Immune Monitoring

or

Why study human immune responses to cancer, what should be examined, and how?

Lisa H. Butterfield, Ph.D.
Associate Professor of Medicine, Surgery and Immunology
Director, UPCI Immunologic Monitoring and Cellular Products Laboratory
University of Pittsburgh, UPCI

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 Javier Mestas and Christopher C. W. 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	Expressed 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?

Issues of Variability

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

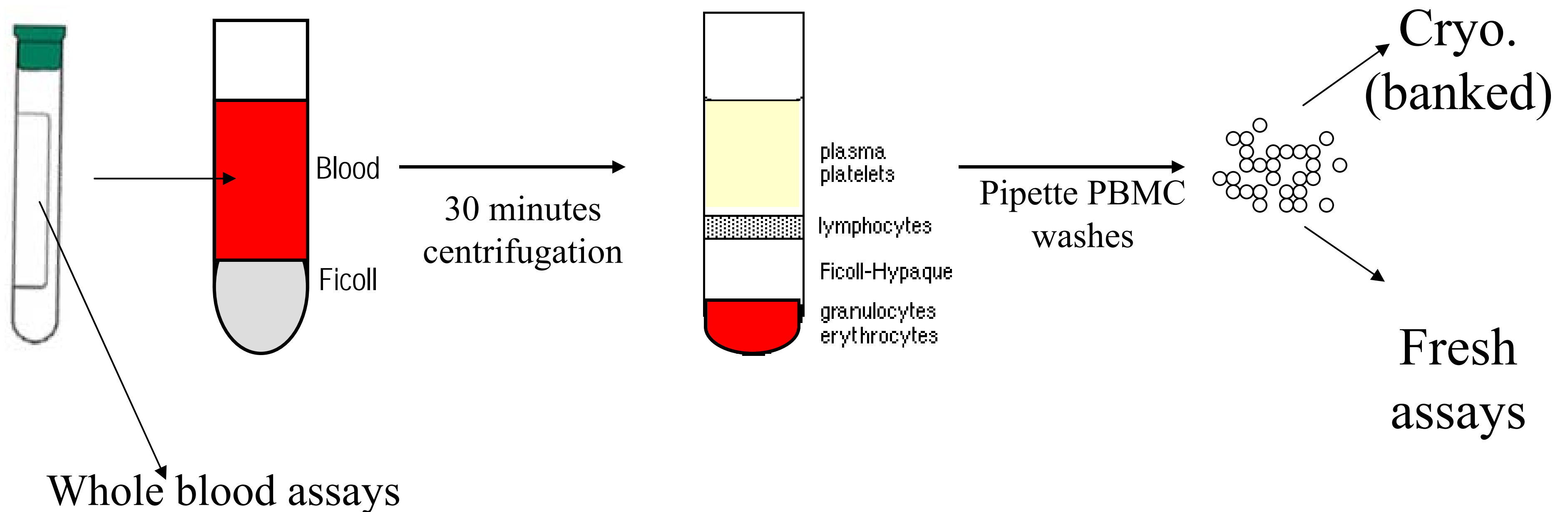
Survey of different types of assays, limitations

Example of clinical trial analysis design issues

Standardization and Validation

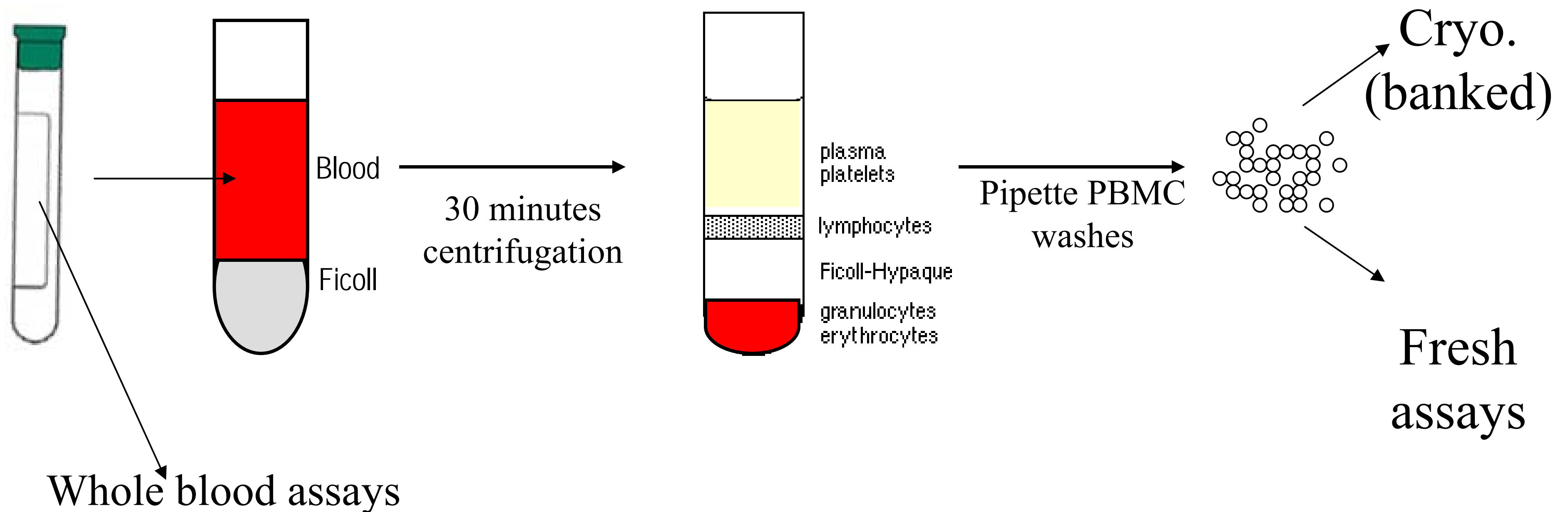
Peripheral Blood: easy to obtain at multiple time points

Separation of blood components on a Ficoll-Hypaque gradient.



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

Peripheral Blood



Variability: *Hemolysed? Correct anti-coagulant in tube (heparin)? 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

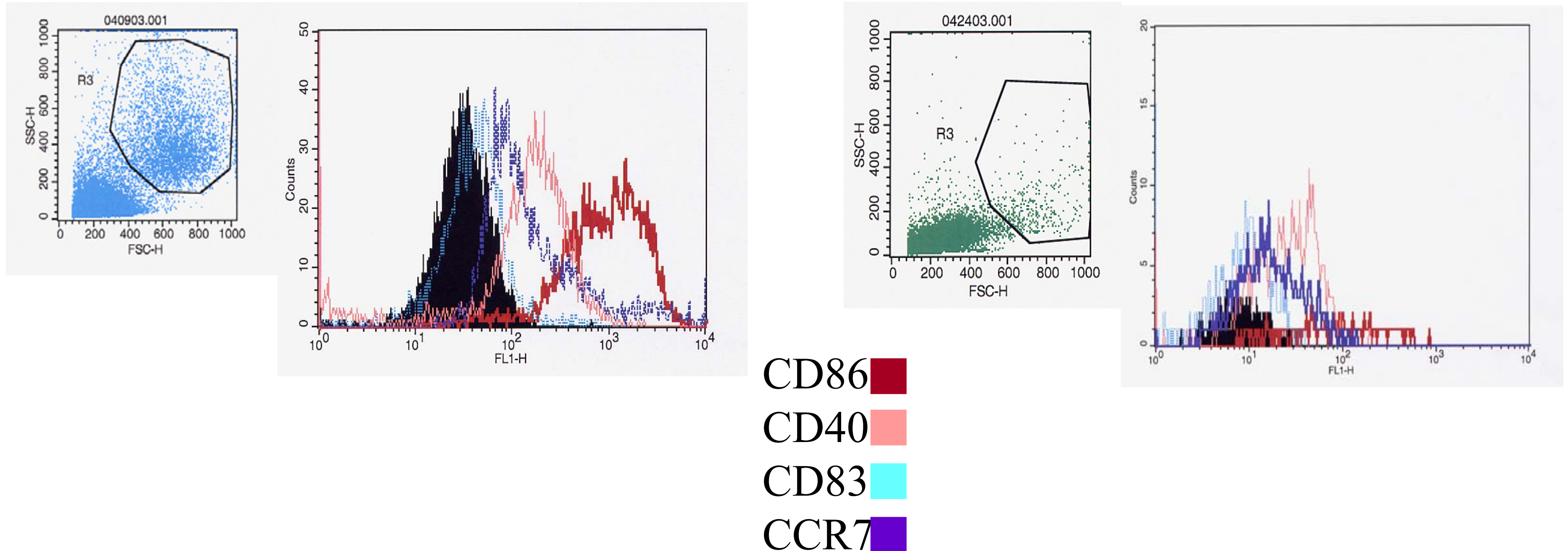
CD3+:	986-2,512 cells/ul	(2.5x)
CD3+/CD4+:	39-67%	(1.7x)
CD3+/CD8+:	9-43%	(4.7x)
CD19:	4-20%	(5.0x)
CD16+/CD56+:	3-16%	(5.3x)

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 “monocyte fraction”: 75-97%
DC recovery: 2-45%

Patient Autologous Vaccine Cells



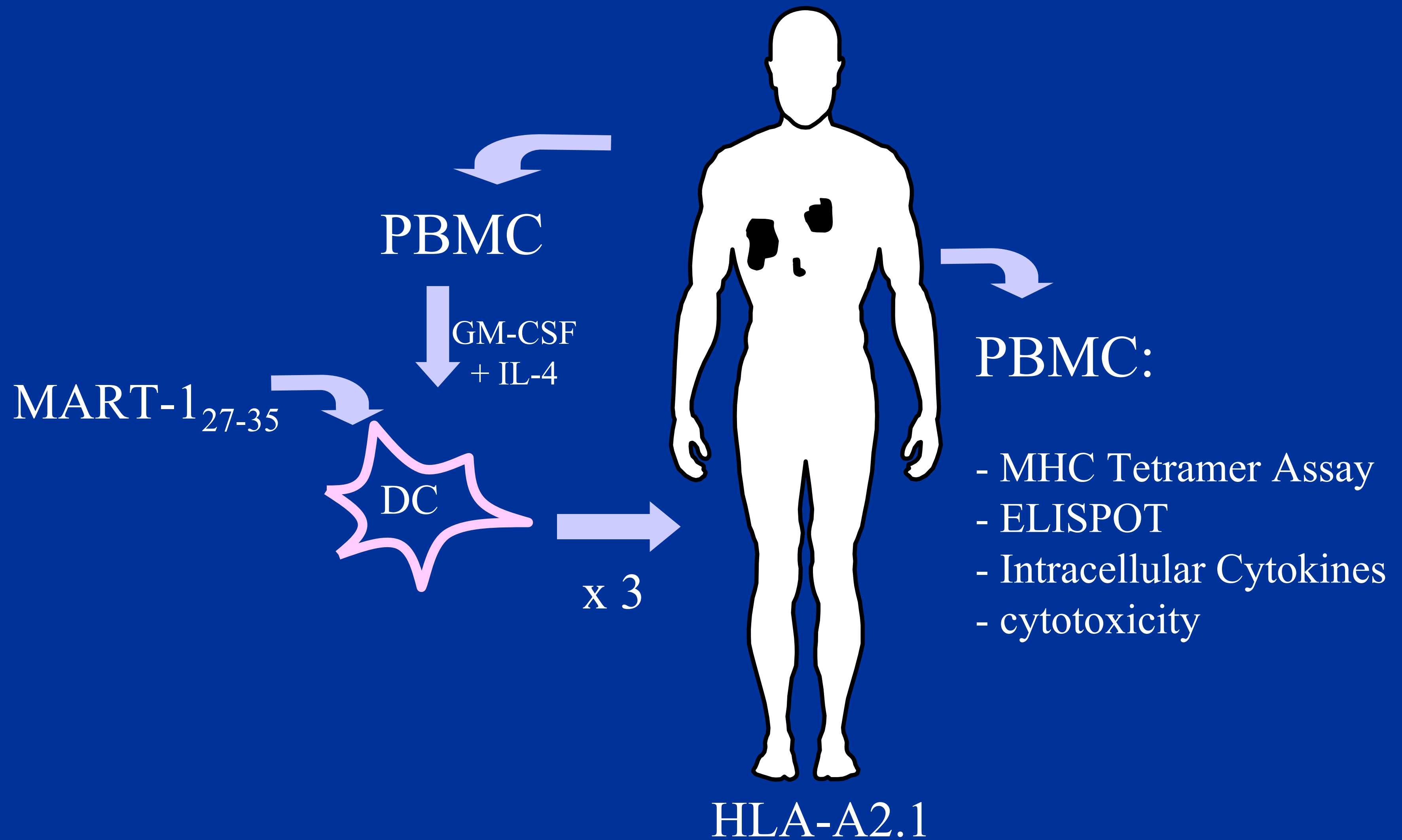
Example from an immunotherapy vaccine study. Some patients were able to expand large numbers of DC bearing cell surface markers CD40, CD83, CD86 and CCR7, but not all. **These 2 patients did not receive the same vaccine.**

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

Real life (historical) example:

Immunotherapy Clinical Trial Monitoring
(circa 1999-2000)

MART-$1_{27\text{-}35}$ Peptide-pulsed DC Clinical Trial Design



CLINICAL TRIAL DESIGN

DESIGN: Phase I/II dose-escalation clinical trial

TREATMENT GROUPS:

Group	No.	DC Dose	Route
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

Day:	Events:
-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. IFNg ELISPOT Cytokine Frequency
3. Intracellular Cytokine Staining (IFNg/IL-4)
4. Cytotoxic T Lymphocyte Assay

Immunological Assay Summary

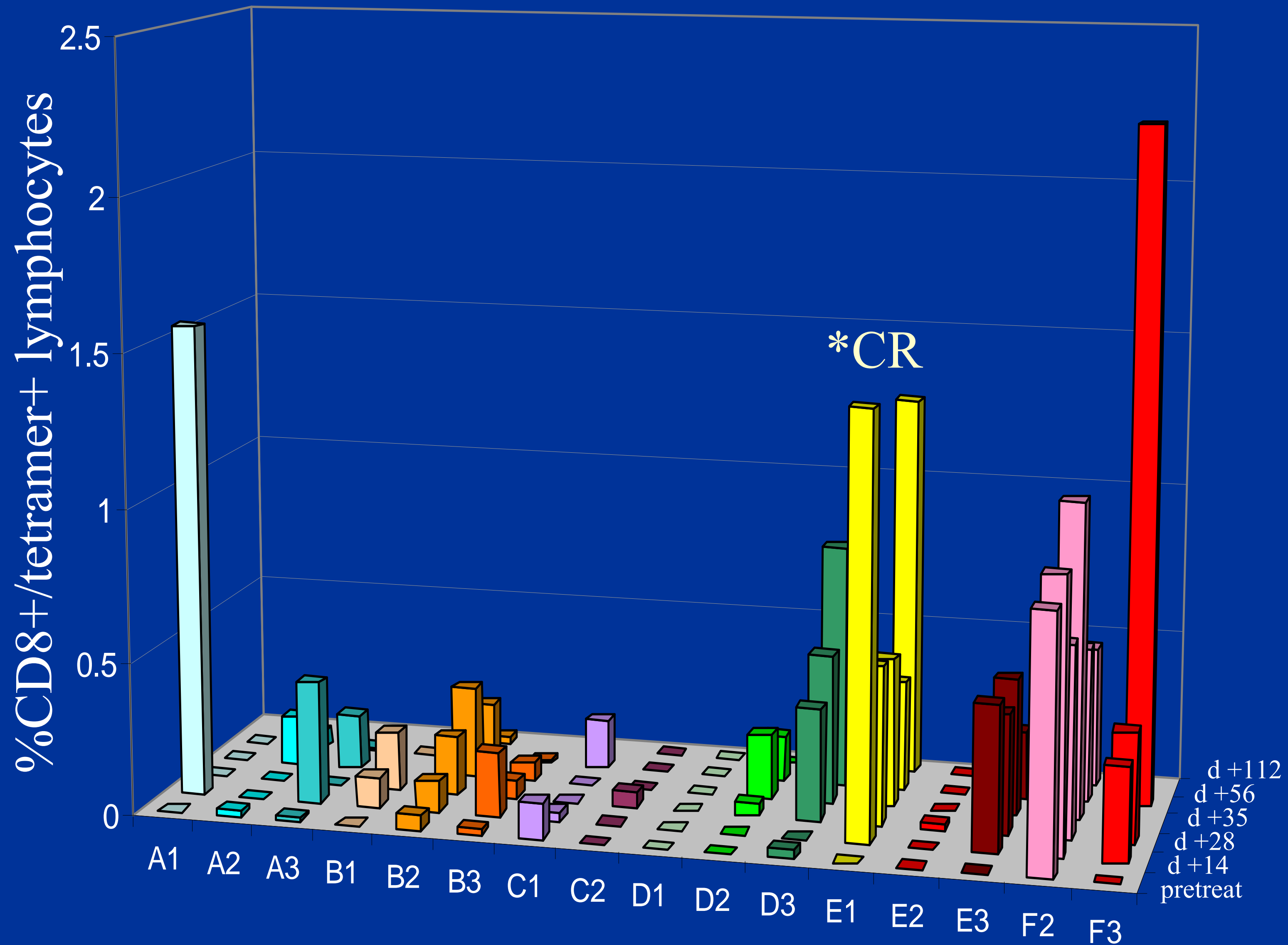
MHC Tetramer: Flow cytometry-based assay to detect MART-1_{27-35} specific T cells.

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

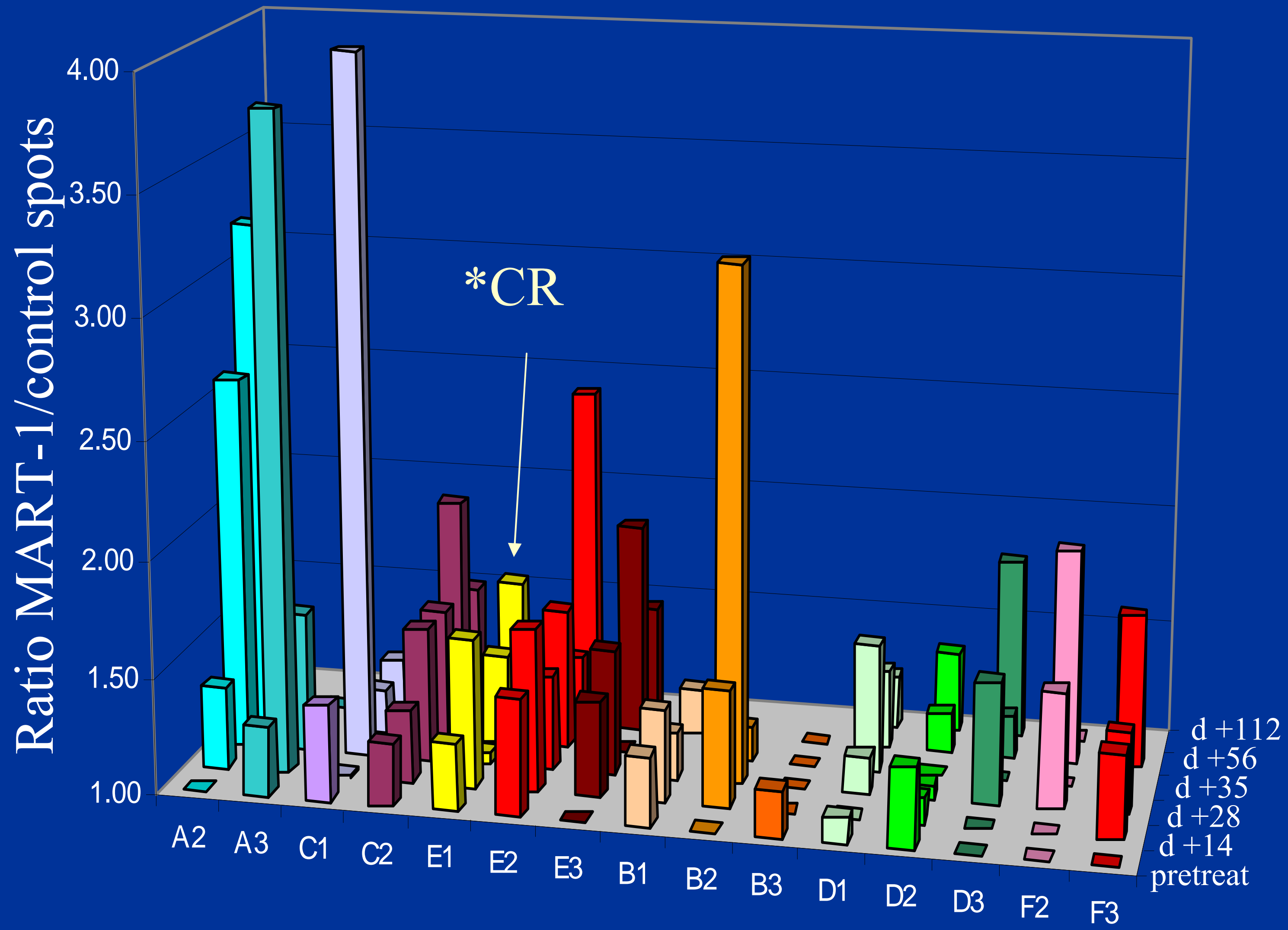
Intracellular Cytokines: Flow cytometry-based functional assay that allows for cell surface phenotyping of responding cells.

