Monitoring of Cancer Immunotherapy Trials

The Methodology, Rationale and Interpretation of Standard Immunological Assays

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Clinical trials design

the role of immunological monitoring

- Clinical endpoints
  - Treatment toxicity
  - Tumor response

Clinical Trial Designs for the Early Clinical Development of Therapeutic Cancer Vaccines
RM Simon et al. JCO (2001)

- Immunological endpoints
  - Tumor antigen specific T cell number
  - Induced T cell function/phenotype

Objective Clinical Responses

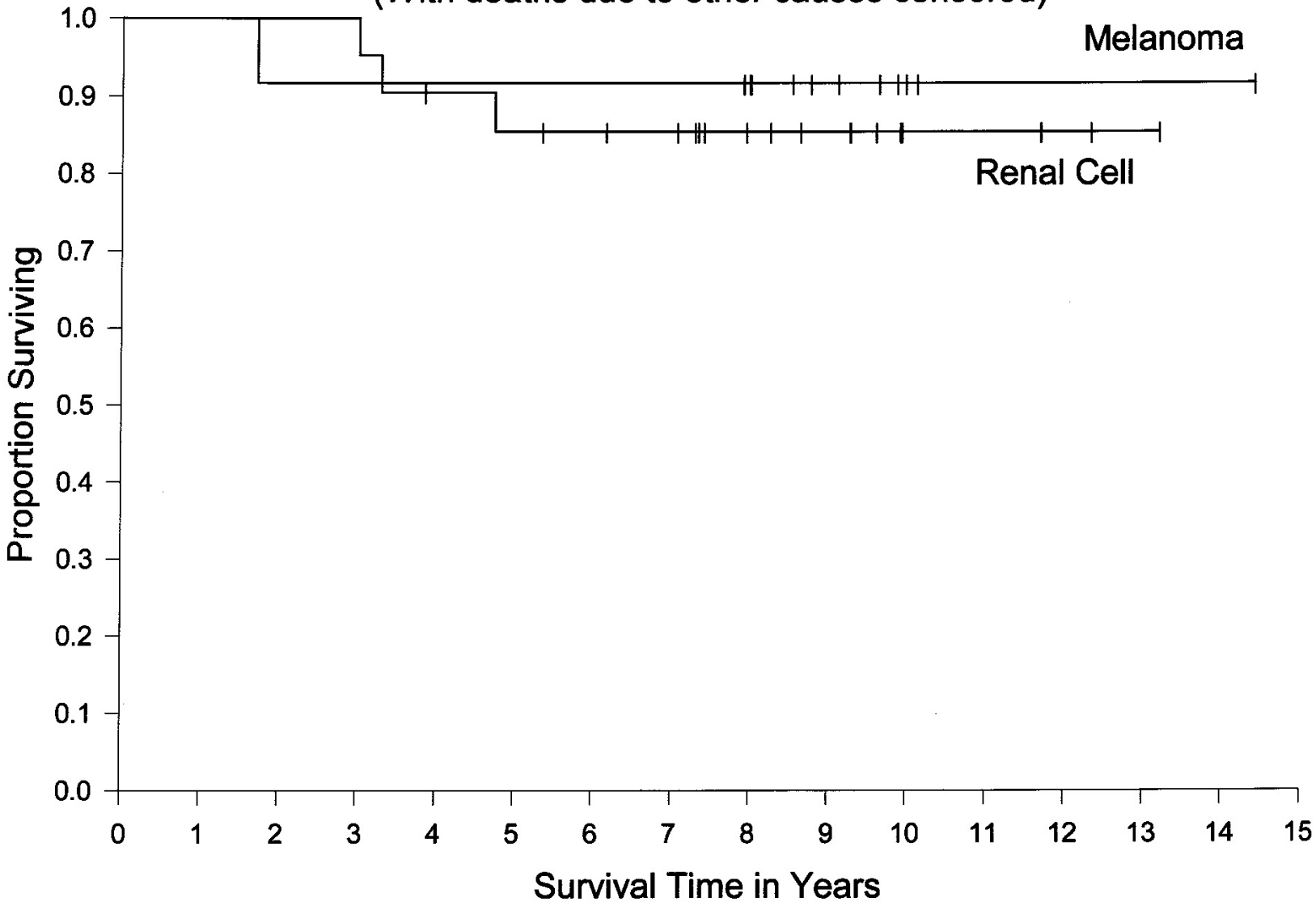
- WHO: 50% decrease in the sum of the products of perpendicular diameters of all lesions with no increase in any lesion and no new lesions
- RECIST: 30% decrease in the sum of the largest diameters of target lesions with no new lesions

Objective clinical responses can result from immunotherapy

- IL-2 (melanoma and renal cell carcinoma)
- Anti-CTLA (Co-stimulation blockade)
- Adoptive Cell Transfer

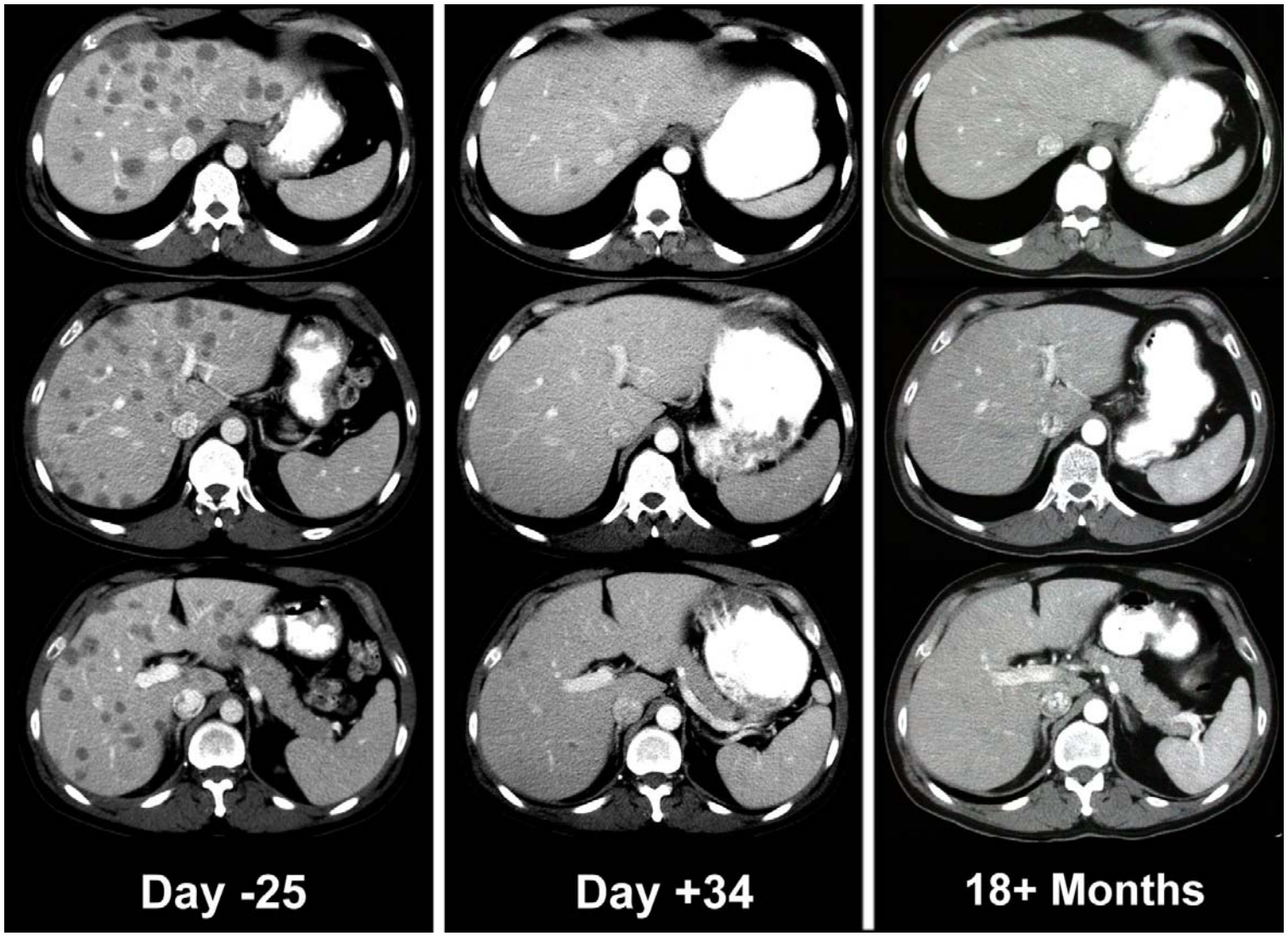
Complete Response to Treatment with High-Dose IL-2

(With deaths due to other causes censored)



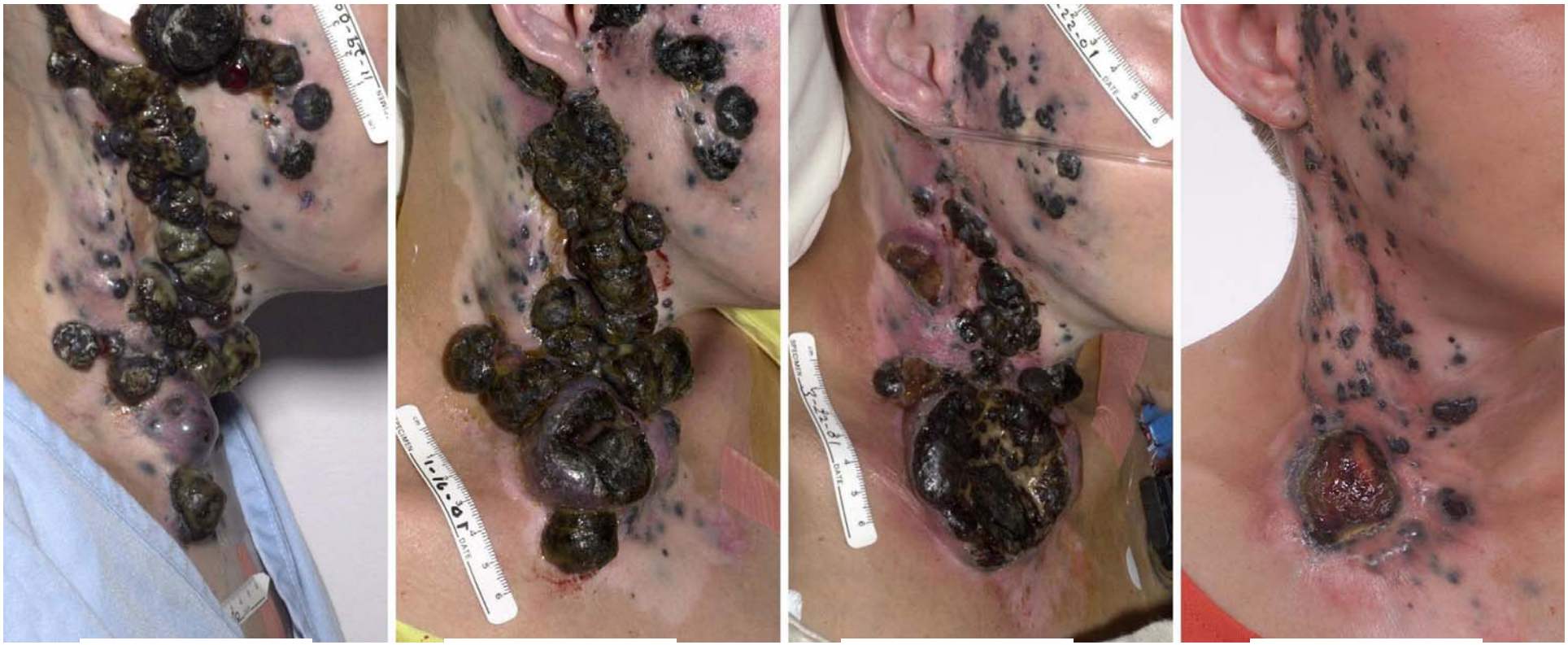
Rosenberg, 2001

Adoptive cell transfer therapy



Rosenberg and Dudley, 2005

Adoptive Cell Transfer Therapy



11-29-00 1-16-01 3-22-01 6-5-01

Rosenberg et al, 2003

Non-standard clinical endpoints - soft criteria

"Tumor necrosis"

