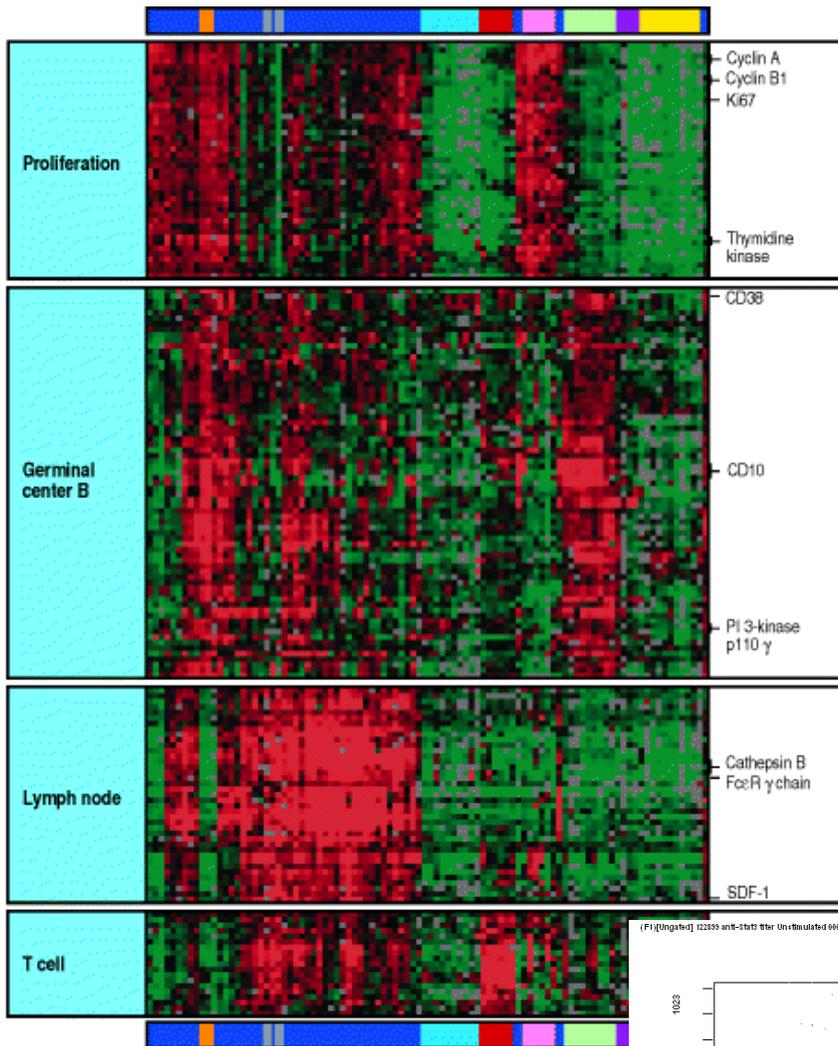
NK cell phenotype

Function:
CD107a, IFN γ

NKG2x
expression



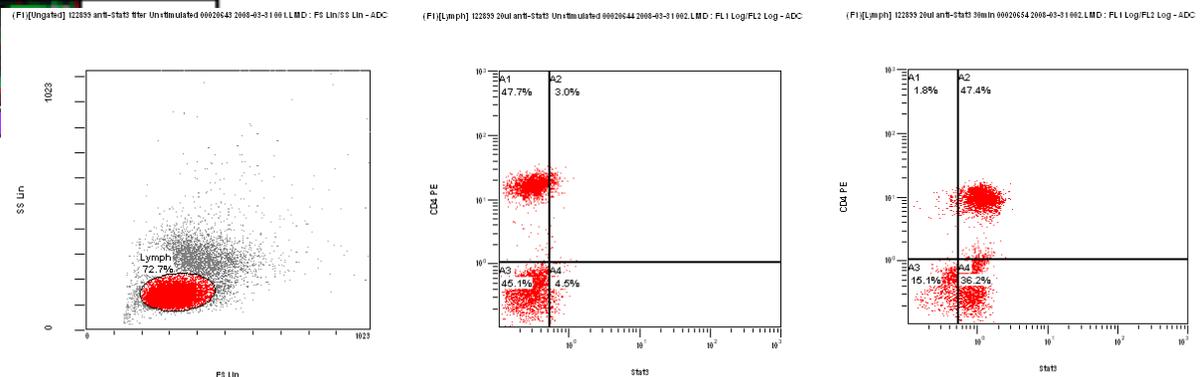
*Vujanovic,
submitted, 2011*



DNA microarrays allow a rapid, simultaneous screening of many genes for changes in expression between different cells.