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

MHC Tetramer Analysis

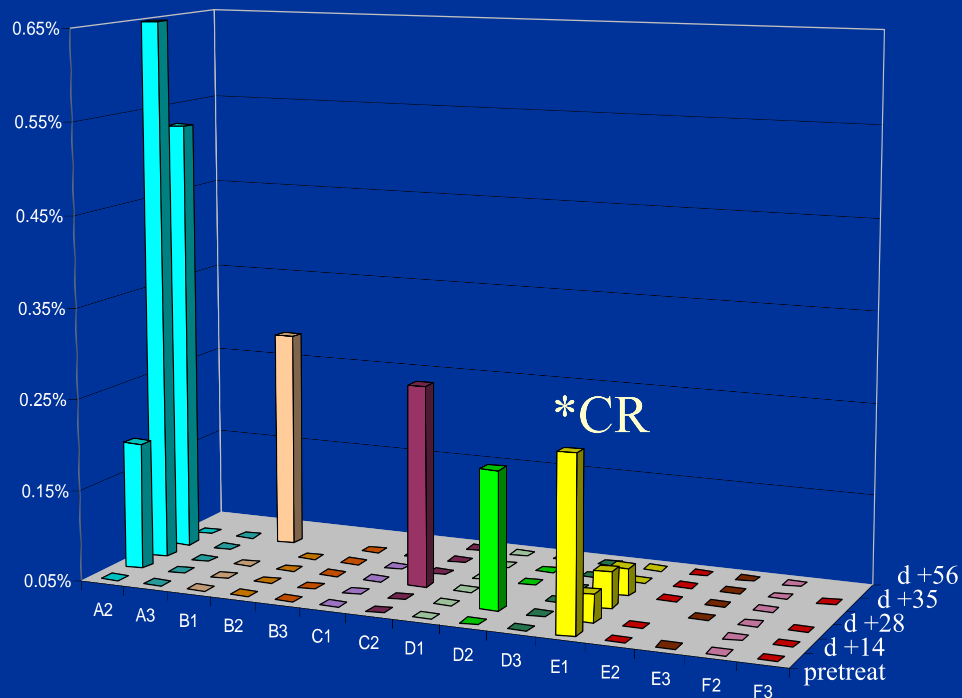


IFNg ELISPOT Analysis

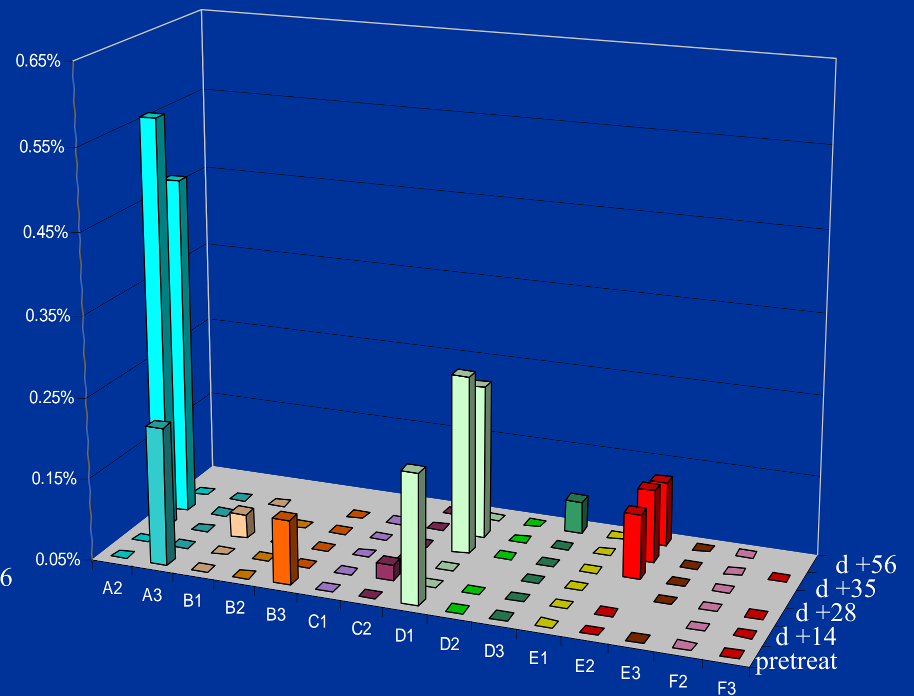


Intracellular Cytokine Analysis

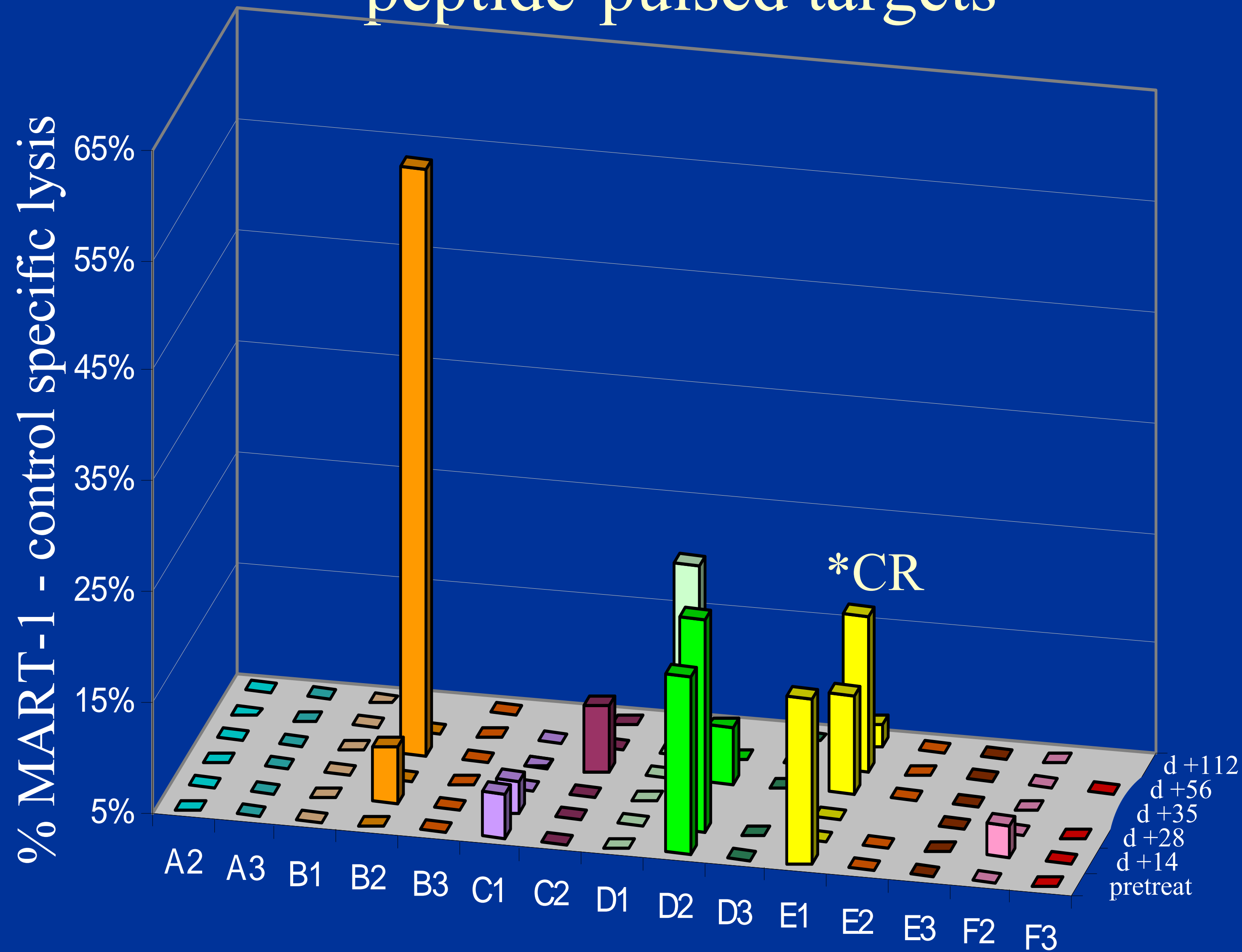
CD3+ MART-1 specific IFNg+ cells



CD3+ MART-1 specific IL-4+ cells

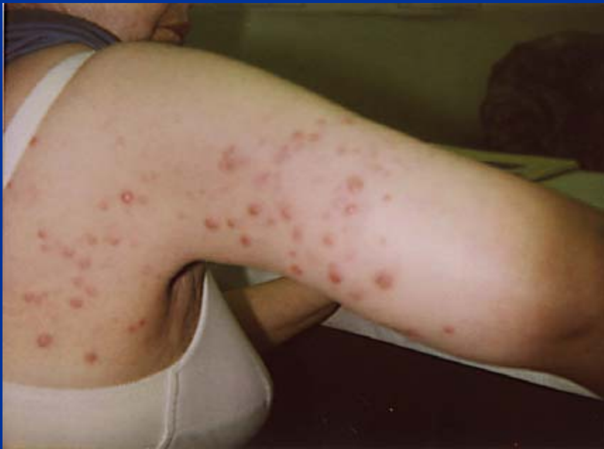


Cytotoxicity Analysis:
peptide-pulsed targets



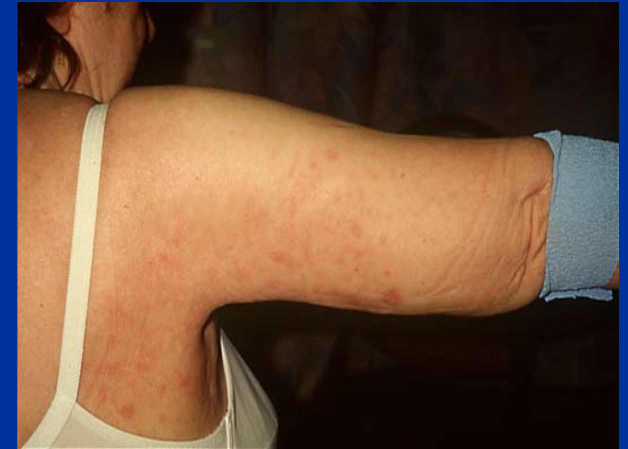
Patient E1 (now 9 years NED)