"Lymphocyte infiltration"

"Antigen loss"

"Stable disease"

"Shrinkage of some lesions"

"Symptom improvement"

"Survive longer than expected"

These characterizations promote confusion because each can occur in the natural course of the disease

“Objective” immunological monitoring of clinical trials

- Standardized criteria
  - Appropriate patient samples available
  - Validated methodology
  - Reproducibility between patients and samples
- Magnitude of response
  - Background activity quantified
  - “Positive” results quantified and objectively defined
- Specificity of activity
  - Immunological changes coincident with treatment
  - Appropriate specificity controls included
    - Specificity of responding cells
    - Specificity of antigen stimulation

Example: Cell transfer therapy for a patient with metastatic melanoma

Protocol 99-C-0158 – patients 9 and 10

Histologically confirmed metastatic melanoma

Refractory to high dose IL-2 therapy

Prior treatments included surgery, immunotherapy, chemotherapy

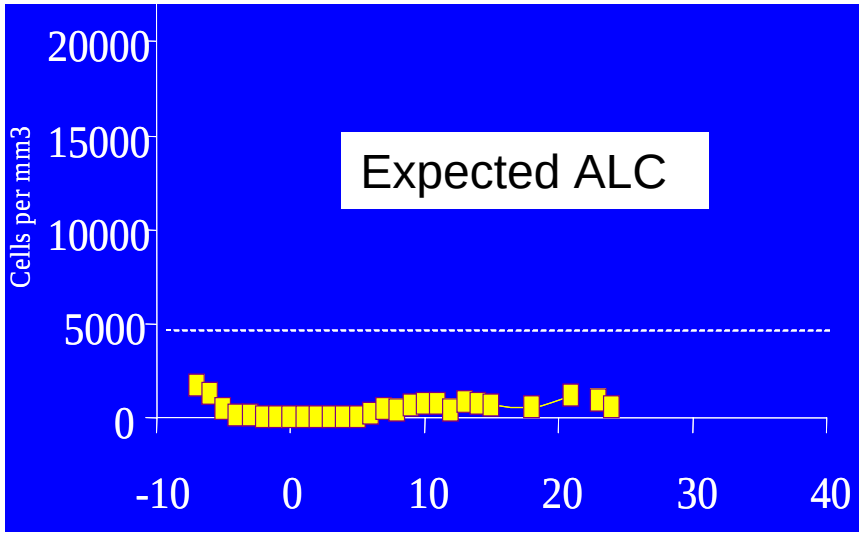
Patients received cell transfer treatments consisting of:

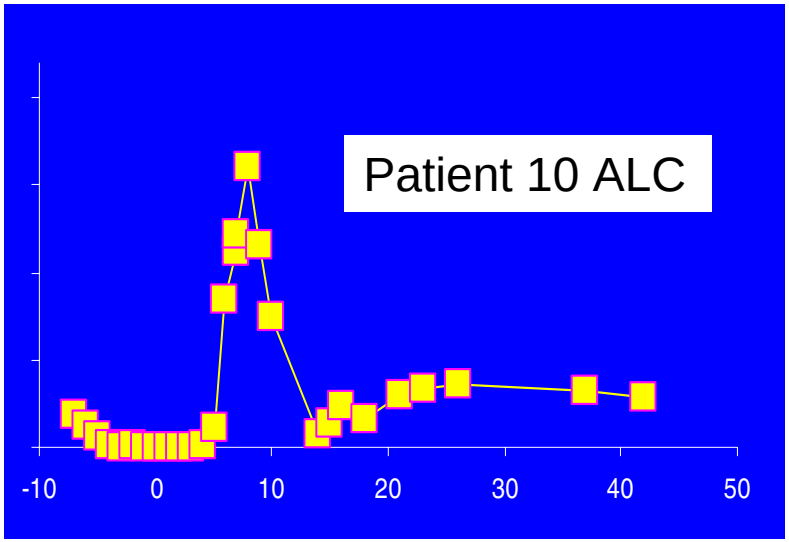
- Non-myeloablative chemotherapy conditioning followed by
- Tumor infiltrating lymphocyte cultures that contained MART-1 specific cells that were highly activated and expanded in vitro
- High dose IL-2 therapy

Dudley et al, 2002

Dudley et al, 2005

Patients 9 and 10 – Unexpected lymphocytosis following treatment with autologous cell transfer

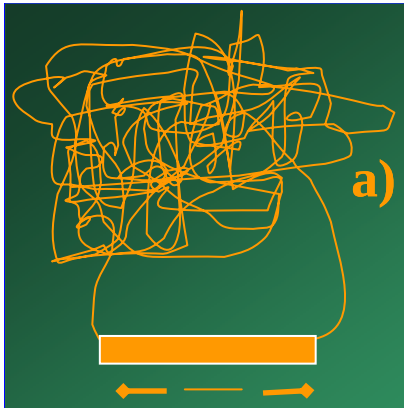




Monitoring T cells during lymphocytosis in patients 9 and 10

- Enumerating antigen-specific T cells
  - FACS analysis
  - TCR specific PCR
  - ELISpot
- Evaluating T cell function and phenotype
  - FACS
  - Cytokine release assays
  - Lysis assays
  - Proliferation assays

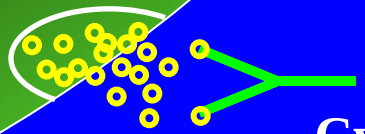
Tracking T cells in vivo



a) **Gene marking**

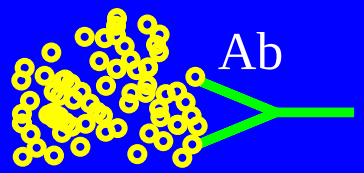
T cell

b) **TCRβ RT-PCR**

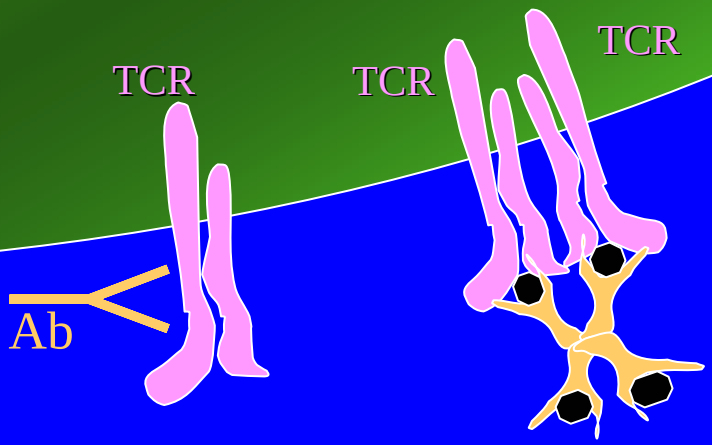


Cytokine release

OR



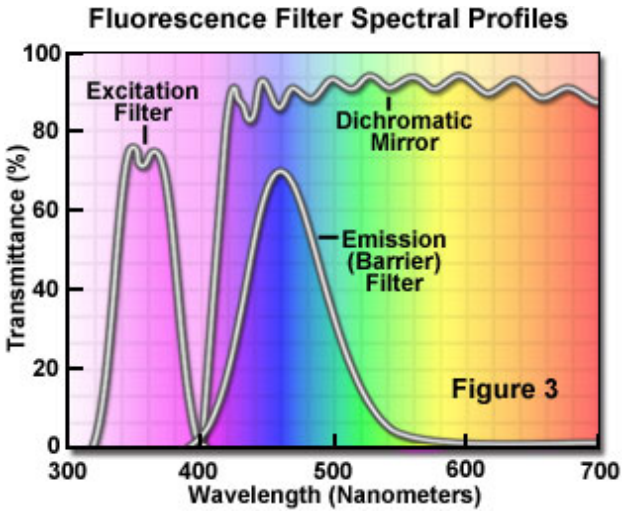
ELISpot



c) **FACS** Labeled antibody (Ab) OR Labeled HLA-peptide tetramers

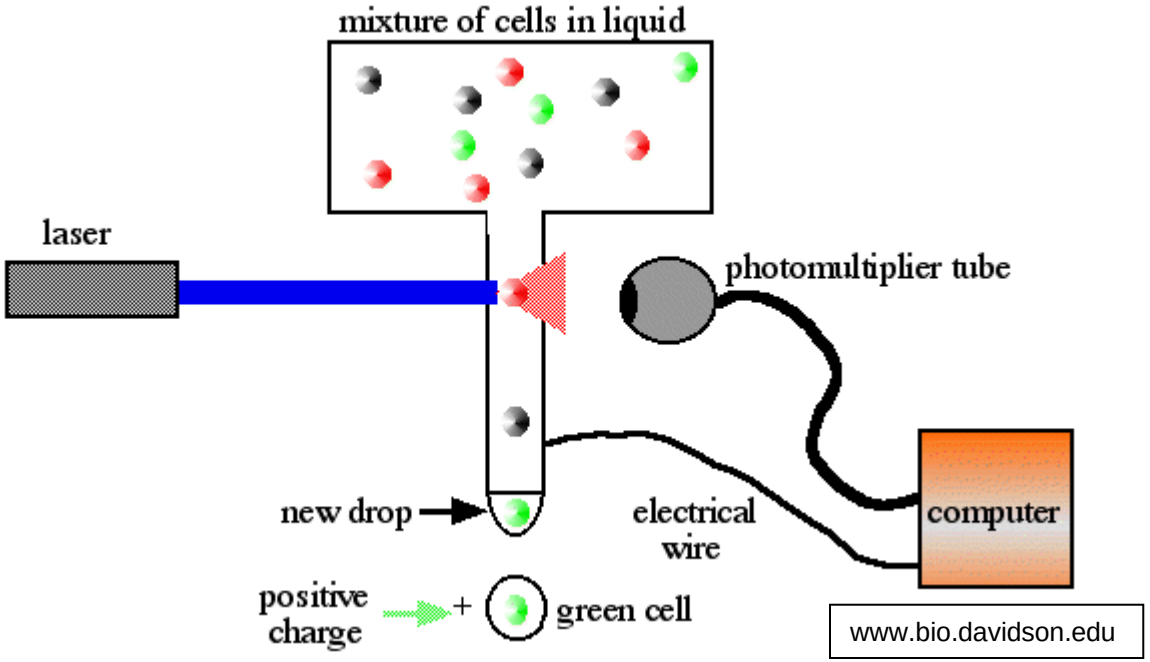
FACS Analysis

- Fluorochrome conjugated reagents
  - Absorbance in one wavelength, fluorescence in a distinguishable wavelength
  - Simultaneously multiple color evaluation