cDNA clones made from lymphoid cells and tumors were arrayed horizontally across the chip. mRNA was hybridized from normal cells and lymphoid tumor cells

STAT-3 Phospho-flow assay:



FS vs. SS Lymphs

Un-stimulated CD4/Stat3

30 minutes stimulation

Design of Hypothetical Clinical Trial Immunologic Monitoring

Practical Considerations

Cells (Blood/PBMC, TIL):

What can you reasonably access, at multiple time points, from patients?

Blood: *yes.*

Tumor: *is a FNA sufficient, or do you need a surgical biopsy?*

is it standard of care to access tumor (IRB)?

lab--clinic logistics and relationship?

will patients consent to the tumor biopsy?

Culture of cells (Blood/PBMC, TIL):

Ex vivo /"direct" assay: either thaw and test or short restim. (4-6 hours)

Overnight restimulation

In vitro culture/ *in vitro* stimulation (IVS)

Culture of cells:

Ex vivo /"direct" assay: either thaw and test
or short restimulation (6 hours to O/N)
+snapshot of actual state of cells
-may not allow function assessment

Overnight restimulation

+not enough culture to drastically change function
-may not allow detection of very rare activity

In vitro culture/*in vitro* stimulation (IVS)

+allows amplification of cells (-or death of cells)
-drastically changes activity of cells
(what they *can be*, not what they are)

Antigen Presenting Cells:

PBMC count on poor APC (B, MΦ) or low frequency DC

Dendritic Cells require 5-7 days culture +/-maturation and autologous cells, can present whole antigen
Most potent APC

T2 TxB hybrid with TAP transporter deficiency, HLA-A2.1+, presents signal peptides (weakly) and exogenous (pulsed) peptides

T2/DR4 same, transfected with HLA-DR4 for specific MHC class II peptide presentation

K562/A2.1 erythroleukemia cells, deficient in HLA MHC I/II (NK target), transfected with A2.1
lower background—no other HLA on surface

Subset Purification:

Total PBMC: ~40% CD4, 10-20% CD8, B cells, NK, monocytes

Non-adherent PBMC: Remove monocytes/macrophages for DC

CD4+: test only helper T cells (and Treg)

CD8+: test only effector T cells

PBMC with CD4+ removed: remove helpers/Tregs

Purified subsets: +clearly identify source of activity
+ eliminate cell—cell interactions
- loss of cells from purification
- test for % purity of subsets

Which therapies are superior and should be moved forward?

IFN γ /CD8+ ELISPOT of 20 spots/10e5

vs.

IFN γ /CD8+ ELISPOT of 200 spots/10e5?

IFN γ +IL-2+TNF α multi-functional CD8+ T cells

vs.

IFN γ +TNF α vs. TNF α expressing CD8+ T cells?

Highest IFN γ ELISA results after 7+ day IVS?

Broadest immunity to antigens/determinant spreading?

Scientific Goal:

To identify important immune biomarkers which might be predictive of clinical outcome, or ability to respond to an intervention:

Need: reliable, standardized measures of immune response.

CLIA (Clinical Laboratory Improvements Amendments) rules:

Test Accuracy (close agreement to the true value),

Precision (agreement of independent results: same day, different day),

Reproducibility (intra-assay and inter-assay)

Reportable range (limits of detection)