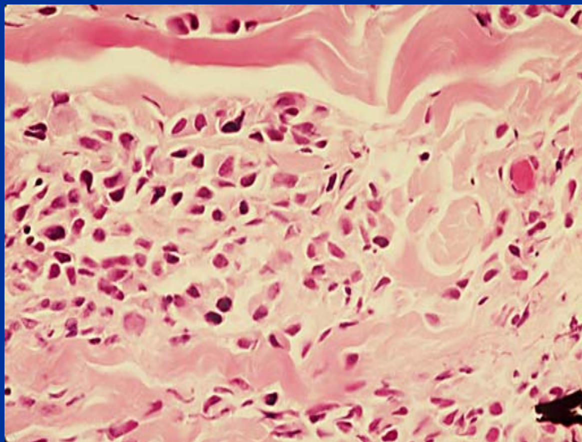
Pretreatment



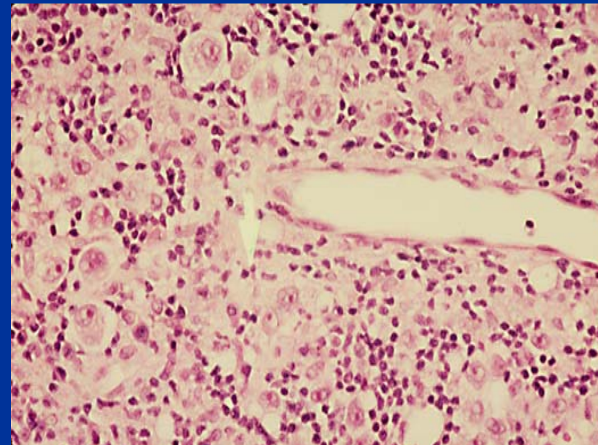
+ 56 days

+ 130 days

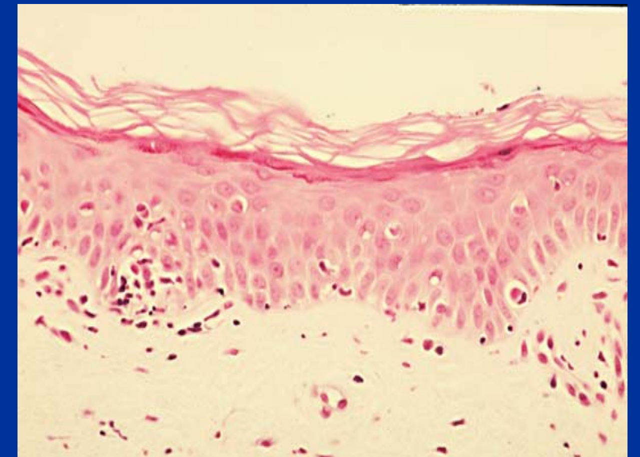




Melanoma Tumor



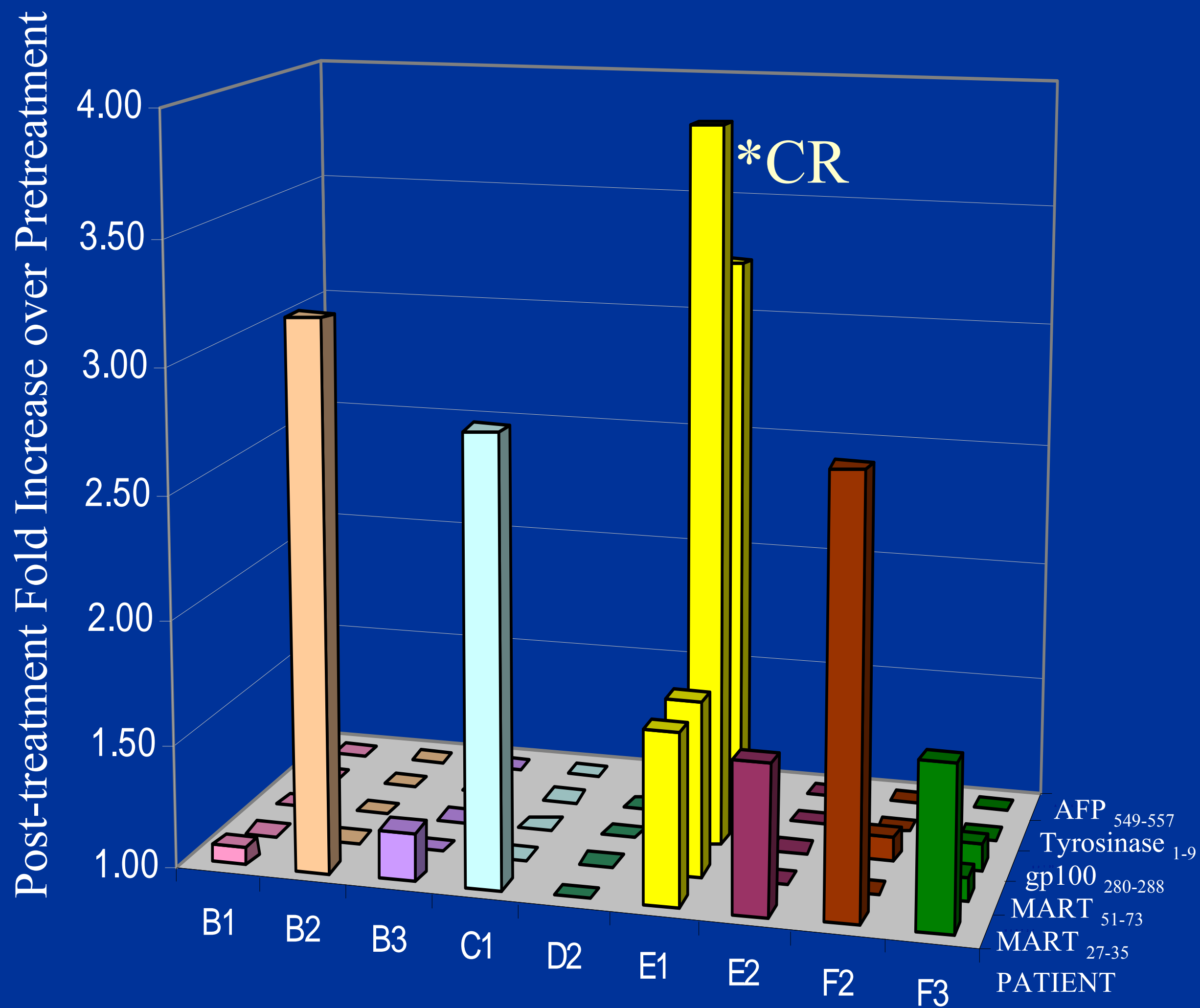
Lymphocytic Infiltrate

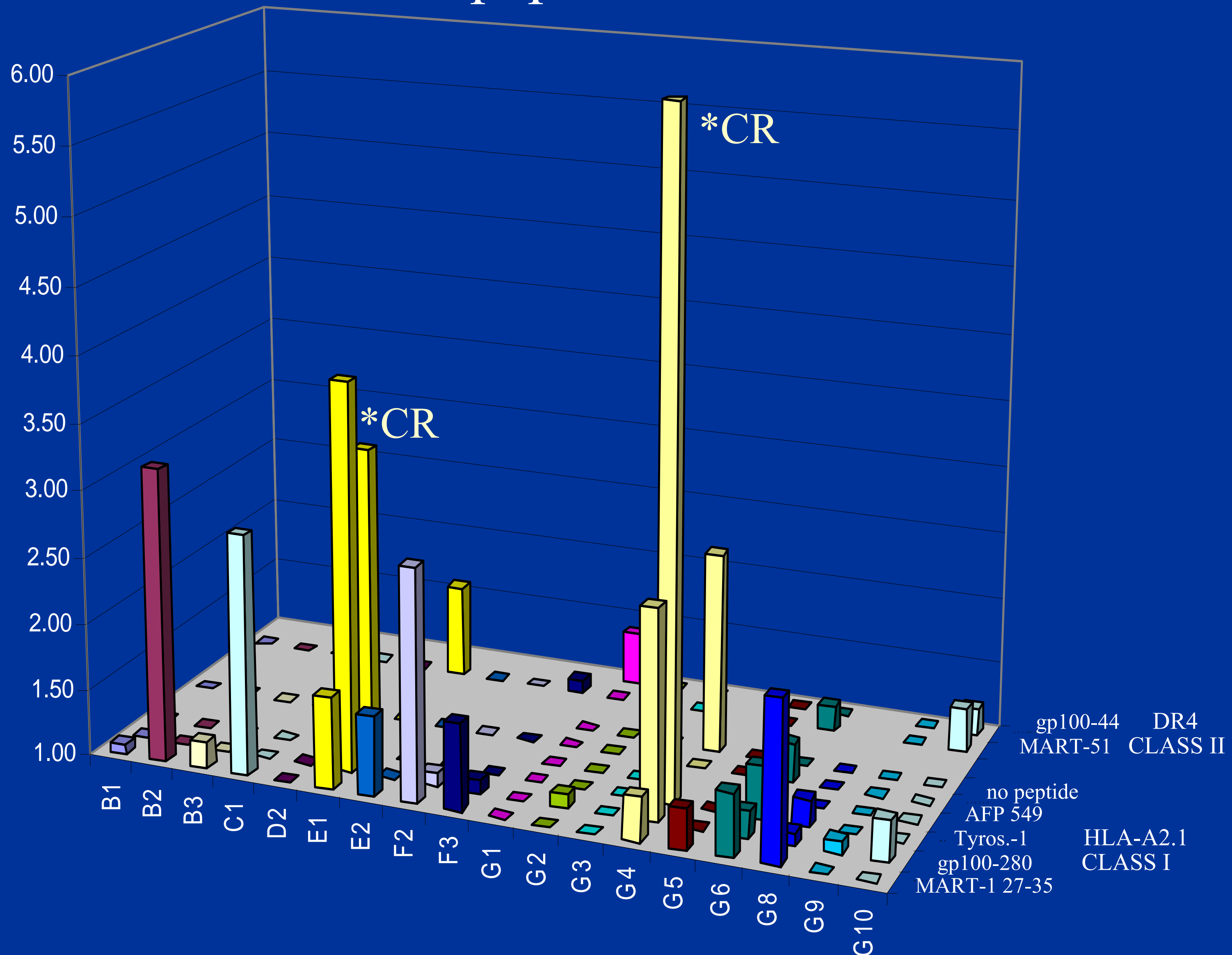


Absence of Melanoma

Patient E1: Determinant Spreading

IFNg ELISPOT




DETERMINANT SPREADING
MART-1 peptide/DC Trial
Post-treatment Fold Increase over Pretreatment
6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
*CR
*CR
B1
B2
B3
C1
D2
E1
E2
F2
F3
G1
G2
G3
G4
G5
G6
G8
G9
G10
gp100-44 DR4
MART-51 CLASS II
no peptide
AFP 549
Tyros.-1 HLA-A2.1
gp100-280 CLASS I
MART-1 27-35

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.

Which vaccine has superior efficacy and should be moved forward?

Vaccine A: IFNg/CD8+ ELISPOT of 20 spots/10e5

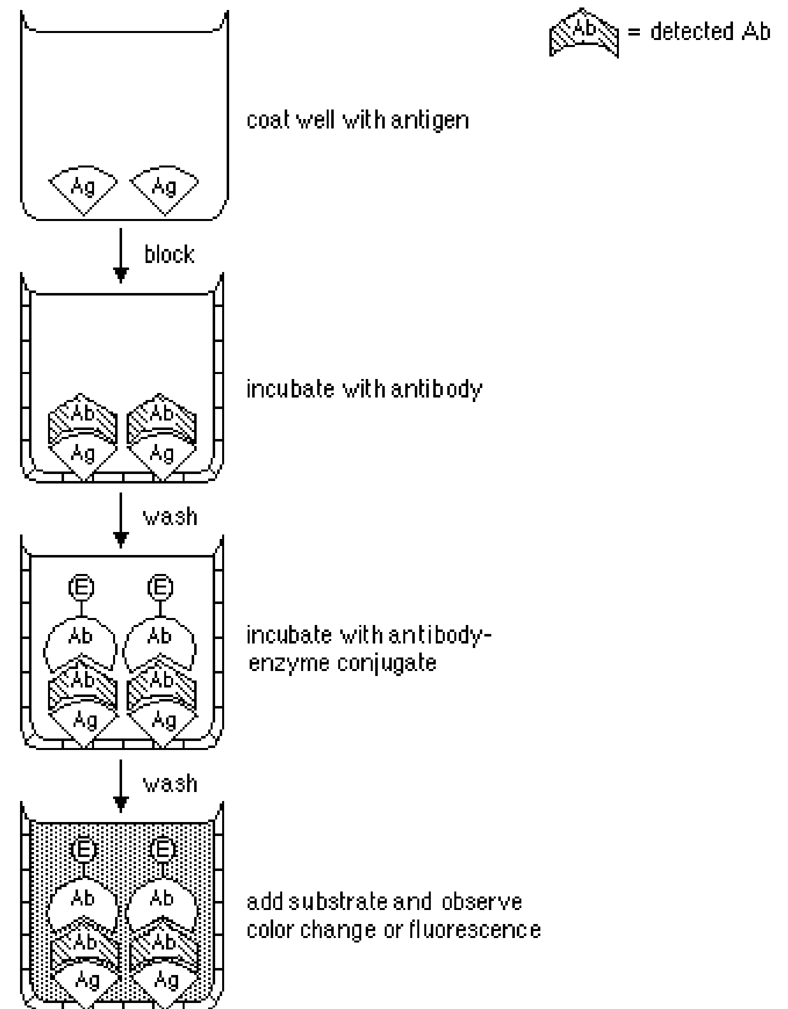
Vaccine B: IFNg/CD8+ ELISPOT of 200 spots/10e5?

Can you tell from the publications?

Survey of Assays

Enzyme-Linked ImmunoSorbent Assay (ELISA)

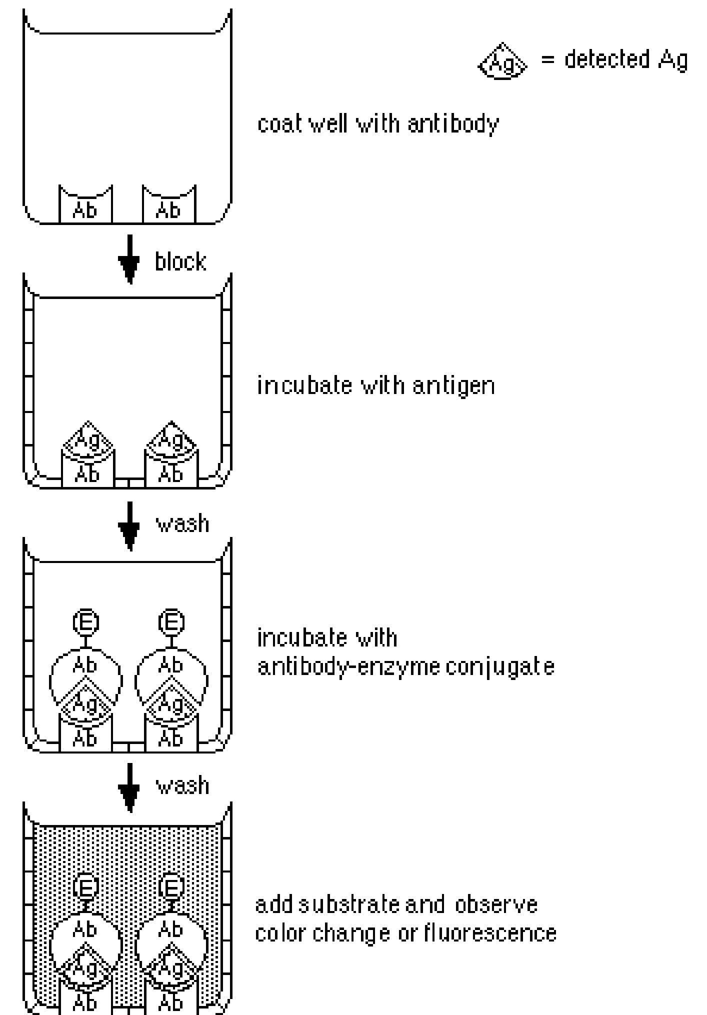
Indirect ELISA to detect specific **antibodies**.



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-1β, 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-1α, MIP-1β, IP-10, MIG, Eotaxin, RANTES, MCP-1, VEGF, G-CSF, EGF, FGF-basic, and HGF

in serum, plasma, or tissue culture supernatant.

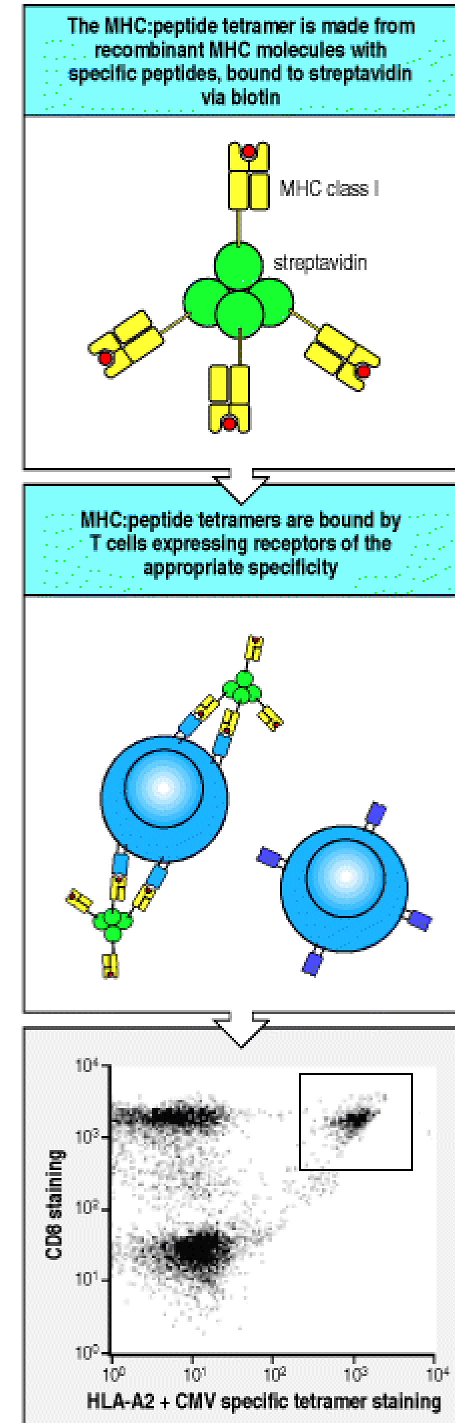
Sample size: 50 ul

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

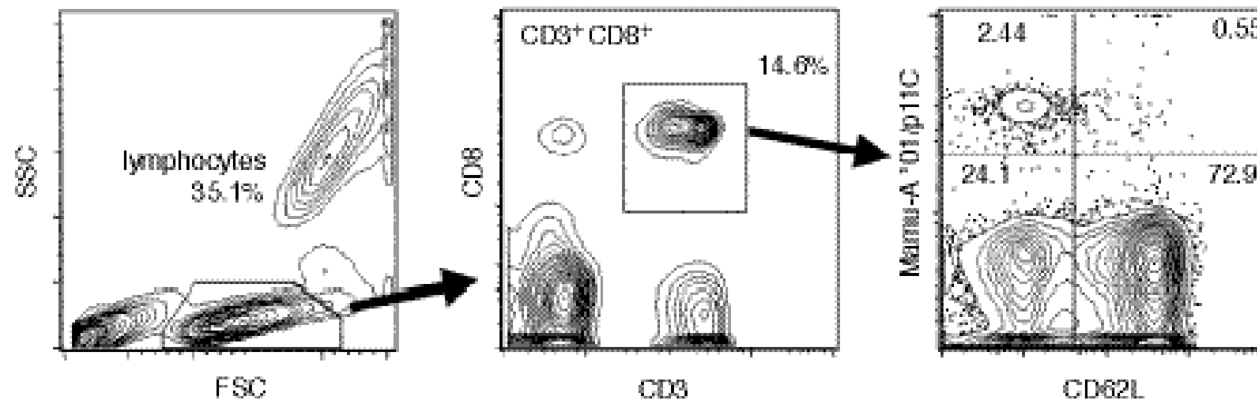
MHC:peptide tetramers are formed from recombinant refolded MHC:peptide complexes containing a single defined peptide epitope. The MHC molecules can be chemically derivatized to Streptavidin via biotin, which creates a tetramer of MHC:peptide complexes.
While the affinity between the T-cell receptor and 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