http://www.olympusmicro.com/primer/lightandcolor/fluoroexcitation.html

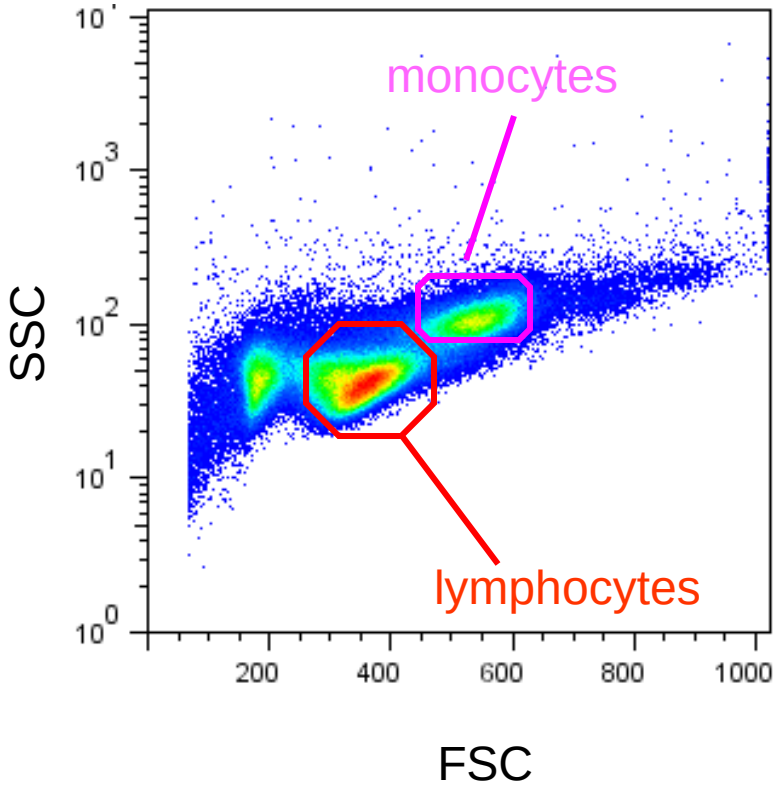
- Flow analysis principles
  - Single cells pass through a laser
  - Light diffraction and fluorescence measured
  - High throughput, quantitative results



www.bio.davidson.edu

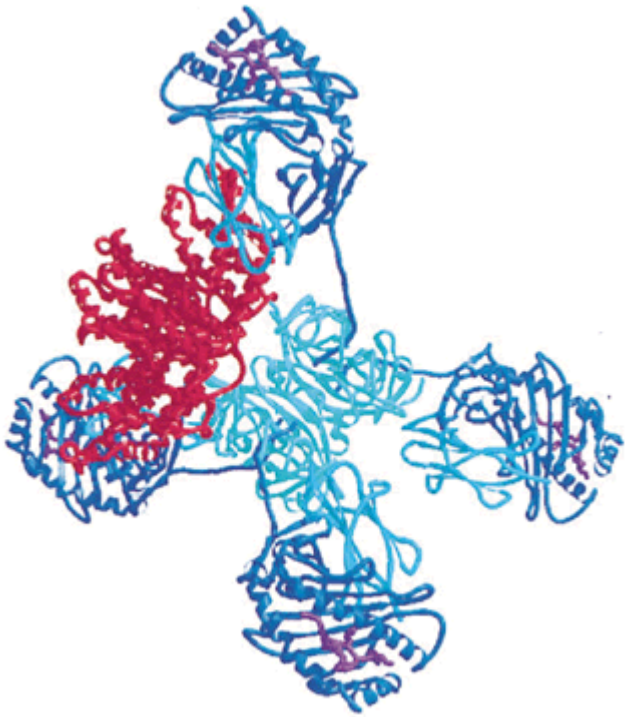
FACS analysis – basics of data acquisition and interpretation

- Logical Gating
  - Peripheral blood mononuclear cell sub populations (e.g. lymphocytes) based on side scatter and forward scatter profiles
  - viable vs dead cells
- Setting markers
  - Negative controls – isotype matched non-specific reagents
  - Different populations within the experimental sample



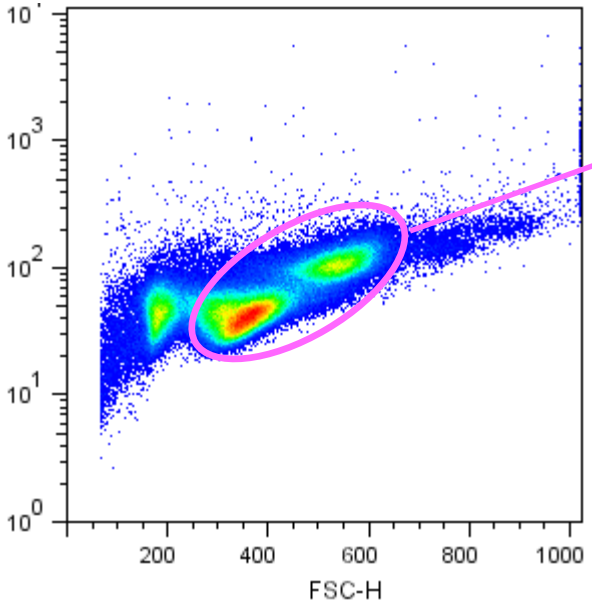
Quantifying antigen specific T cells with FACS based on T cell receptor binding

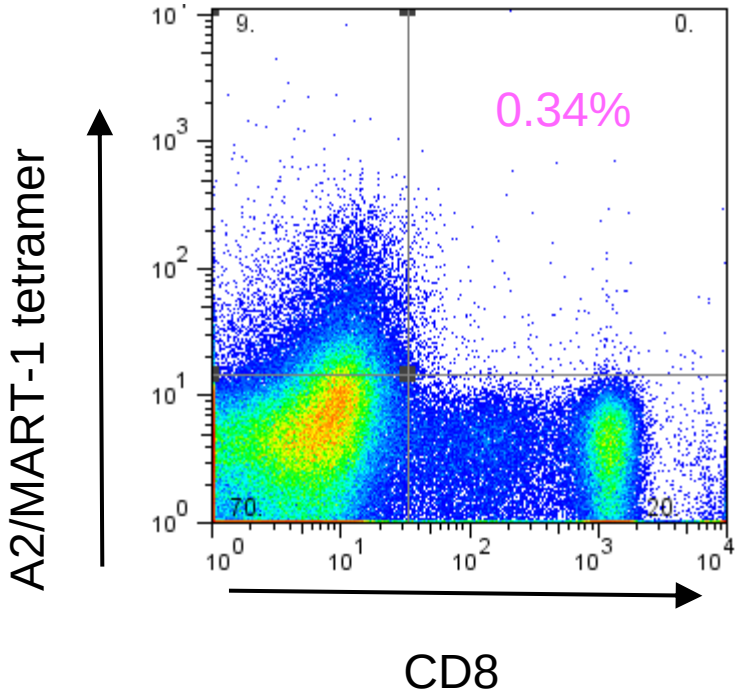
- HLA/peptide tetramers
  - Soluble recombinant HLA-biotin (CD8 independent)
  - Refolded around a peptide antigen
  - Multimerized with Avidin-fluorochrome
  - Multiple binding sites increase avidity and stability of staining
- Binds T cells through TCR
  - Cell surface binding
  - Antigen specificity of T cells individually determined
  - Protocols compatible with simultaneous antibody binding