Normal ranges (pools of healthy donors, accumulated patient samples),

Personnel competency testing

Equipment validation, monitoring

Reagent tracking

Central Immunology Laboratory

Clinical Site

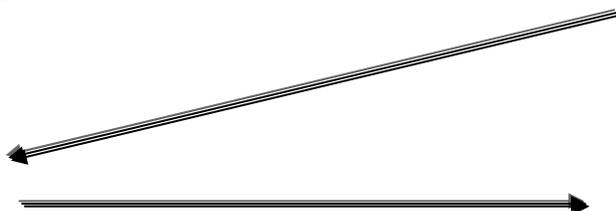
Central Lab

Screen or enrollment:
fax blood kit request



Kit prepared and
shipped ground

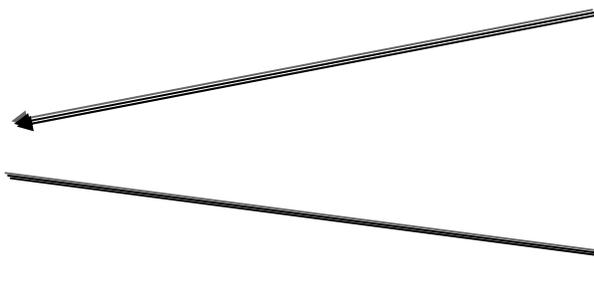
Pt. blood draw
mailed O/N to lab



Blood processed and
banked according to
SOPs within 24 hours



gather data;
biostatistics

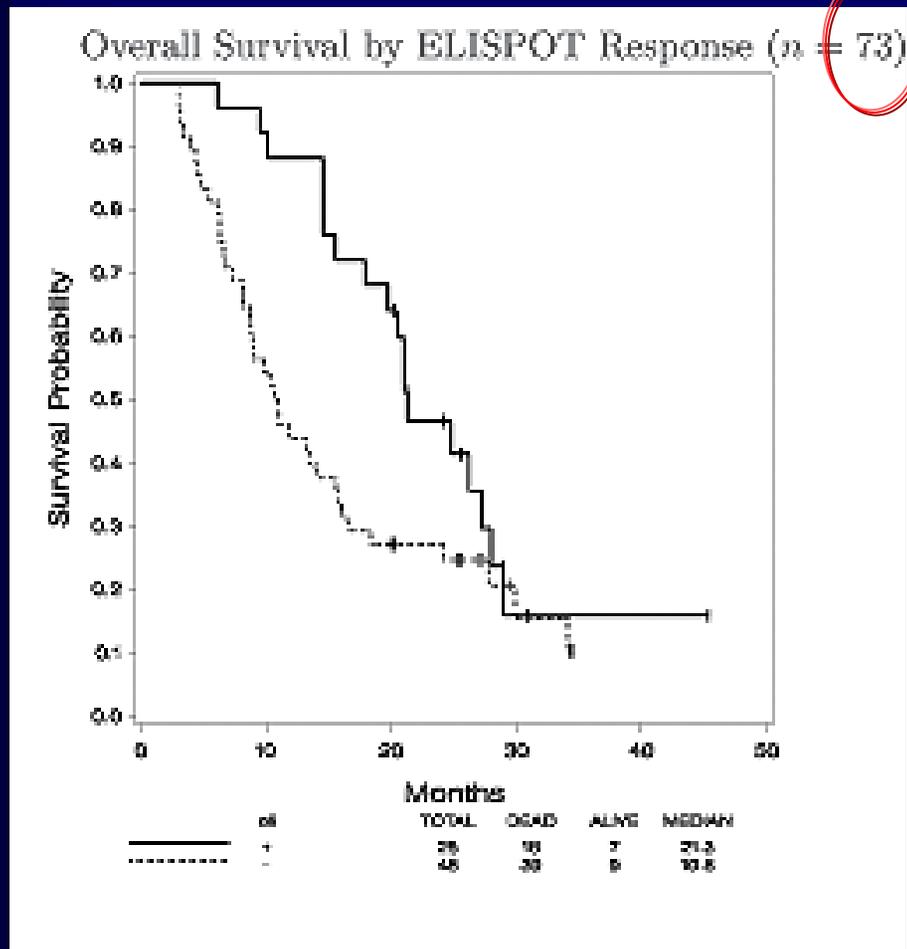


Assays performed per
SOPs, send results

Results to PI,
publish!