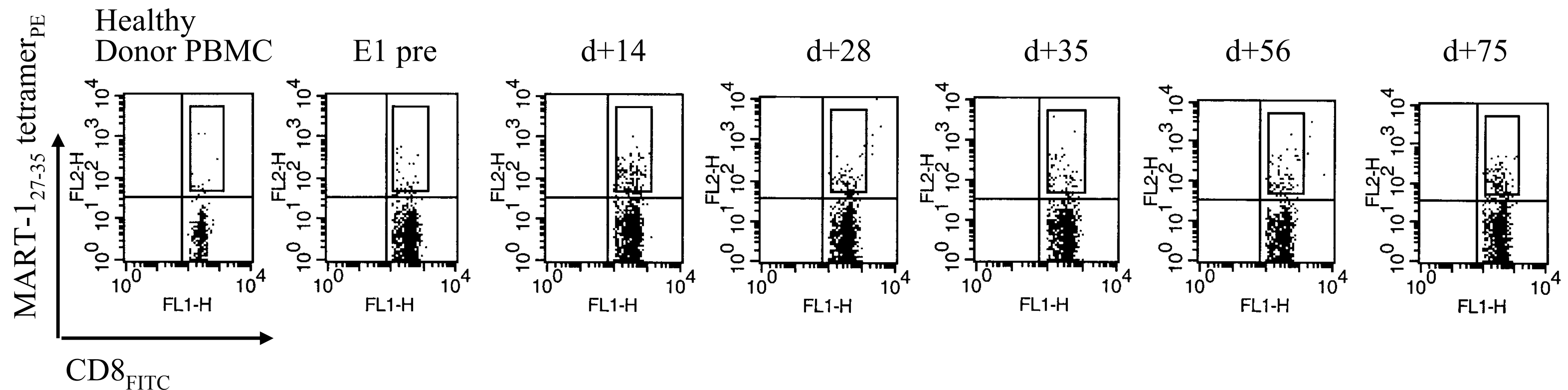


MHC-Peptide Tetramers to Visualize Antigen-Specific T Cells



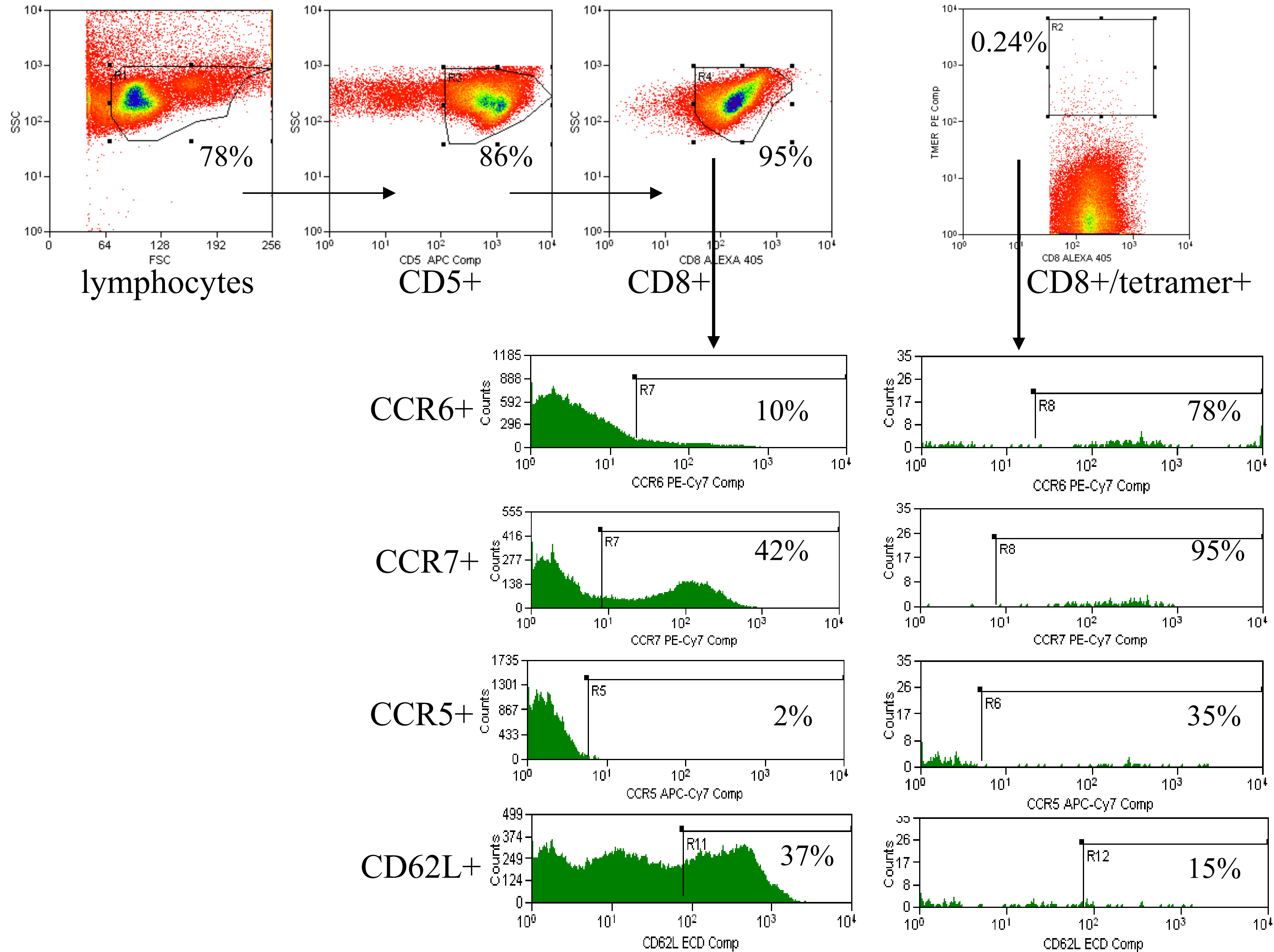
Data shows full gating strategy
Clear, distinct tetramer+ (or multimer+) population

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?

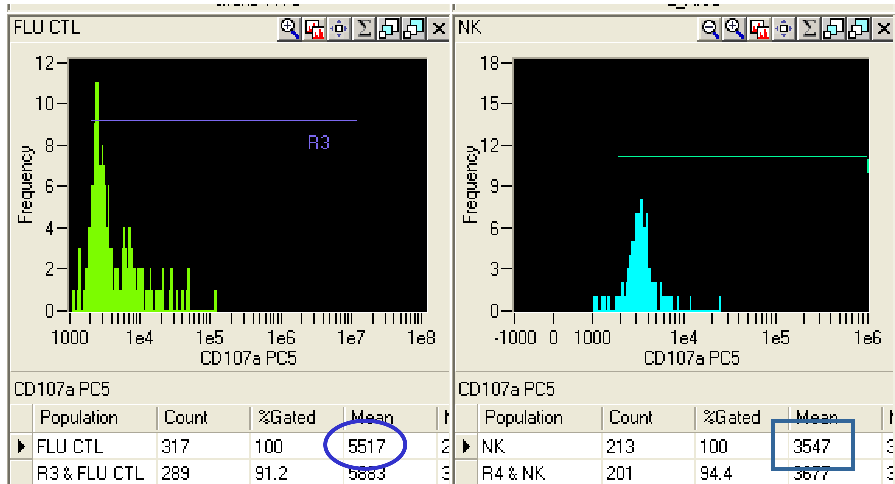
Patient B10 AFP_{137} Tetramer Phenotype Analysis Strategy



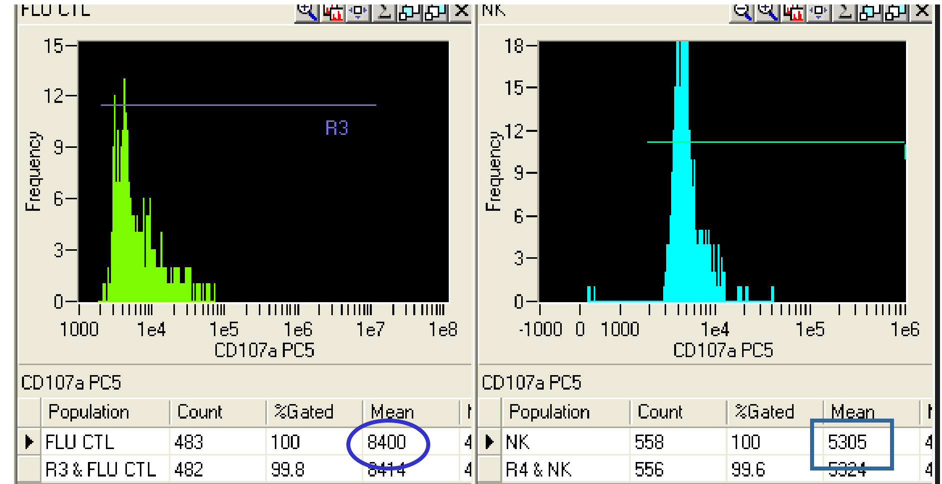
Granzyme B and CD107a Profiles—CTL and NK Cells (Amnis)