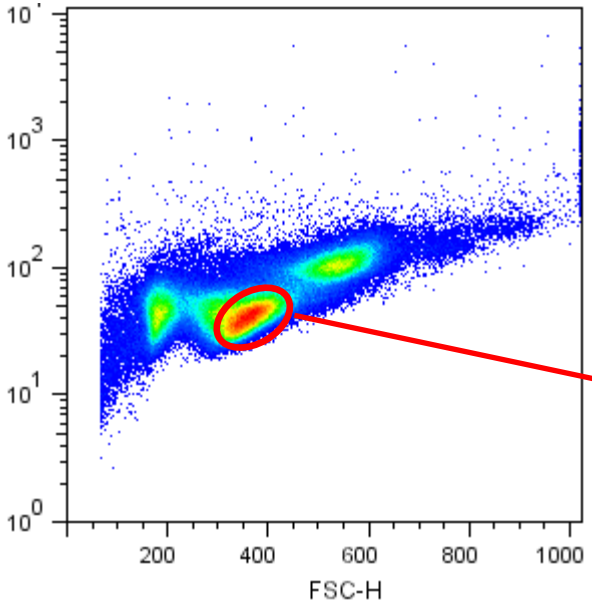
probes.invitrogen.com

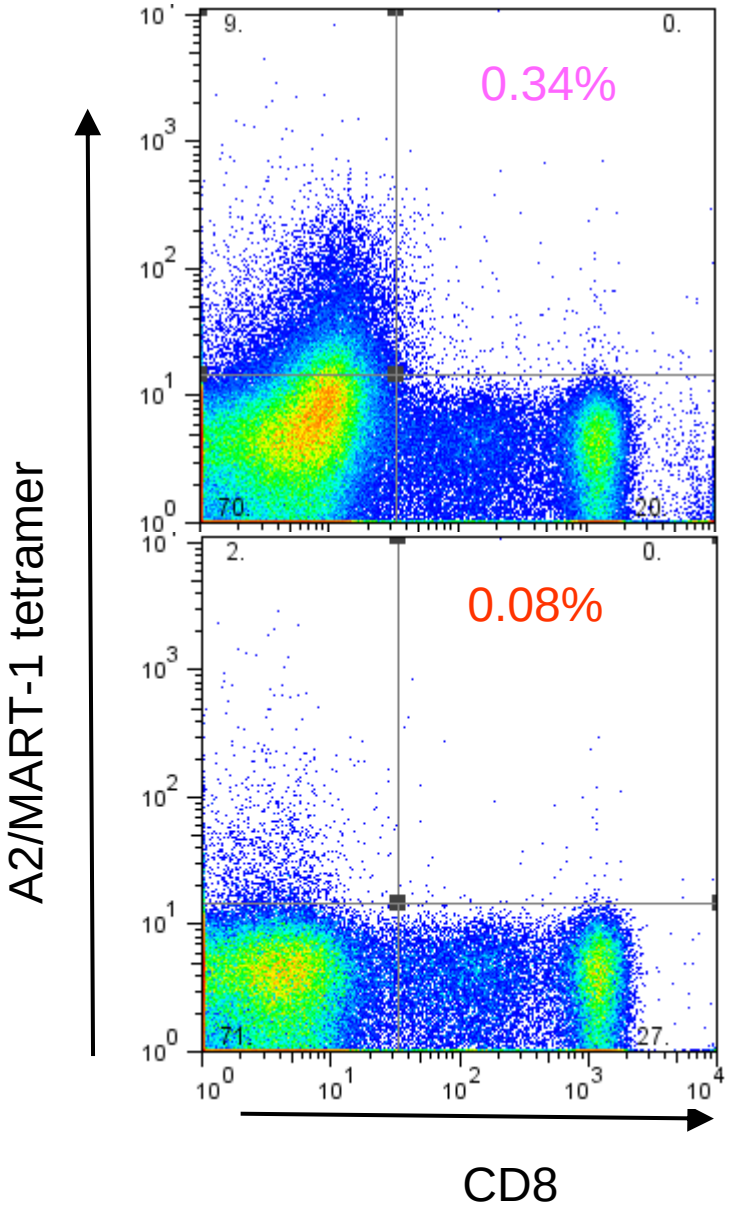
Setting appropriate gates – background



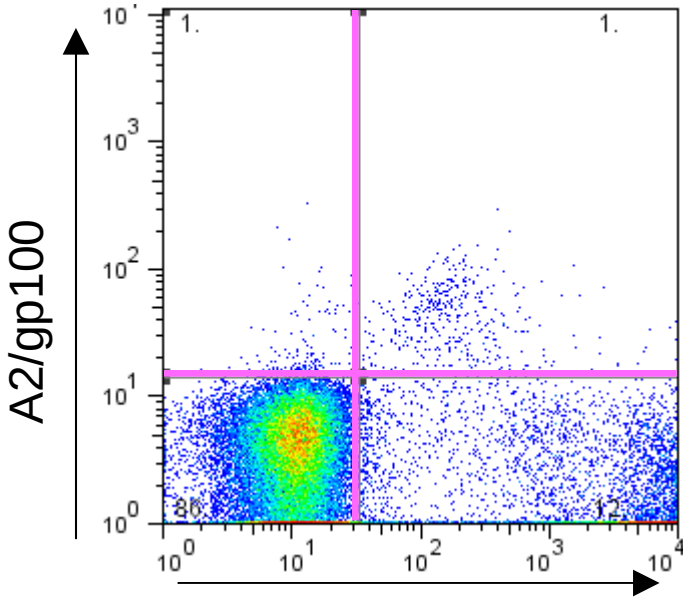


Setting appropriate gates – background

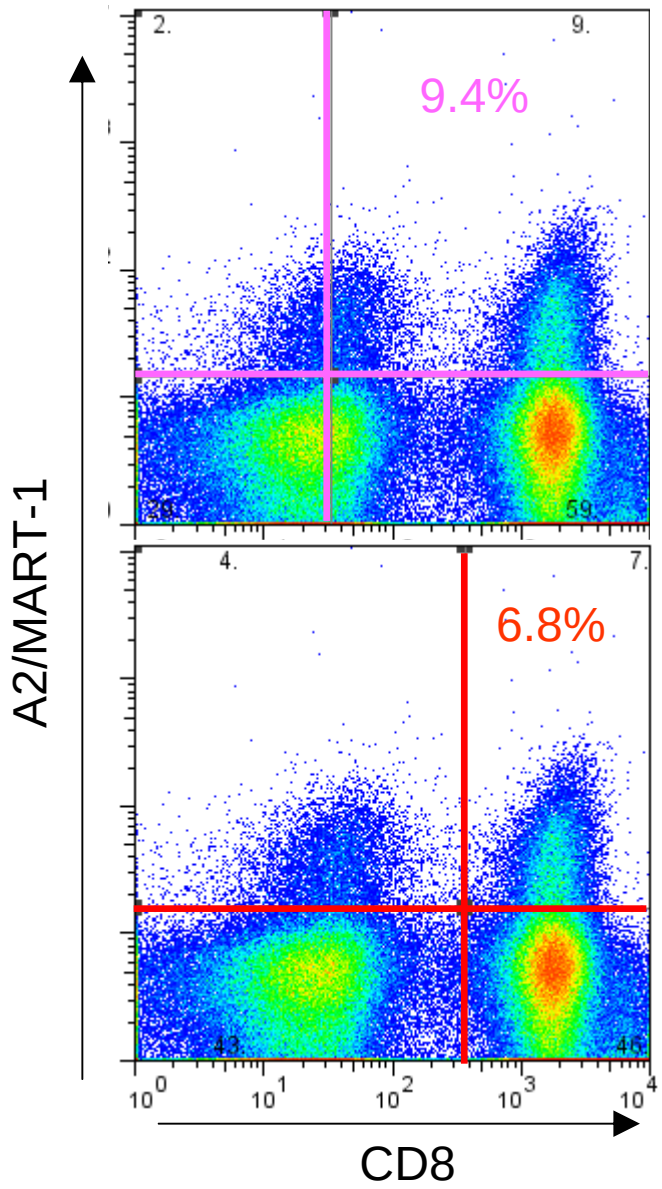




Setting appropriate markers - background

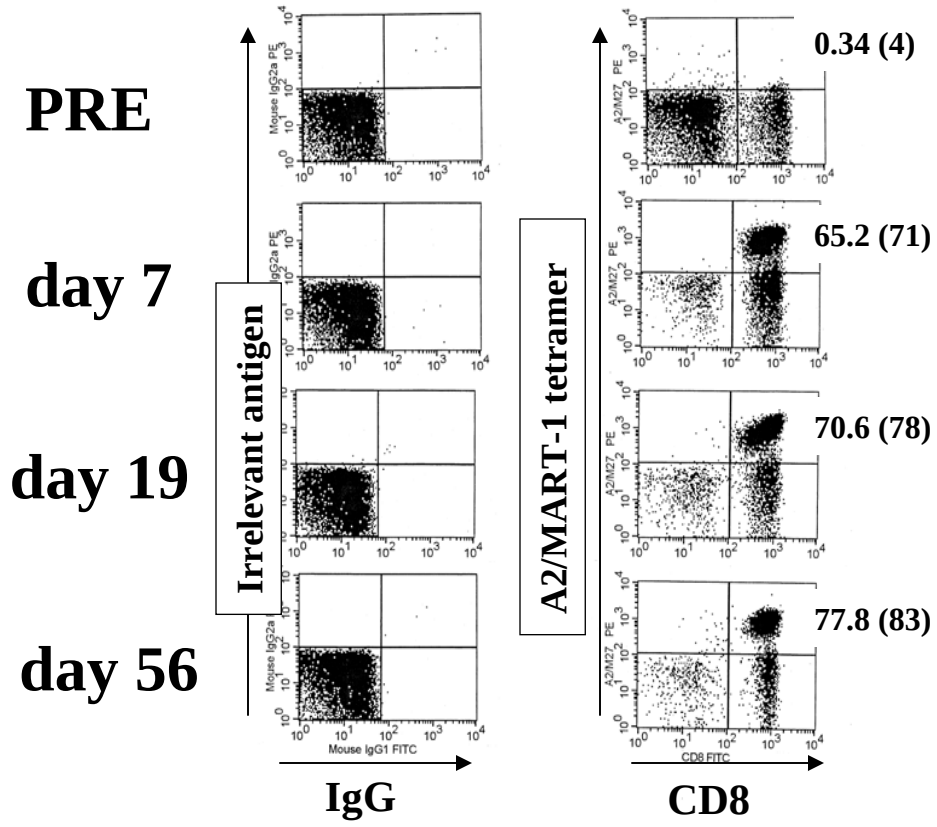


Gated on viable lymphocytes



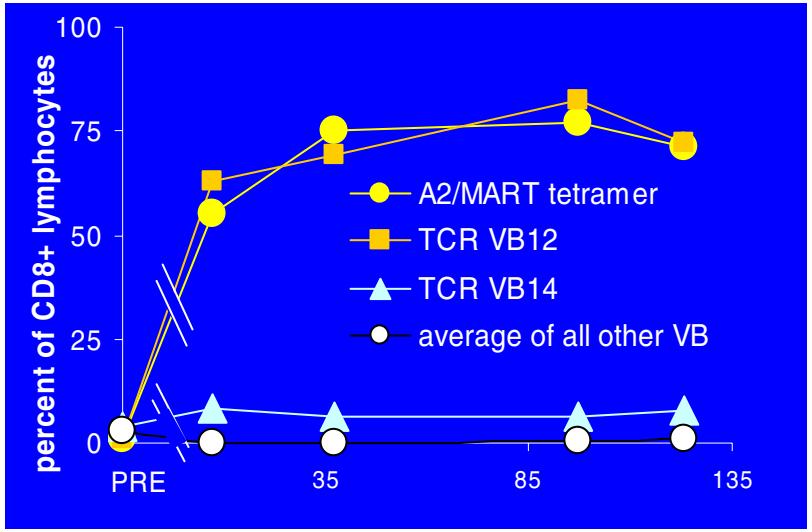
Patient 9 – lymphocytosis of a MART-1 reactive population