Immune Response Correlates with Overall Survival

Multiple melanoma antigen peptide vaccine \pm GM-CSF \pm IFN α 2b



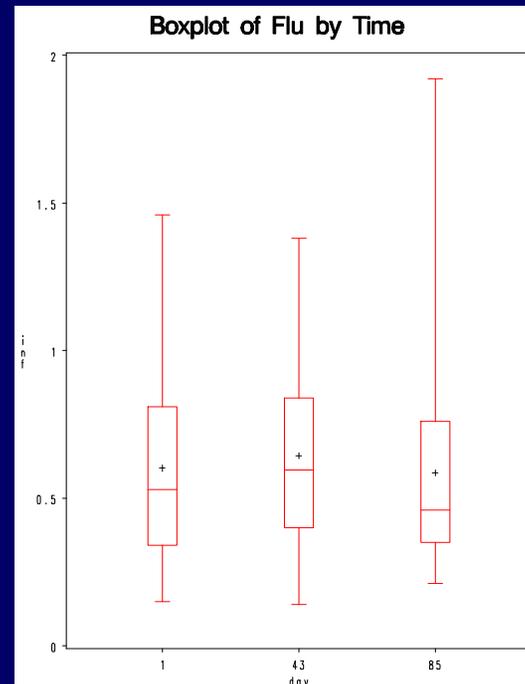
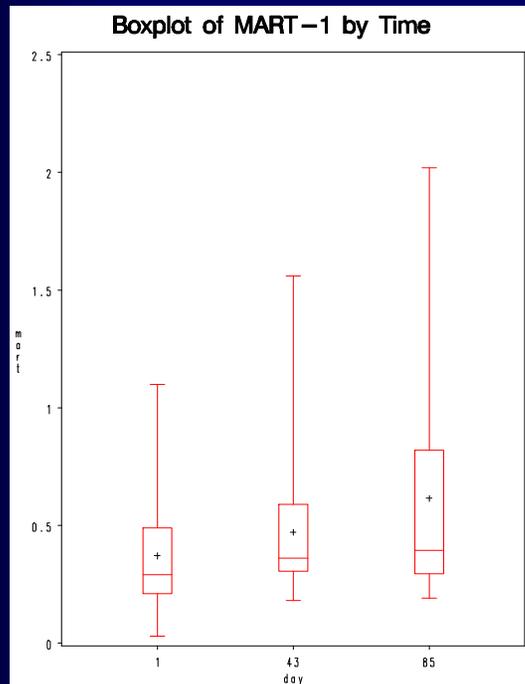
The Kaplan-Meier plot for OS by immune response status is shown for E1696 (Phase II).

There was a significant difference in OS by immune response status. Immune responders lived longer than the non-immune responders (median OS 21.3 versus 10.8 months, $p=0.033$).

(Kirkwood, J.M., Clin. Cancer Res. 2009)

Immune Response: E1696

Melanoma antigen peptide-specific CD8+ T cells



%MART-1
CD8+ cells: .29% .36% .39%

.53% .53% .43%

%effector
cells: 10% 16% 18%
(p=0.048)

17% 17% 16%
(p = ns)

MHC Tetramer Analysis:

The frequency of vaccine peptide-specific CD8+ T cells was measured by MHC tetramers, showing significant increases for all 3 melanoma antigen peptides (not Flu).

The MART-1 and gp100-specific cells differentiated towards effector cells with vaccination.

ELISPOT Assays

E4697 (*n=20, 2008-2009*)

	<u>spontaneous</u>	<u>PMA/I (+)/OKT3</u>
Healthy control	ave.: 4.9 (54%CV)	304 (19.2%CV <i>intra-assay</i>) (48% CV inter-assay)
Patient	ave.: 0.7 (35%CV)	81 (38.7 %CV)

E1696 (*n=20, 2002-2003*)

	<u>spontaneous</u>	<u>PMA/I (+)/PHA</u>
Healthy control	ave.: 5.4 (56%CV)	284 (15.5%CV <i>intra-assay</i>) (51% CV inter-assay)
Patient	ave.: 19 (40%CV)	171 (18.8 %CV)

ImmunoAssay Proficiency Panel Program

About the ImmunoAssay Proficiency Panel Program

The program's objectives are:

1) to offer an external validation program, and 2) to enhance assay harmonization.

The Proficiency Panel Program seeks to identify:

- issues and deficiencies of current assay practices;
- sources for assay variability within and among institutions;
- protocol details that optimize assay performance.

Define criteria for and support harmonization & validation of the assay for individual member laboratories.

Determine the value of resulting criteria in the cancer immunotherapy community.

Offer training programs to the cancer immunotherapy community to enhance assay performance and comparability between laboratories.

Establish immune assays as standard monitoring tools, if feasible.