CTL + no treatment

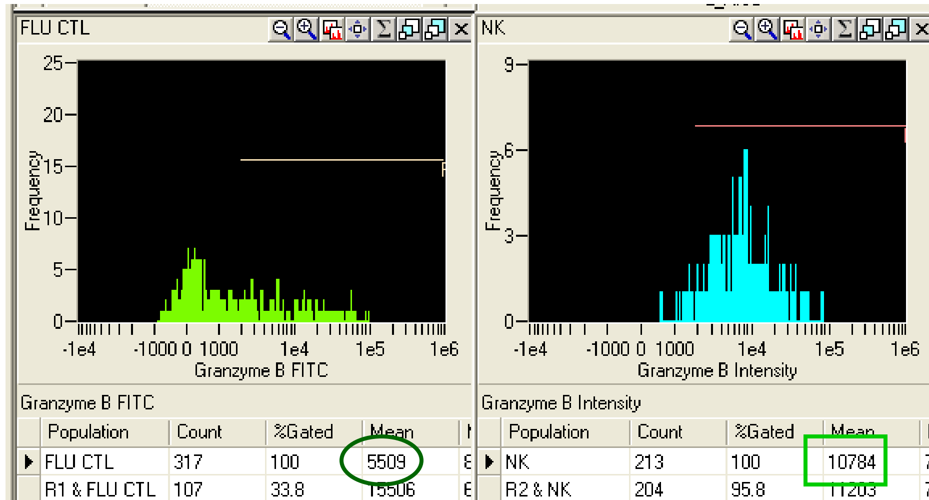
CTL + OKT3/IL-2

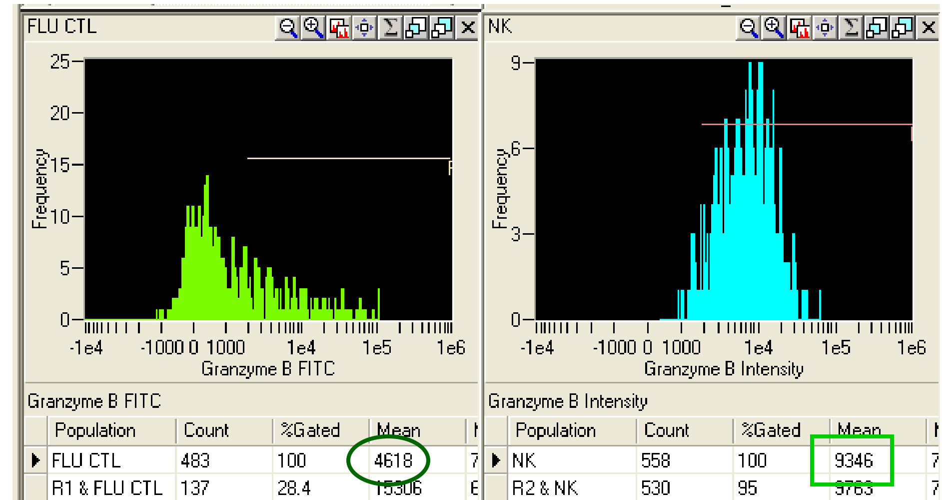


CTL NK



Increased CD107a in Flu CTL and NK cells

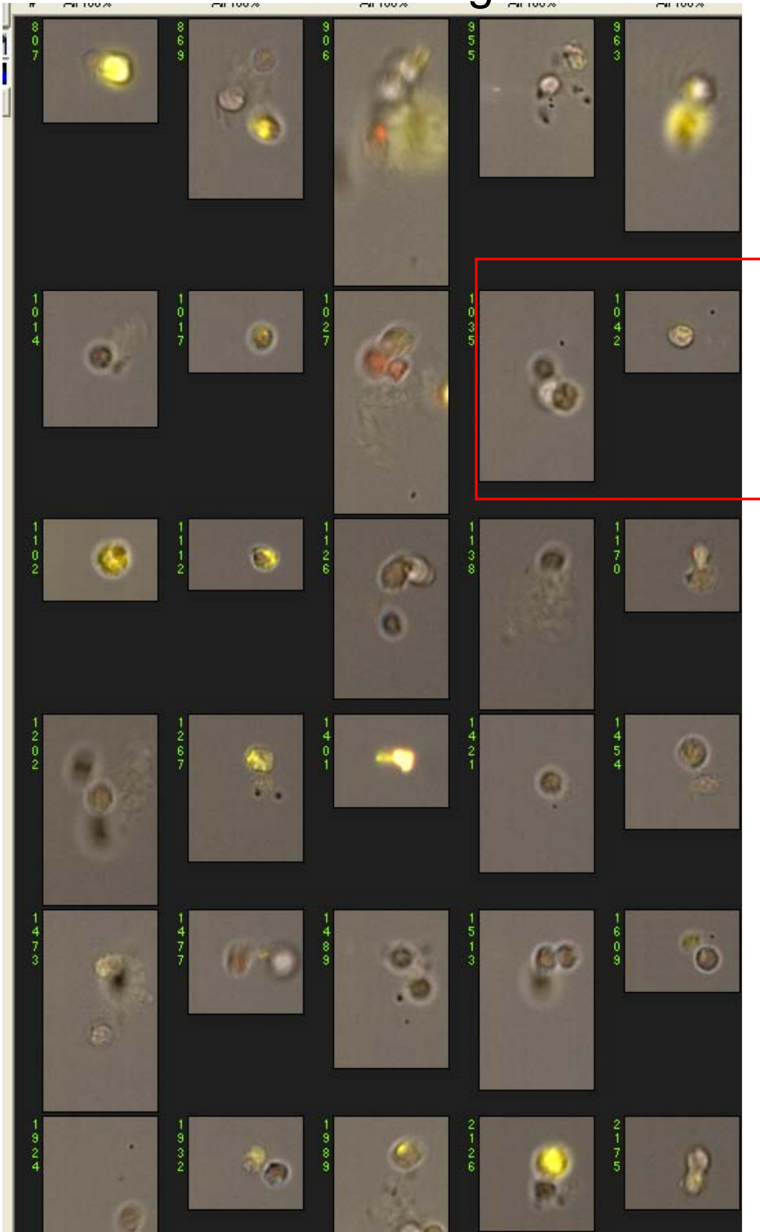




Decreased granzyme B in Flu CTL and NK cells

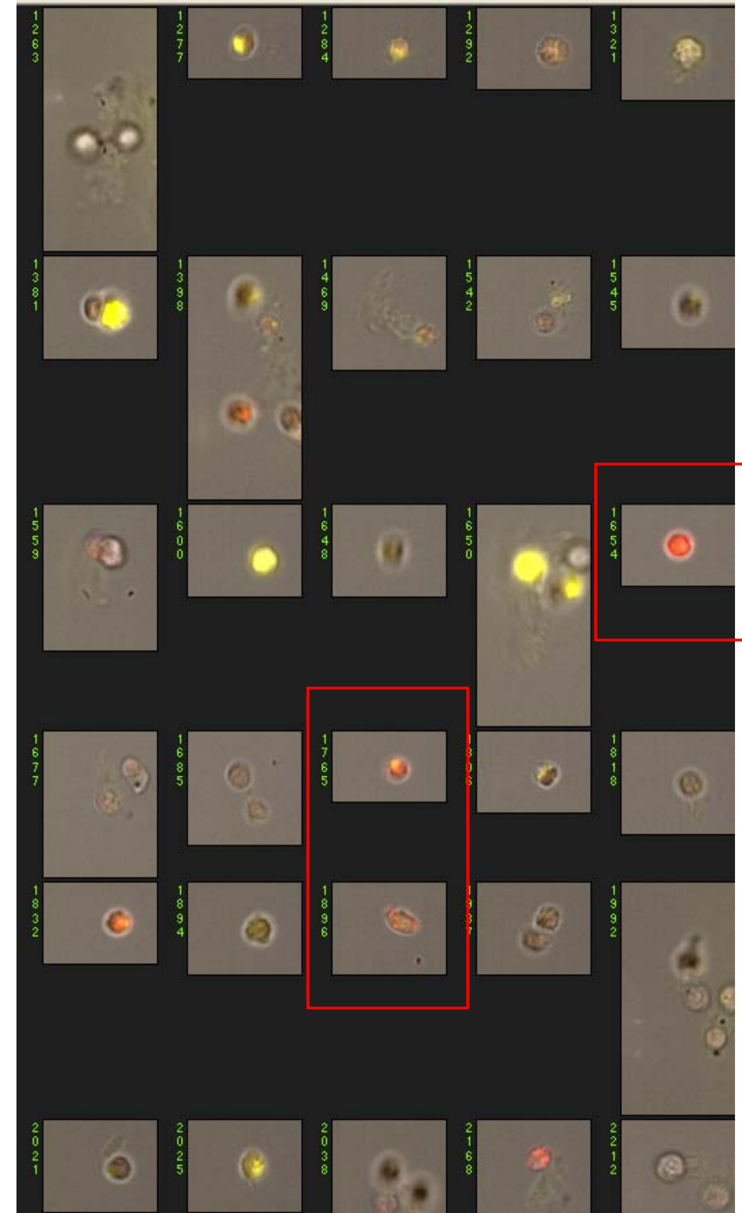
CTL (CD5+/CD8+) Granzyme B+/CD107a+ Cell Images

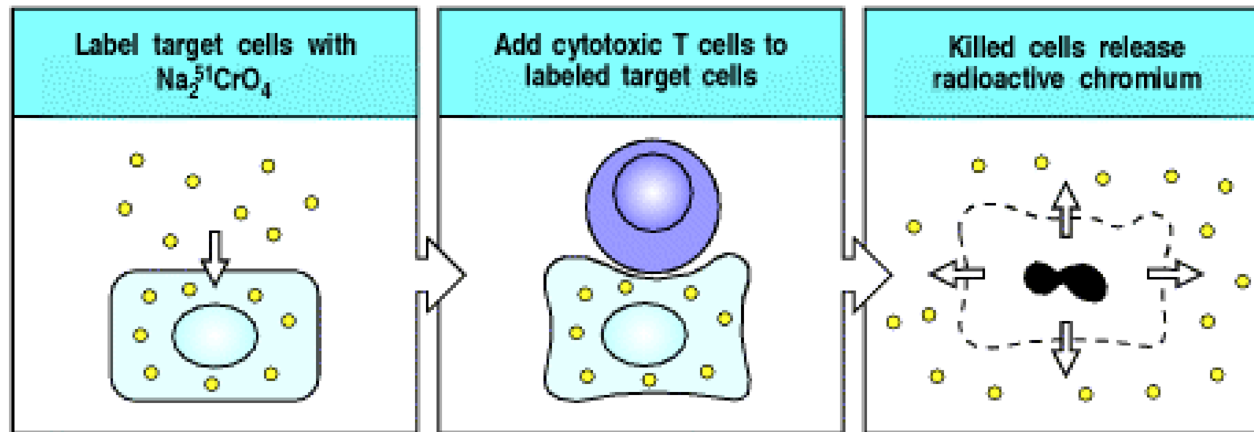
CTL + nothing



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 $Na_2{}^{51}CrO_4$, 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 TNFα.

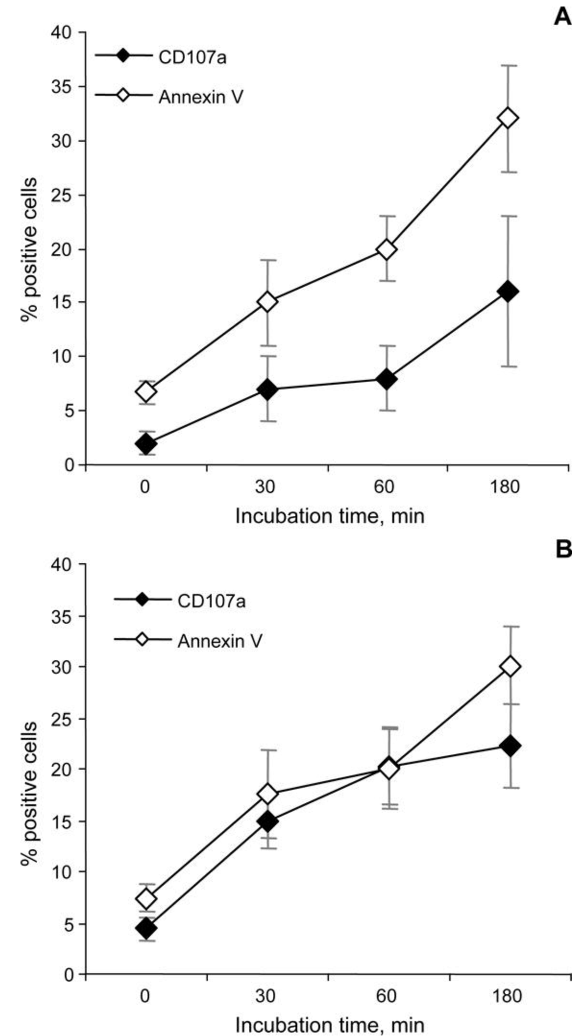
E:T ratios go as high as 100:1
Often requires in vitro stimulation to detect
Variably affected by cryopreservation

A Novel Flow Cytometric Assay for Evaluating Cell-Mediated Cytotoxicity

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

CD107a expression by anti-peptide CTLs correlates with *annexin V binding by specific targets* as measured in the flow cytometric assay.

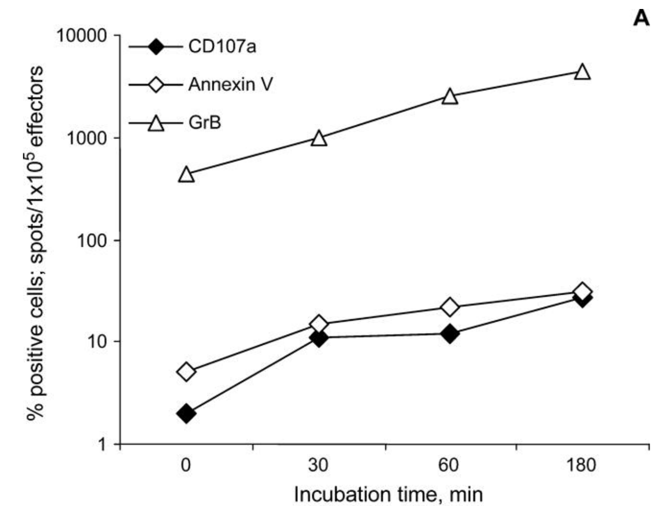
As CD107a appears on the surface of effector CTL over 3 hours, the pre-apoptotic marker Annexin-V appears on the surface of specific target cells.

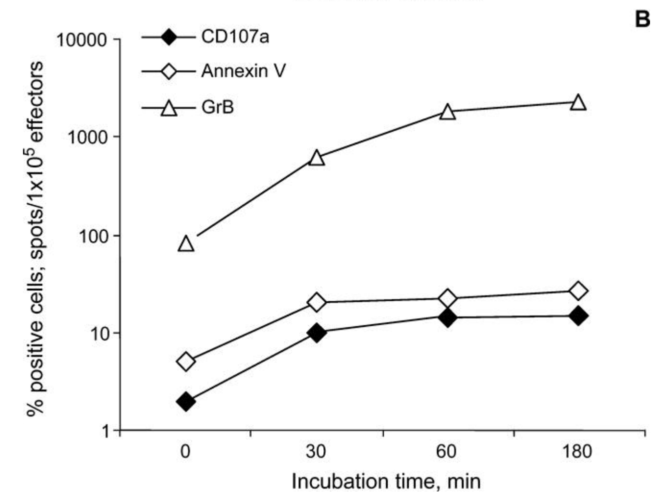


A Novel Flow Cytometric Assay for Evaluating Cell-Mediated Cytotoxicity

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.





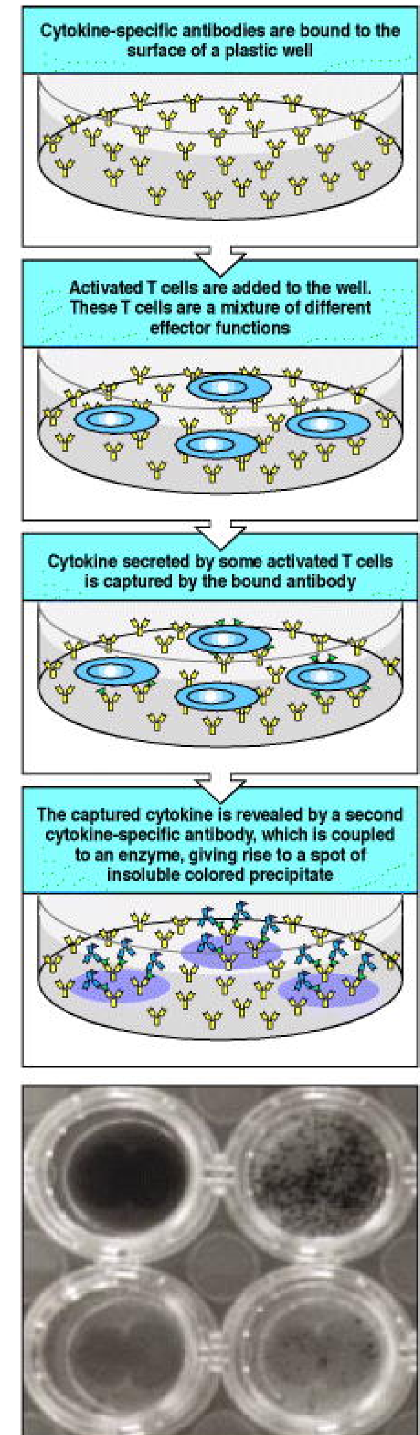
CD107a surface expression also correlates with Granzyme B release detected by ELISPOT.

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.