Gated on viable peripheral blood lymphocytes

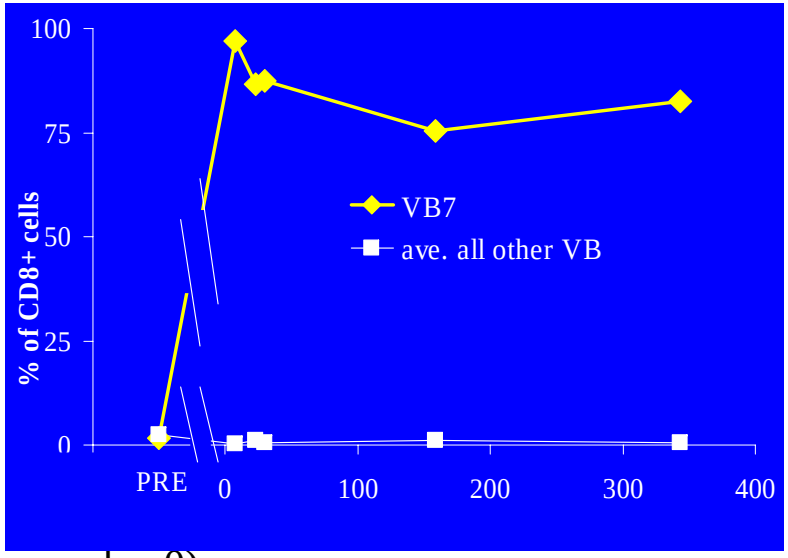


Clonal re-population and prolonged persistence

Patient 9

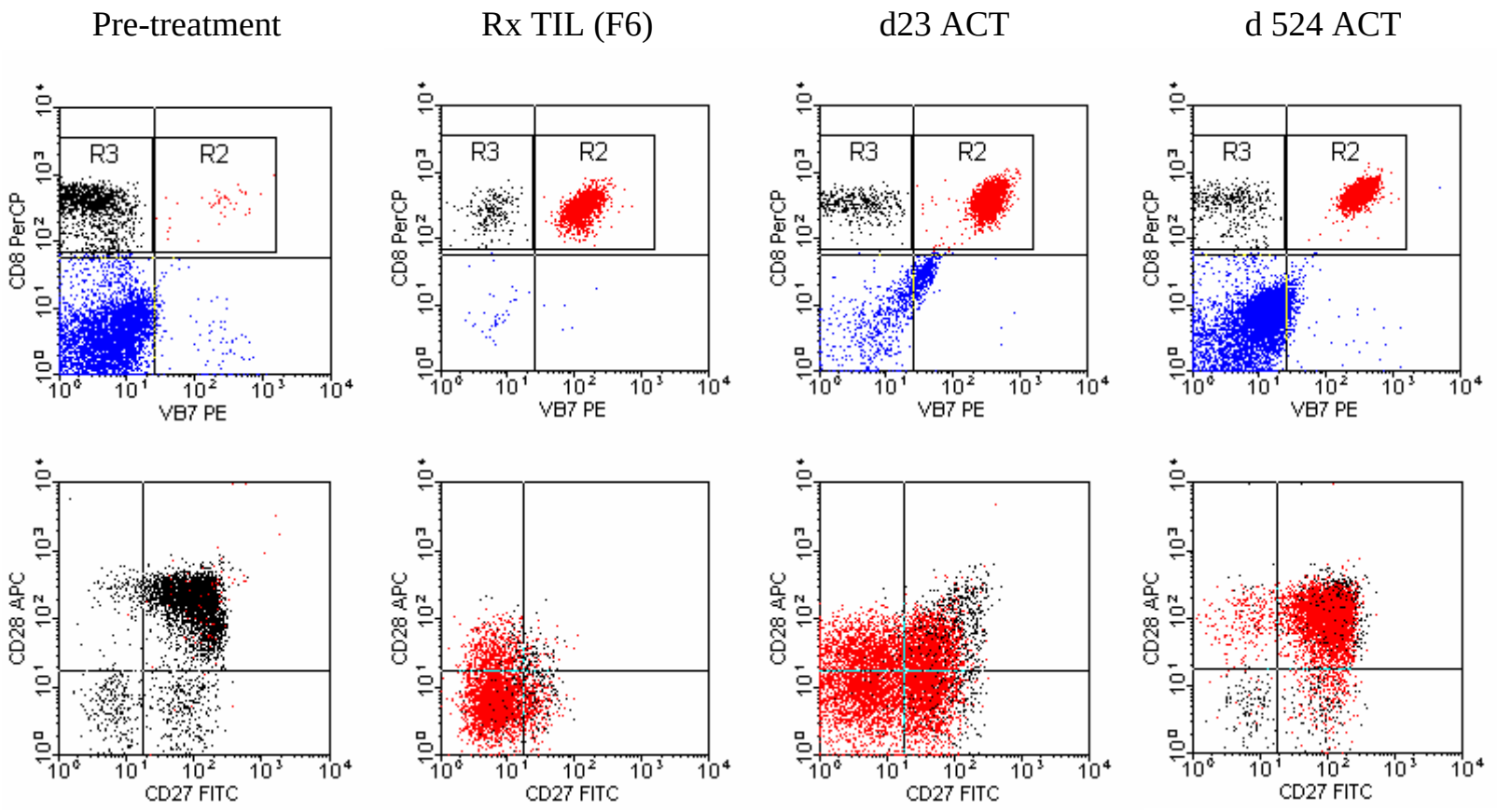


Patient 10



days (relative to TIL infusion on day 0)

Changes in expression of costimulatory receptors in vivo following ACT

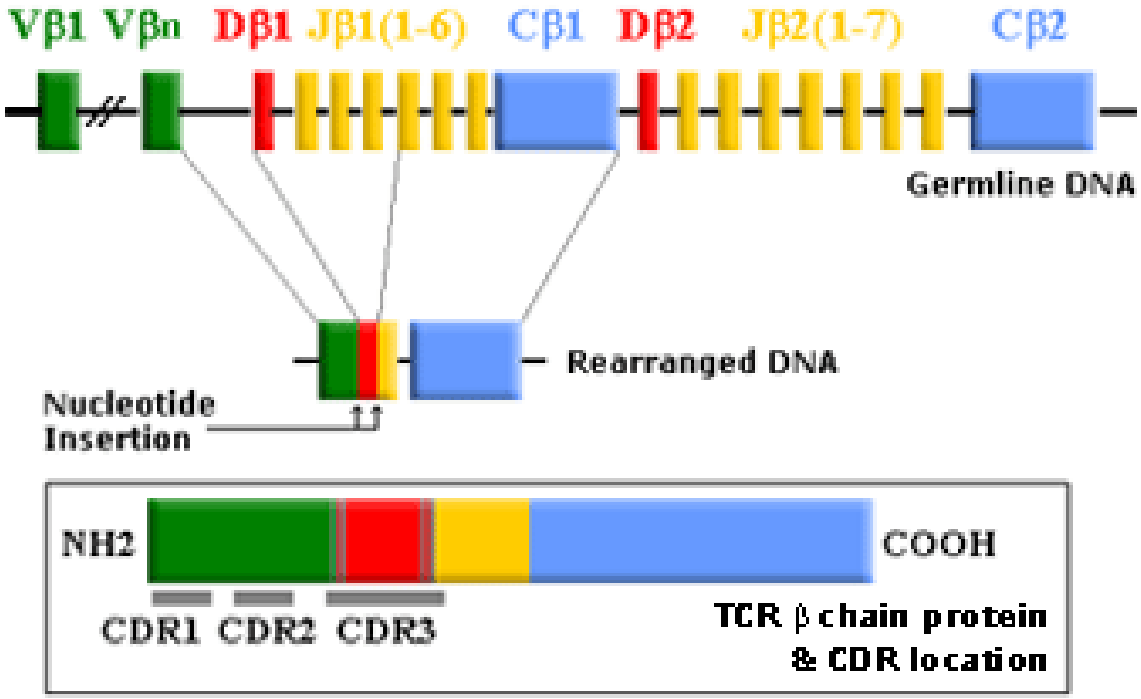


Summary – FACS analysis

- Highly sensitive, specific, quantitative method
- Interpretation of results depends on gating and marker placement
- Low frequency cell populations require highly controlled experiments
  - Blinded data acquisition and analysis
  - Appropriate control reagents and cell populations

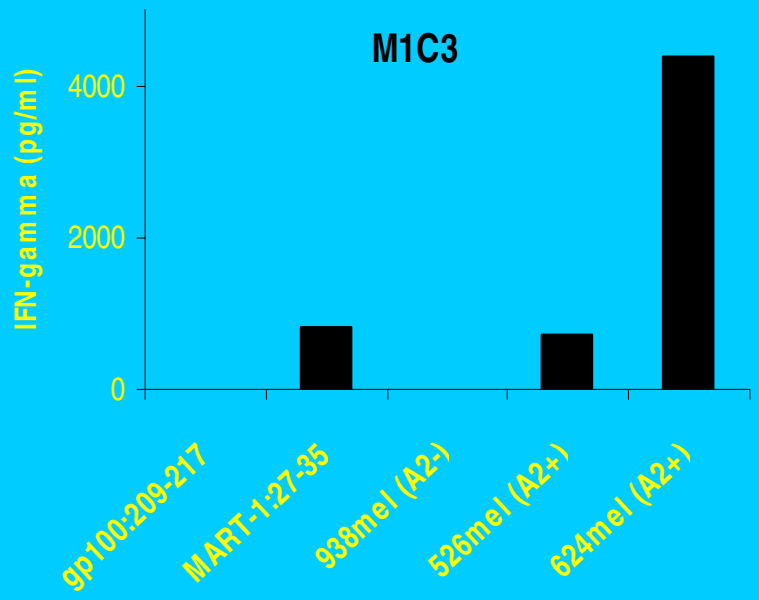
T cell clonal identification based on nucleotide sequence analysis

- The T cell receptor (TCR) locus genetic organization and expression permits identification of T cell clones based on unique TCR sequences

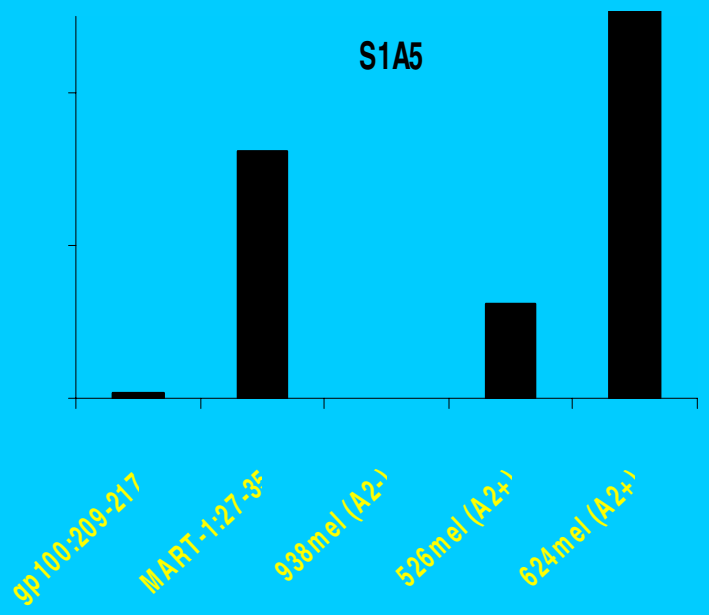


Cloned T cells from the infused TIL treatment specifically recognize MART-1

Patient 9



Patient 10



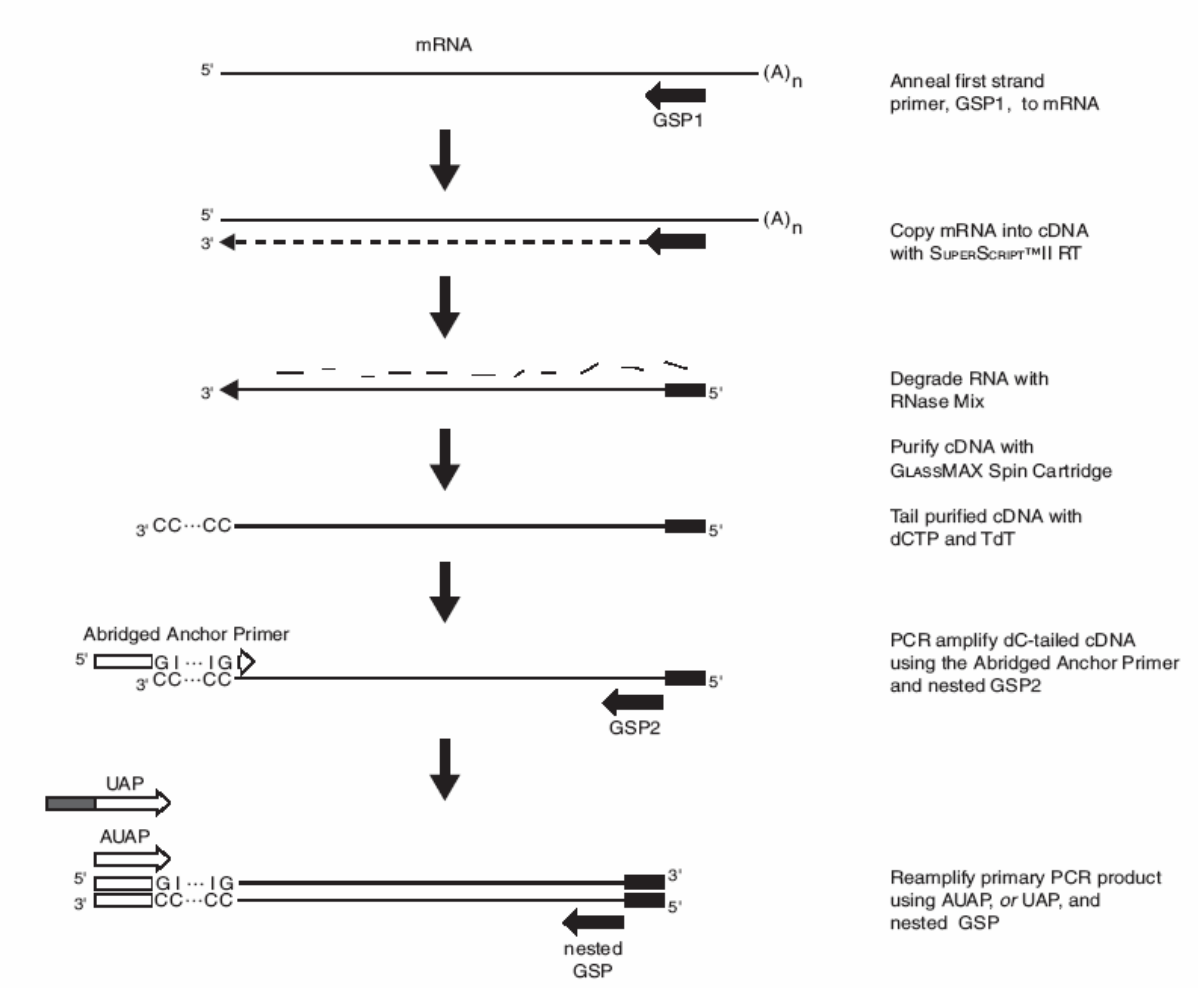
Patient 9 - MART-1 specific TIL clone

V12	D1	J1S5
…gcc atc agt gag	gta ggg gtt ggg	cag ccc cag cat...
A I S E	V G V G	Q P Q H