ELISPOT -- Dr. Sylvia Janetzki, ZellNet Consulting

ICS -- Dr. Lisa McNeil, Wyeth

Multimer (Tetramer/Pentamer) Staining -- Dr. Pedro Romero, Ludwig Institute for Cancer Research, Lausanne, Switzerland, and Dr. Cedrik Britten, University of Leiden, The Netherlands

Central laboratory services, cells, and shipping are provided by the Immunology Quality Assurance Center (IQAC) of the Duke Human Vaccine Institute (Durham, NC) under the leadership of Dr. Thomas Denny.

Contact Assay Working Group coordinator Sylvia Janetzki: sylvia@zellnet.com, (201) 346-0710.



To facilitate development of innovative immunotherapy approaches, there is a need to develop and validate tools to identify patients who can benefit from a particular form of immunotherapy.

Despite substantial effort, we do not yet know which parameters of anti-tumor immunity to measure and which assays are optimal for those measurements.

SITC, FDA and NCI partnered to address these issues for immunotherapy of cancer.

Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers

Lisa H. Butterfield, A. Karolina Palucka, Cedrik M. Britten, Madhav V. Dhodapkar, Leif Håkansson, Sylvia Janetzki, Yutaka Kawakami, Thomas-Oliver Kleen, Peter P. Lee, Cristina Maccalli, Holden T. Maecker, Vernon C. Maino, Michele Maio, Anatoli Malyguine, Giuseppe Masucci, Graham Pawelec, Douglas M. Potter, Licia Rivoltini, Lupe G. Salazar, D.J. Schendel, Craig L. Slingluff, Jr., Wenru Song, David F. Stronck, Hideaki Tahara, Magdalena Thurin, Giorgio Trinchieri, Sjoerd H. van Der Burg, Theresa L. Whiteside, Jon M. Wigginton, Francesco Marincola, Samir N. Khelif, Bernard A. Fox, Mary L. Disis
Clin Cancer Res; 17(10) May 15, 2011

SITC/iSBTc Cancer Immunotherapy Biomarkers Resource Document

Davide Bedognetti, James M. Balwit, Ena Wang, Mary L. Disis, Cedrik M. Britten, Lucia Gemma Delogu, Sara Tomei, Bernard A. Fox, Thomas F. Gajewski, Francesco M. Marincola, Lisa H. Butterfield
J. Translational Medicine, 2011

Participating Organizations:

- Association for Immunotherapy of Cancer (CIMT)
- Biotherapy Development Association (BDA)
- Cancer Vaccine Consortium (CVC (now CIC)) of the
Cancer Research Institute (CRI)
- Food and Drug Administration (FDA)
- Italian Network for Tumor Biotherapy (NIBIT)
- Japanese Society of Cancer Immunology (JSCI)
- National Cancer Institute (NCI)
- National Institutes of Health (NIH)
- Nordic Center for Development of Antitumour Vaccines
(NCV-network)

Variability:

1. Patient
2. Blood draw
3. Processing/cryo/thaw
4. Cellular product
5. Assay choice
6. Assay conduct
7. Assay analysis
8. Data reporting
9. Next cool new assay

Recommendations:

1. Save DNA/RNA/cells/tumor; include healthy donor control
2. Standardized procedures
3. Standardized procedures
4. Functional assays to characterize/develop potency
5. Standardized, functional
6. SOP
7. Appropriate biostatistical methods
8. Full details, controls, QA
9. Sufficient blood/tissue to interrogate the samples *now*, as well as *later*, to generate new hypotheses.



Analysis of antigen-specific T cell responses has become an increasingly used tool in immune monitoring from basic research to clinical trial settings. Although an increasing number of promising data from correlative studies has been published, the lack of conformity on reporting such data hampers the ability to objectively evaluate data.

Minimal Information About T cell Assays (“MIATA”): we propose to generate recommendations on the minimum information required to allow an objective and thorough interpretation of published results from immunological T cell assays. Any recommendation in the proposed Modules is strictly based on supporting published data.

MIATA has two main goals that will be addressed in a two-step approach:

Mid-term goal of MIATA: Establishment of a minimal information reporting framework for human T cell immune monitoring methods in scientific publications.

Long-term goal of MIATA: adapt the reporting framework for annotations of immune monitoring data sets from human studies for a central database, possibly in the context of a Human Immunity Project. This may include structured database vocabulary.