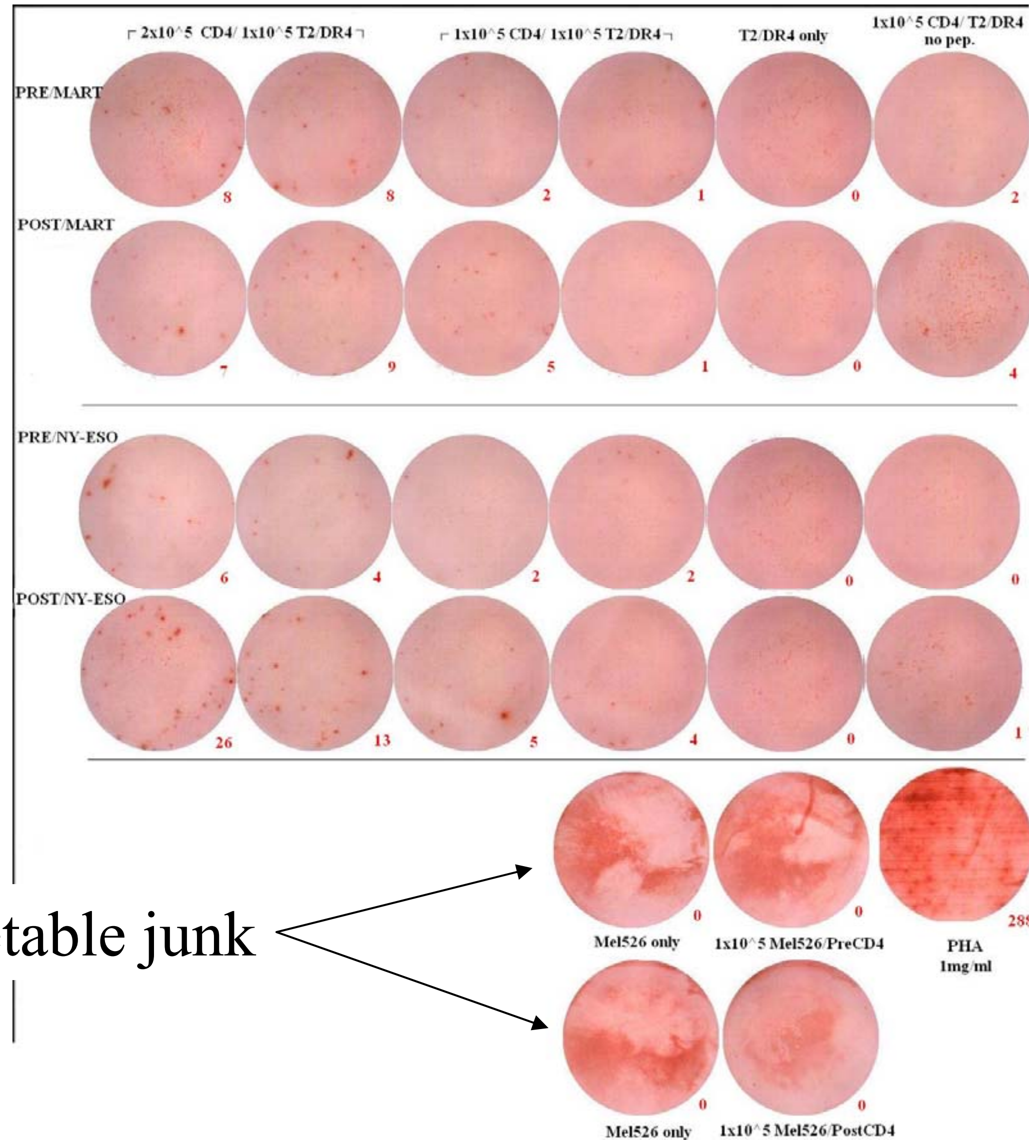
IFNg ELISPOT

Melanoma Antigen Peptides, CD4+ T cells

Same frequency
pre vs. post

Increased frequency
Post vaccine

Un-interpretable junk

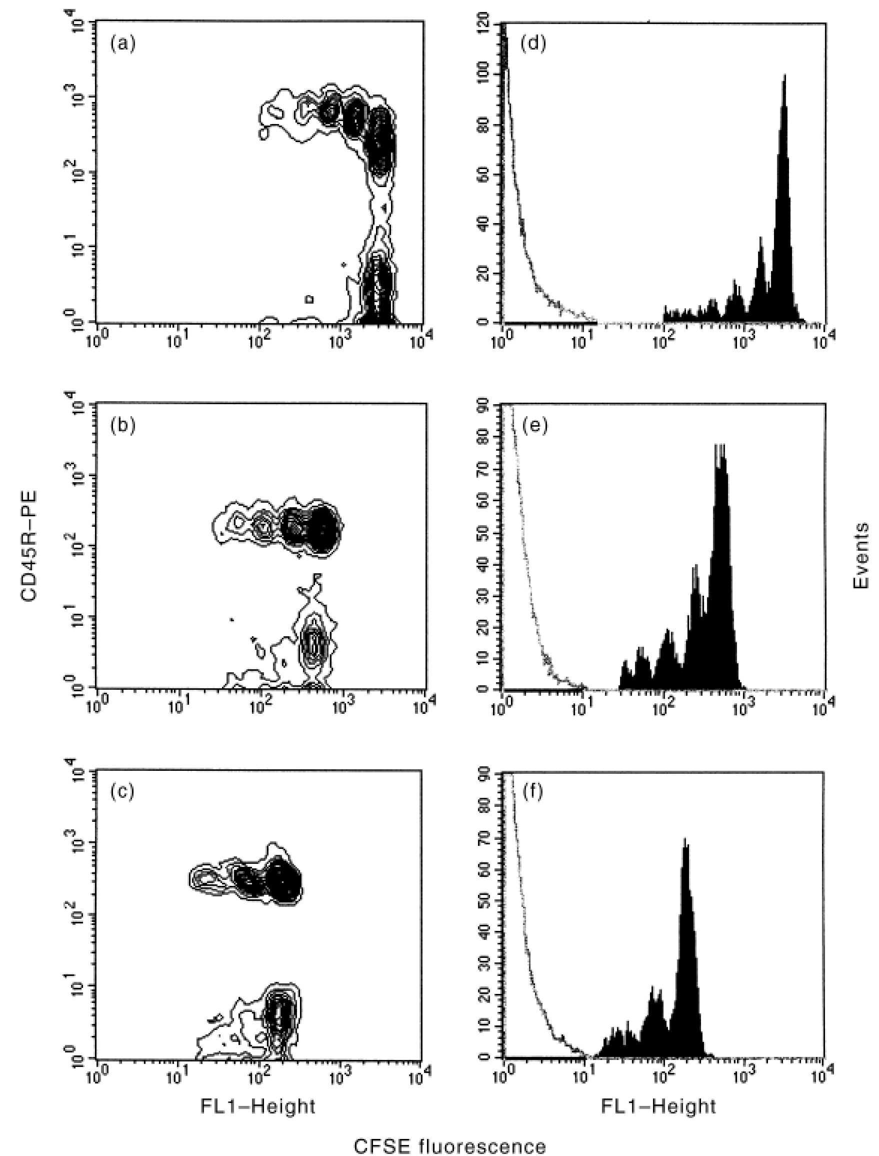


CFSE Proliferation

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

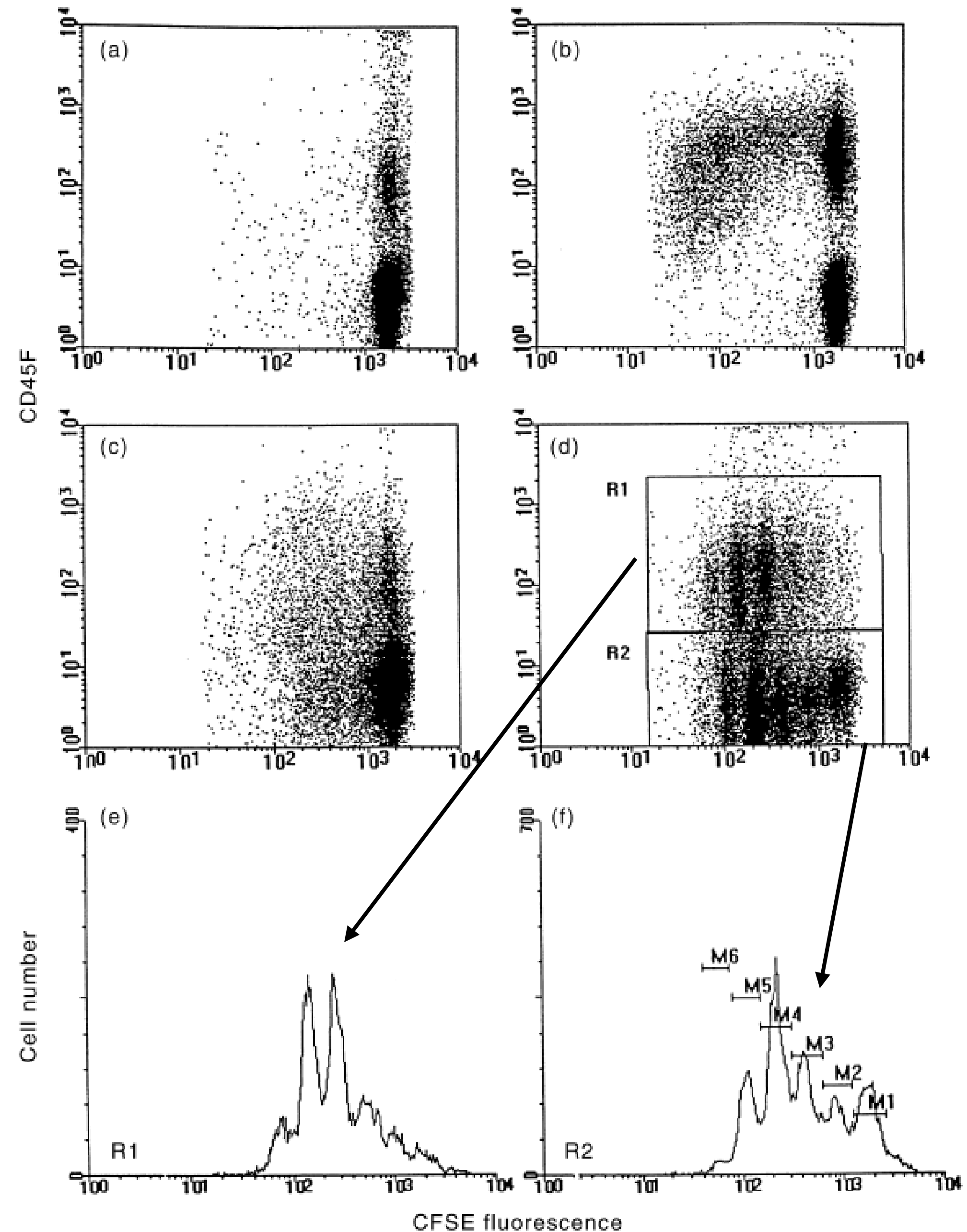
This is a cell division analysis procedure based on the quantitative serial halving of the membrane permeant, stably incorporating fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE), that covalently attaches to free amines of cytoplasmic proteins.

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

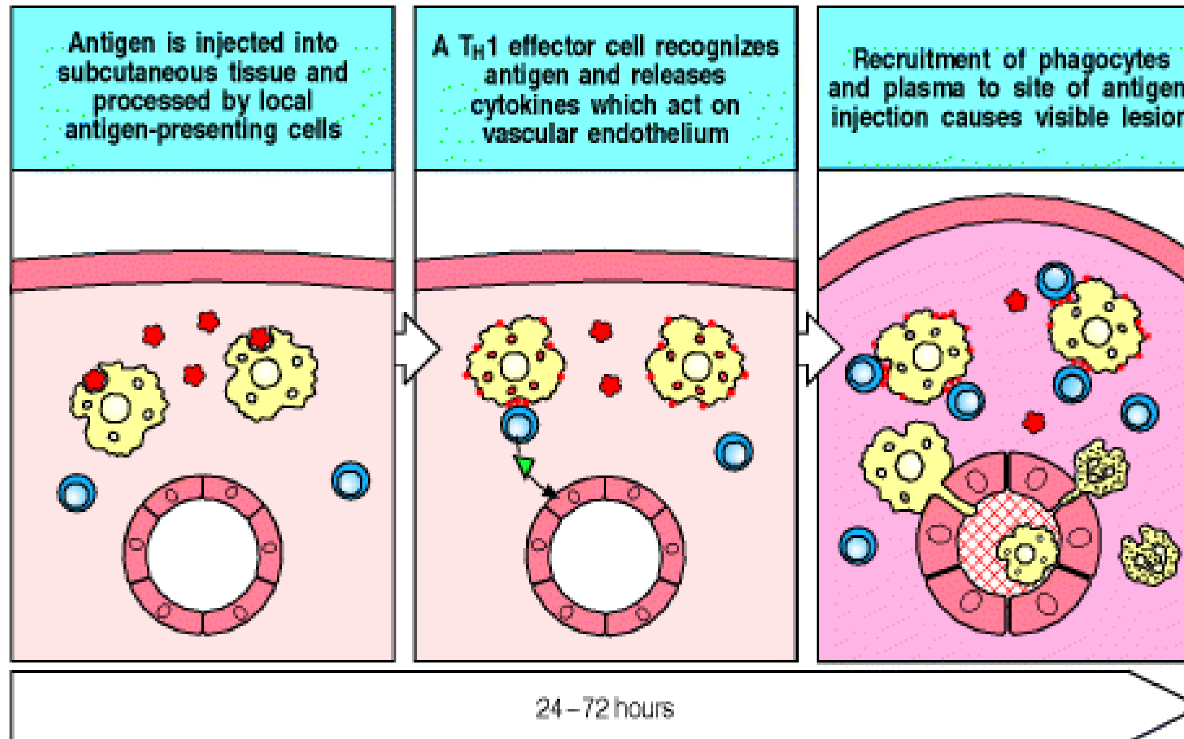


CFSE Proliferation/Cell Surface/Function

Can follow cells which proliferate and have surface markers CD3, CD4 CD8, etc., or have function (IFNg)