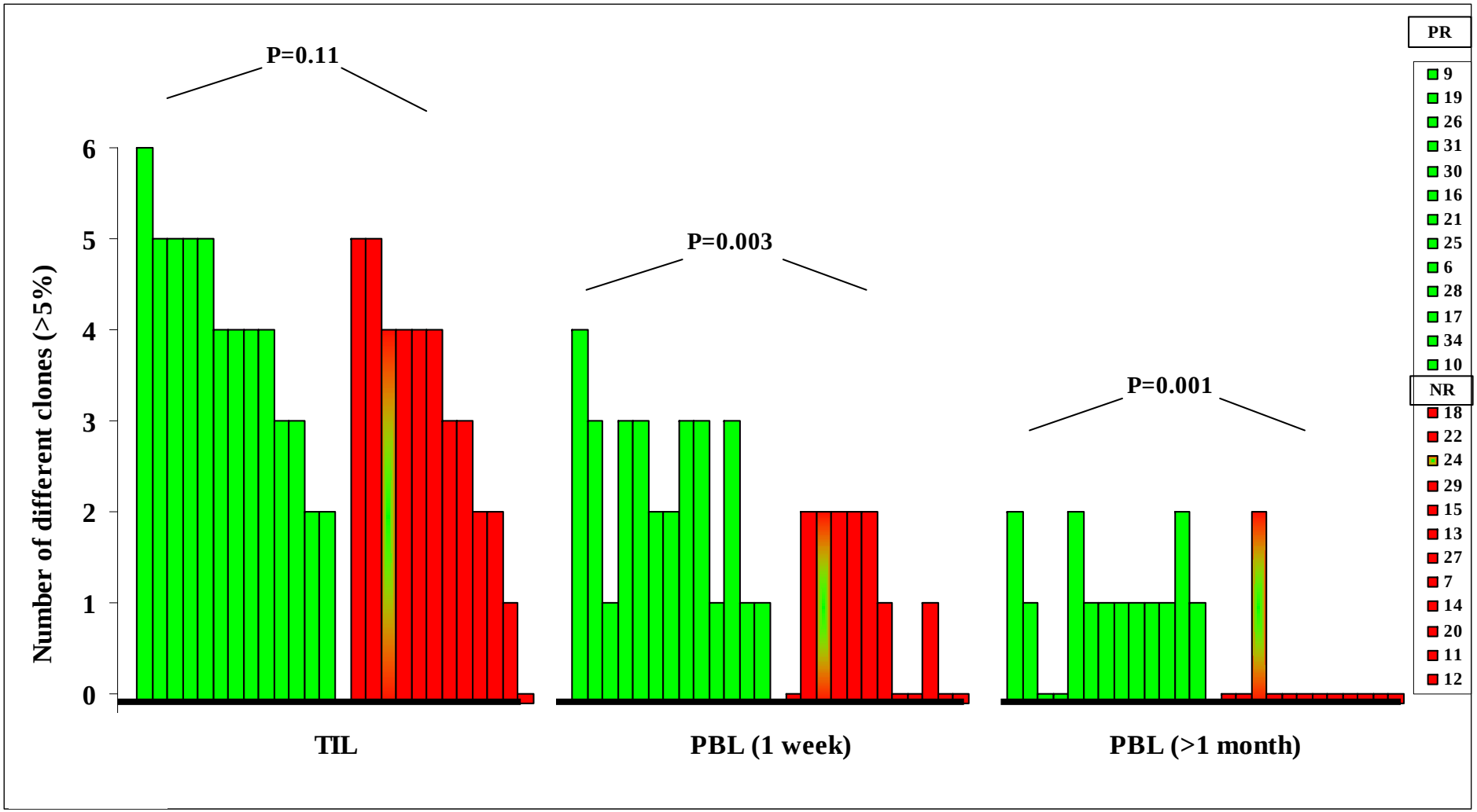
Patient 10 - MART-1 specific TIL clone

V7	D1	J1S1
...gcc agc agc caa	tca tgg acc ttc t	tg aac act gaa.. .
A S S Q	S W T F	L N T E

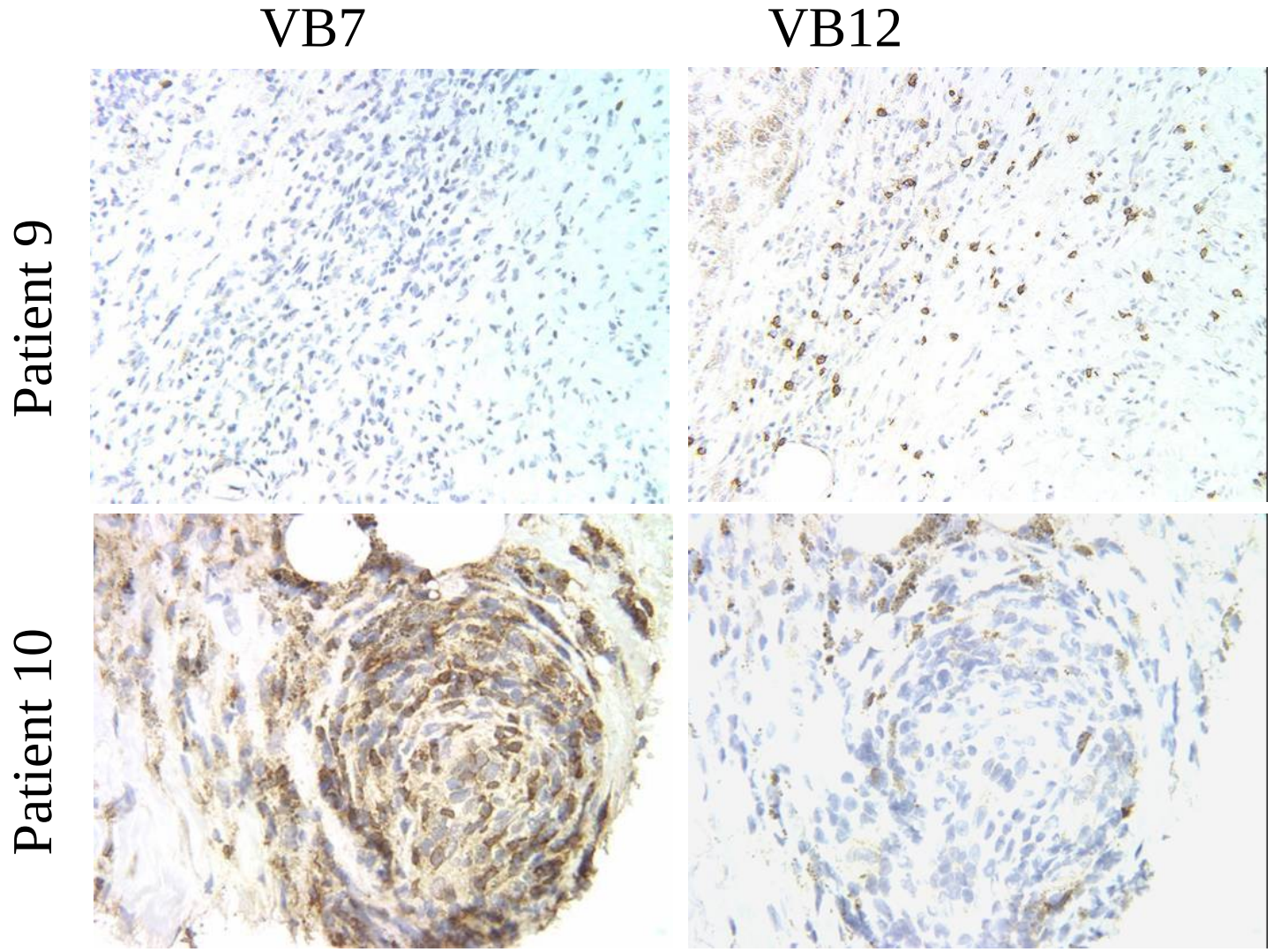
RACE analysis of T cell persistence following adoptive cell transfer



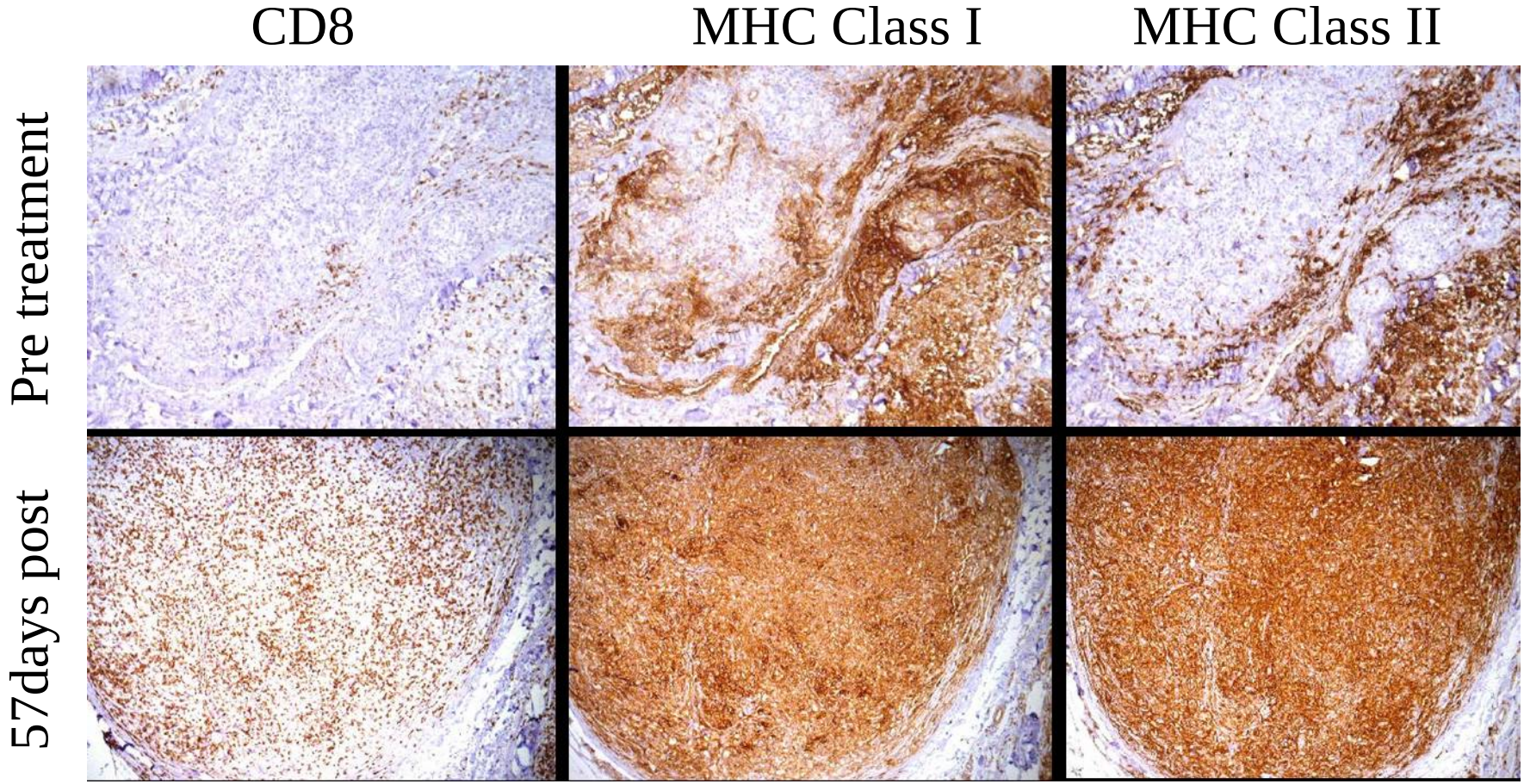
Responding Patients demonstrate Persisting Clones from Transferred TIL



Transferred TIL trafficked to tumor



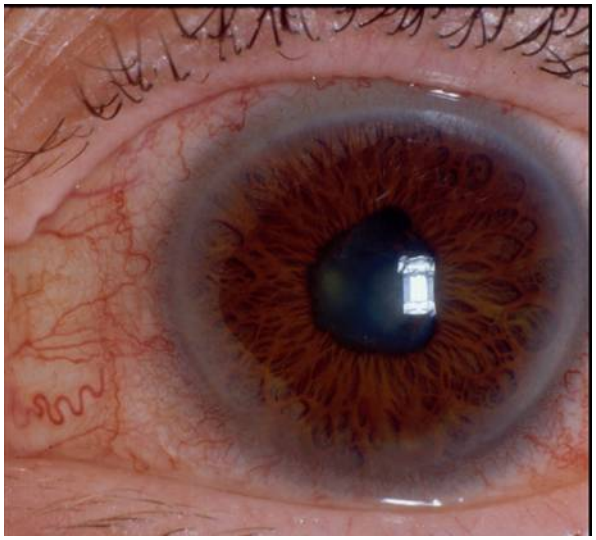
High level expression of HLA by post-treatment tumor cells *in vivo*

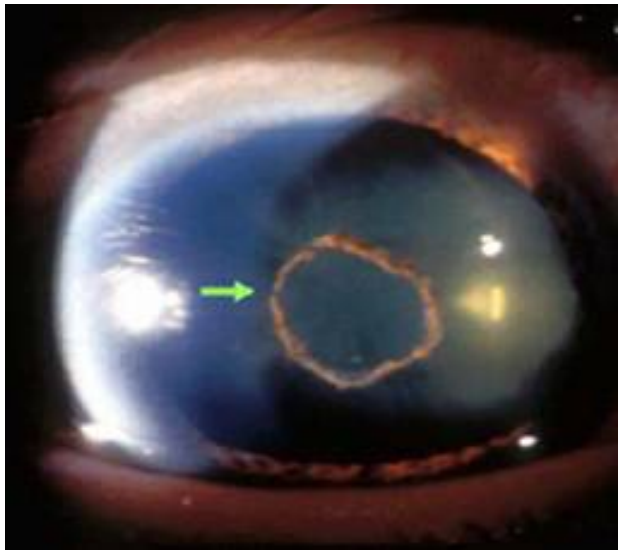


Patient 10: tumor regression and autoimmunity

>99% tumor reduction, PR (14 months)

Autoimmune uveitis: bilateral anterior uveitis controlled with steroid eye drops





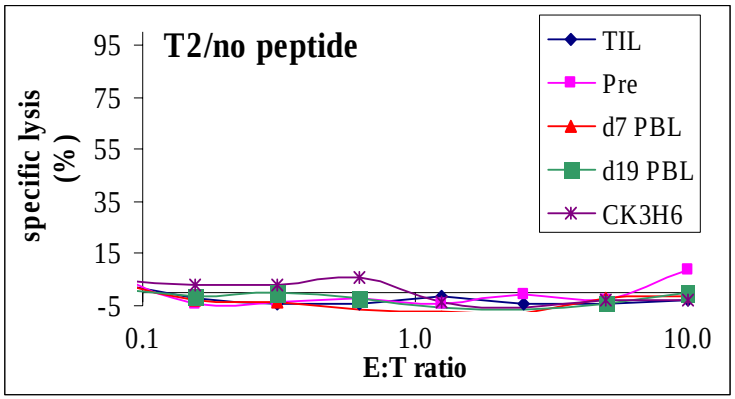
Functional characterization of T cells in patient peripheral blood

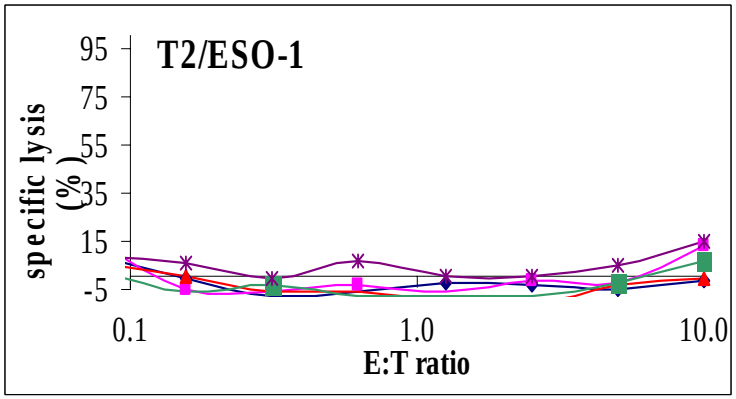
- Lysis assays
- Cytokine release assays
- ELISpot assay
- Proliferation assays

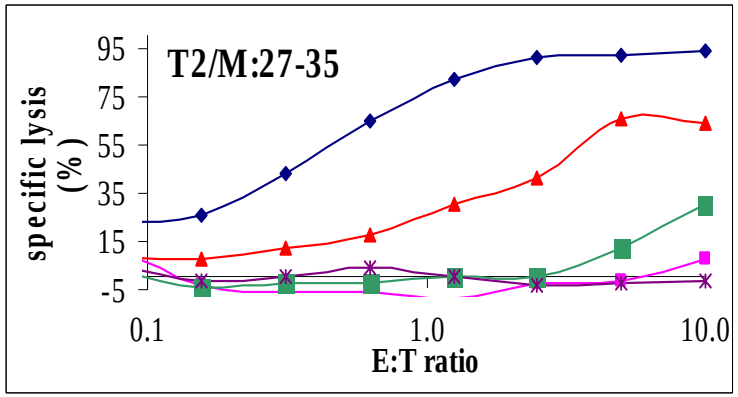
Lymphocyte functional assays: Cell mediated lysis

- Label Targets with Cr-51
  - Antigen loaded cells
  - Tumor cells
- Add Effector cells
  - Multiple dilutions generate multiple E:T ratios
  - Controls for maximum and spontaneous Cr-51 release
- Positive and negative controls for specificity
  - Targets
  - Effectors
- Evaluate Cr-51 release in supernatant

Antigen-specific lysis by PBL of Patient 9 directly ex vivo







Lymphocyte functional assays: Cytokine release

- Coculture format
- Stimulator cells
  - Antigen pulsed
  - Tumor cells
- Responder cells
- Positive and negative controls for specificity
  - Stimulators
  - Responders
- Evaluate secreted cytokines in supernatant by capture ELISA assay

Example: hTERT vaccinated patients – a cautionary tale

Cytokine release assay (Interferon gamma, pg/ml)

Target	Cell type	HLA A2	hTERT	Pt. #5	Pt. #11
Media	**none**	**-**	**-**	71	116
T2 + 1 μg/ml HBVc:18	**T-B hybrid**	**+**	**-**	450	153
T2 + 1 μg/ml hTERT:540	**T-B hybrid**	**+**	**-**	**4,210**	**15,004**
T2 + 0.01 μg/ml hTERT:540	**T-B hybrid**	**+**	**-**	**3,582**	**9,858**
293-UT	**Renal cell**	**-**	**+**	222	51
888mel	**melanoma**	**-**	**+**	299	82
938mel	**melanoma**	**-**	**+**	120	64
C1R-A2	**B lymphoblast**	**+**	**+**	**766**	**1270**
IM-9	**EBV-B cell**	**+**	**+**	**579**	**1078**
1978E	**EBV-B cell**	**+**	**+**	**1168**	**1274**