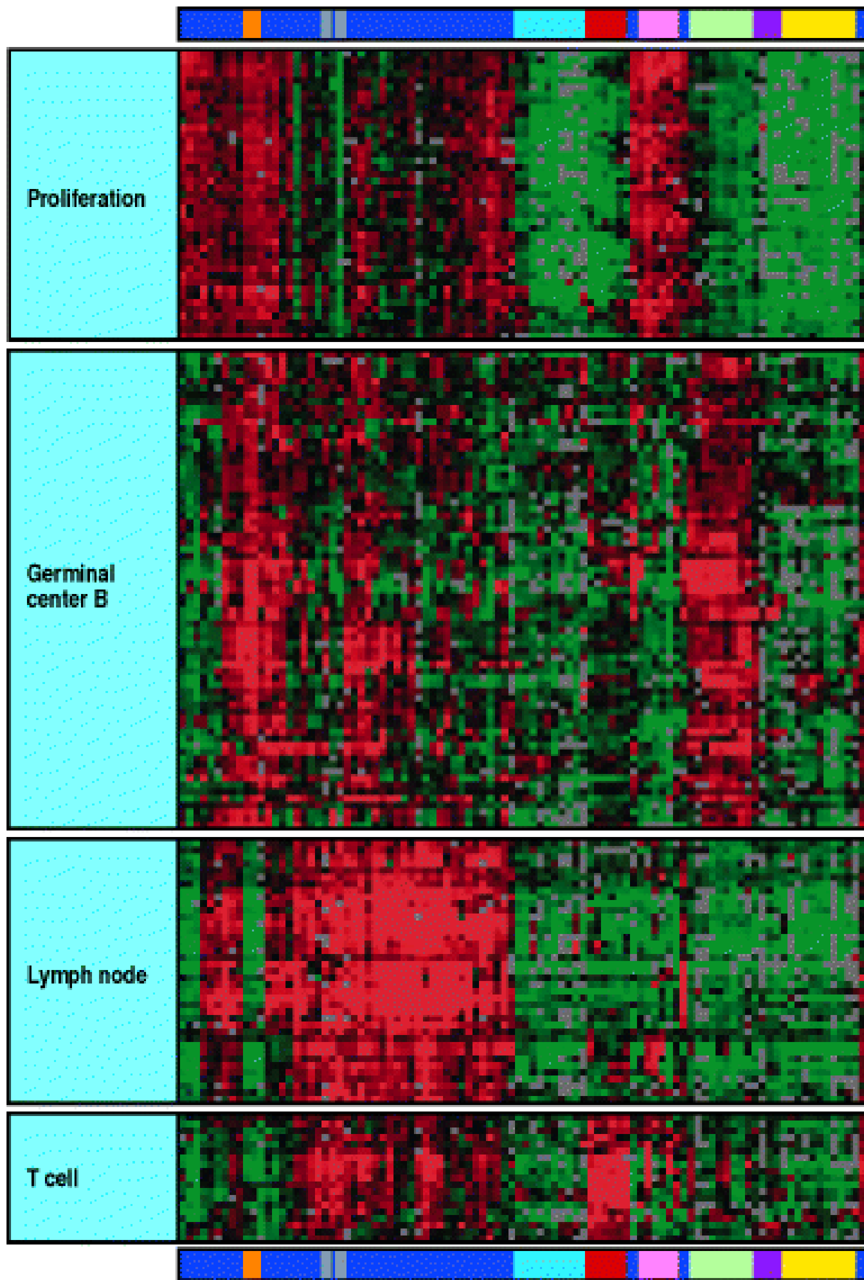
Delayed Type Hypersensitivity Reaction



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.



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

Green is expressed at lower levels than in a control cell, while red represents higher expression.

Design of Hypothetical Clinical Trial Immunological Monitoring

Practical Considerations

Culture of cells (Blood/PBMC):

Ex vivo /"direct" assay: either thaw and test
or short restimulation (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)

Culture of cells:

Ex vivo /"direct" assay: either thaw and test

or short restimulation (4-6 hours)

+snapshot of actual state of cells

-may not allow function assessment

Multimer stain, subset phenotype, ICS

Overnight restimulation

+not enough culture to drastically change function

-may not allow detection of very rare activity

ICS, ELISPOT

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)

ICS, ELISPOT, proliferation, 51Cr cytotoxicity

Antigen Presenting Cells:

PBMC

Dendritic Cells

T2

T2/DR4

K562/A2.1

(Mitogen)

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

Responding Cell Purification:

Total PBMC

Non-adherent PBMC

CD4+

CD8+

PBMC with CD4+ removed

Subset Purification:

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

Non-adherent PBMC: Remove monocytes/macrophages for DC

CD4+: test only helper T cells (and T_{reg})

CD8+: test only effector T cells

PBMC with CD4+ removed: remove helpers/T_{regs}

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?

IFNg/CD8+ ELISPOT of 20 spots/10e5
vs.
IFNg/CD8+ ELISPOT of 200 spots/10e5?

IFNg+IL-2+TNFa multi-functional CD8+ T cells
vs.
IFNg+TNFa vs. TNFa expressing CD8+ T cells?

Highest IFNg 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:

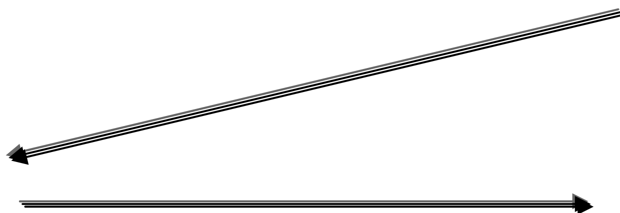
Test Accuracy (close agreement to the true value),
Precision (agreement of independent results: same day, different day),
Reproducibility (intra-assay and inter-assay)

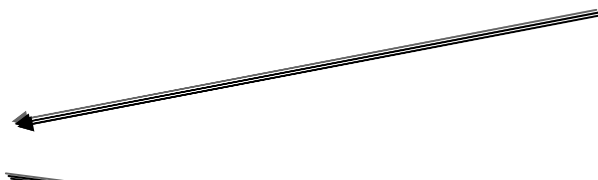
Reportable range (limits of detection)
Normal ranges (pools of healthy donors, accumulated patient samples),

Personnel competency testing
Equipment validation, monitoring
Reagent tracking

Central Immunology Laboratory

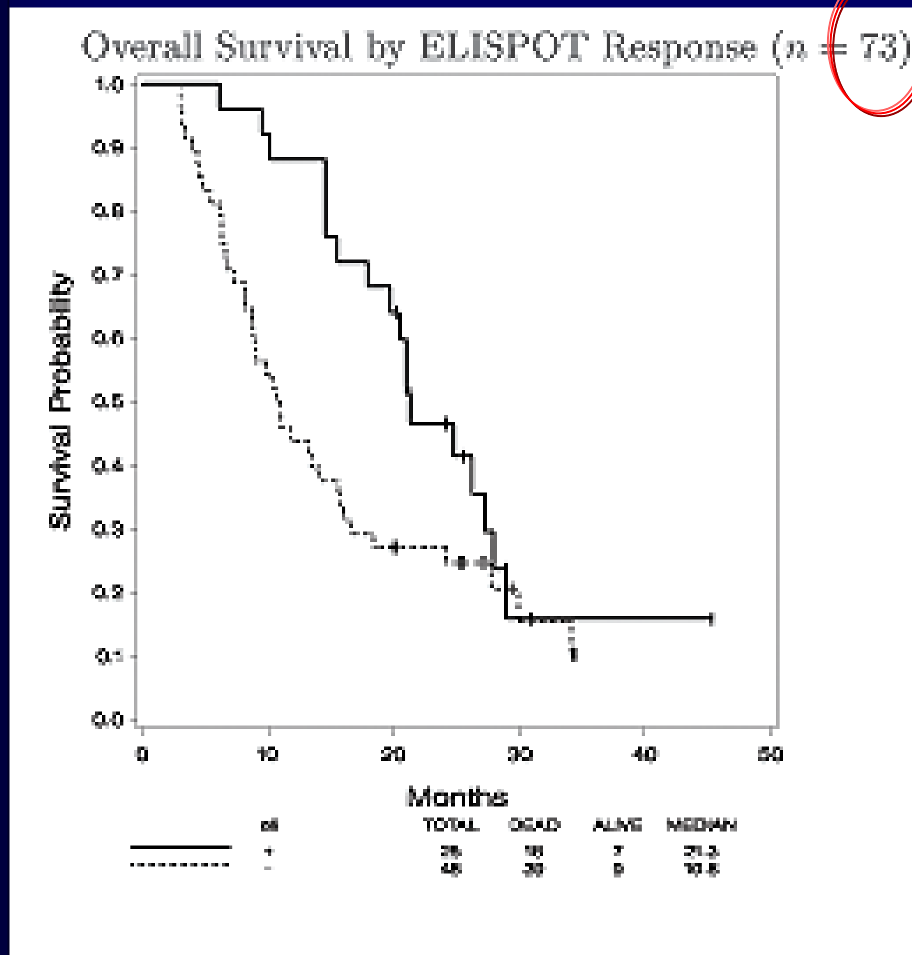
Clinical Site	Central Lab
Screen or enrollment: fax blood kit request	Kit prepared and shipped ground
Pt. blood draw mailed O/N to lab	Blood processed and banked according to SOPs within 24 hours
gather data; biostatistics	Assays performed per SOPs, send results
	Results to PI, publish!





Immune Response Correlates with Overall Survival

Multiple melanoma antigen peptide vaccine ± GM-CSF ± IFNα2b

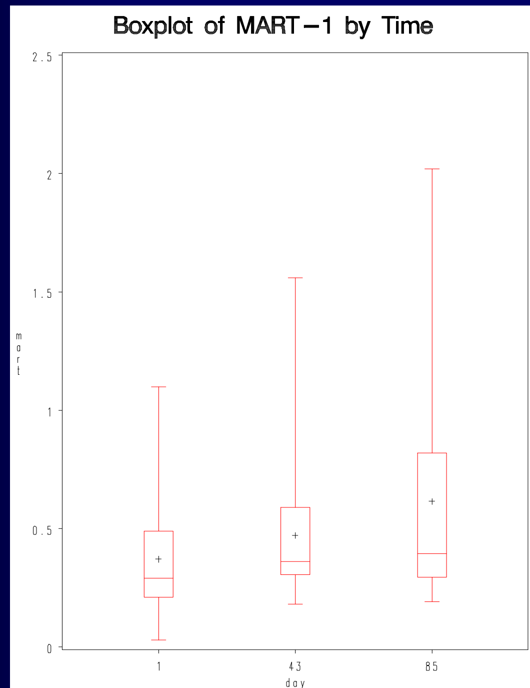


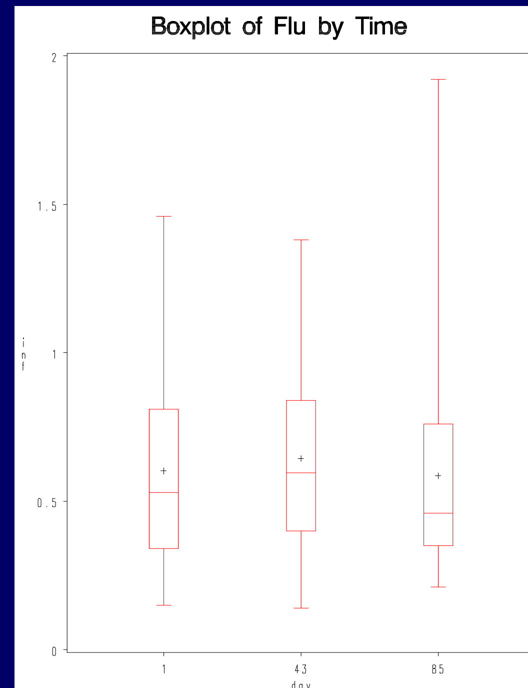
The Kaplan-Meier plot for OS by immune response status is shown for E1696 (Phase II).

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%	17%	17%	16%
		(p=0.048)			(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)*

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

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

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

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.

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

Recommendations from the iSBTc/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. Stroncek, Hideaki Tahara, Magdalena Thurin, Giorgio Trinchieri, Sjoerd H. van Der Burg, Theresa L. Whiteside, Jon M. Wigginton, Francesco Marincola, Samir N. Khlief, Bernard A. Fox, Mary L. Disis

While specific immune parameters and assays are not yet validated, we recommend:

1. Following standardized (accurate, precise and reproducible) protocols
2. Use of functional assays for the primary immunologic readouts of a trial (to address hypothesis being tested)
3. Consideration of central laboratories for immune monitoring of large, multi-institutional trials
4. Standardized testing of several phenotypic and potential potency assays for any cellular product
5. When reporting results, the QA/QC, examples of truly representative raw data and the assay performance characteristics should be included
6. To promote broader analysis of multiple aspects of immunity, in addition to cells and serum, RNA and DNA samples should be banked (under standardized conditions) for later testing
7. Sufficient blood should be drawn to allow for the planned testing of the primary hypothesis being addressed, *and* for testing novel hypotheses (or generating new hypotheses) that arise in the field