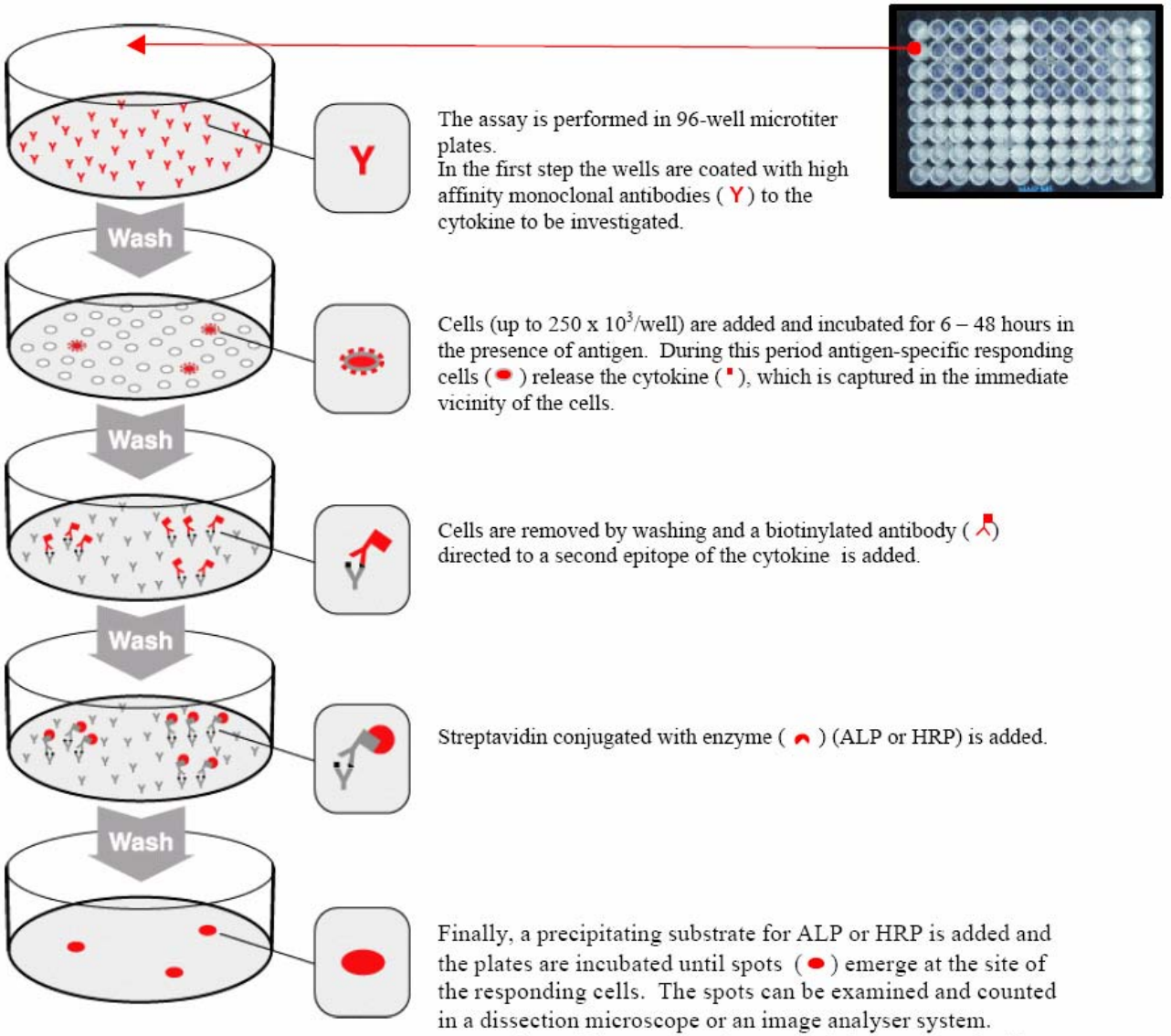
Maria Parkhurst

Example: hTERT vaccinated patients – the rest of the story

Cytokine release assay (Interferon gamma, pg/ml)

				Patient 5		Patient 11	
Target	Cell type	HLA	hTER	pre	post	pre	post
Media	none	-	-	45	71	218	116
T2 + 1 ug/ml HBVc:18	T-B hybrid	+	-	69	450	243	153
T2 + 1 ug/ml hTert	T-B hybrid	+	-	68	**4,210**	218	**1,500**
T2 + 0.01 ug/mlh Tert	T-B hybrid	+	-	77	**3,582**	256	**9,858**
293-UT	Renal cell	-	+	47	222	84	51
293-A2	Renal cell	+	+	49	288	77	48
888mel	melanoma	-	+	51	299	142	82
938mel	melanoma	-	+	46	120	113	64
1861mel	melanoma	+	+	45	82	102	57
624mel	melanoma	+	+	41	79	88	56
Sk23mel	melanoma	+	+	47	142	101	59
N-KH	RCC	+	+	39	75	56	43
S-KH	RCC	+	+	48	70	124	72
C1R	B	-	+	174	247	541	291
C1R-A2	B	+	+	**458**	**766**	**1139**	**1270**
U266	myeloma	+	+	71	**276**	411	178
SKW6.4	EBV-B cell	+	+	**1004**	**422**	**457**	**363**
IM-9	EBV-B cell	+	+	**282**	**579**	**1396**	**1078**
1978E	EBV-B cell	+	+	**477**	**1168**	**1212**	**1274**

Cytokine ELISpot



The assay is performed in 96-well microtiter plates.
In the first step the wells are coated with high affinity monoclonal antibodies (Y) to the cytokine to be investigated.

Cells (up to 250 x 10^3/well) are added and incubated for 6 – 48 hours in the presence of antigen. During this period antigen-specific responding cells ( ) release the cytokine ( ), which is captured in the immediate vicinity of the cells.

Cells are removed by washing and a biotinylated antibody ( ) directed to a second epitope of the cytokine is added.

Streptavidin conjugated with enzyme ( ) (ALP or HRP) is added.

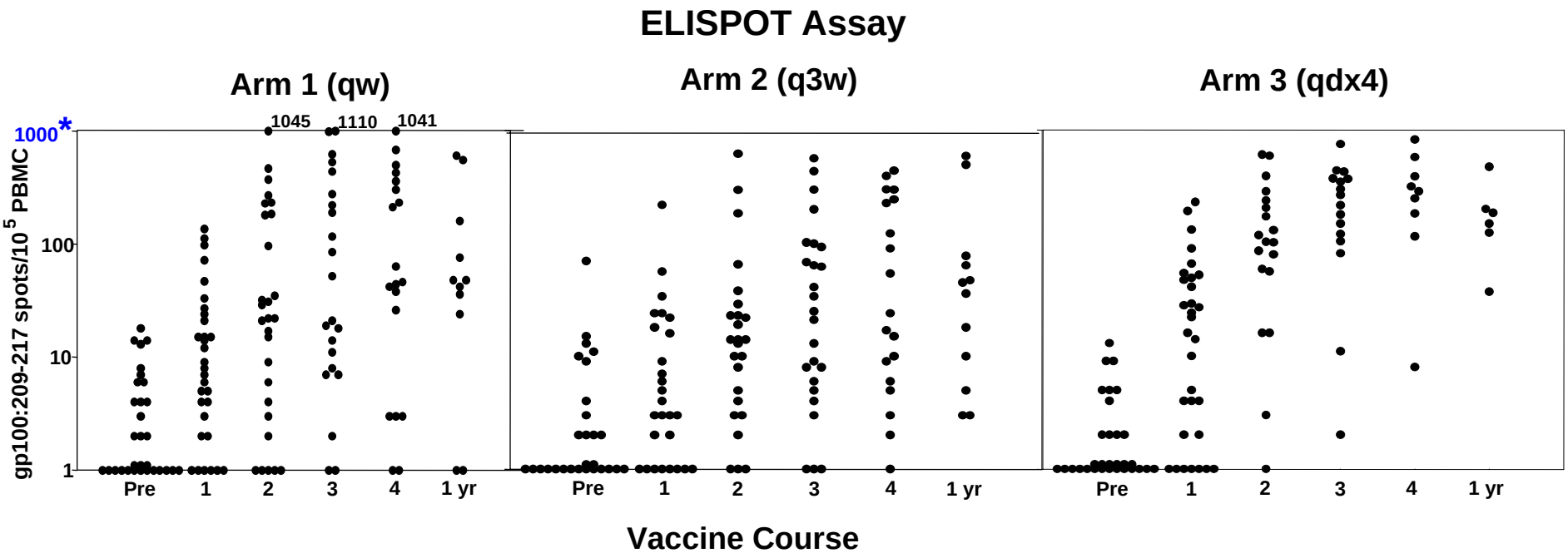
Finally, a precipitating substrate for ALP or HRP is added and the plates are incubated until spots ( ) emerge at the site of the responding cells. The spots can be examined and counted in a dissection microscope or an image analyser system. Comparison of the number of spots with the number of cells added to each well gives the frequency of responding cells.

www.mabtech.se/

Evaluation of a clinical vaccine trials using ELISpot

- Appropriate patient samples for longitudinal analysis
- Cultured lymphocytes vs *ex vivo* PBL
- Appropriate specificity controls
- Signal to noise problem for rare T cells

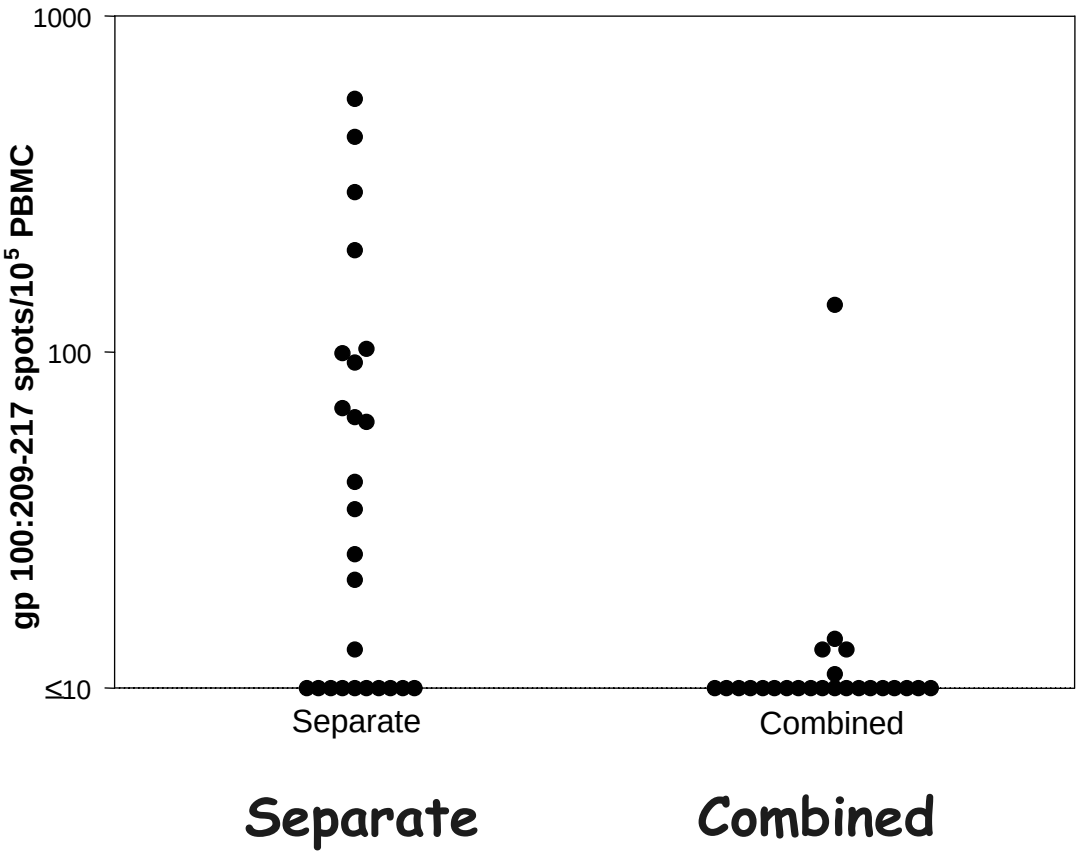
Frequent and Prolonged Peptide Vaccination in the Adjuvant Setting: A Modified Peptide in IFA (GP100: 209-217 [210M])



* *Approx 1 in 10 CD8 cells*

Some patients generate "very high" CTL precursor frequencies after 6-12 months of peptide vaccination

ELISpot analysis comparing immune response in PBL among sequential patient cohorts receiving different vaccine formulations



Summary

- Reporting of clinical responses must be based on objective criteria
- Immunological monitoring must use appropriate methods with:
  - Objective criteria
    - Appropriate patient samples available
    - Validated methodology
    - Reproducibility between patients and samples
  - Significant magnitude of response for the sensitivity of the assay
    - Background accurately quantified
    - “Positive” results objectively defined
  - Specificity of activity evaluated
    - Immunological changes coincident with treatment
    - Appropriate specificity controls included
      - Specificity of responding cells
      - Specificity of antigen stimulation