Standardization of MHC Tetramer and ELISPOT Assays to Quantitate Antigen-Specific T Cell Expansion after CTLA4 Blockade

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Background and Study Goal

- Background:
  - Implementation of cellular immunological assays to monitor immunotherapy require quality control monitoring and assay validation studies (Keilholz *et al*. JIT 2002).
- Goal:
  - Define a “positive immunological response” to immunotherapy taking into account the assay imprecision.

Performance Specifications: LLD and Assay Imprecision

LLD = Mean of a Negative Epitope + 2 Standard Deviations

$$\text{Coefficient of Variation (CV)} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100$$

$$\text{Total Assay Variation} = \sqrt{\text{preCV}^2 + \text{aCV}^2 + \text{pCV}^2}$$

preCV = Pre-analytical variation:
- Sample collection.
- Sample processing.
- Storage conditions.
- Assay conditions.

} Minimized by standardized sample collection and processing, and adherence to SOP of optimized assays.

aCV = Analytical variation.
pCV = Physiological variation.

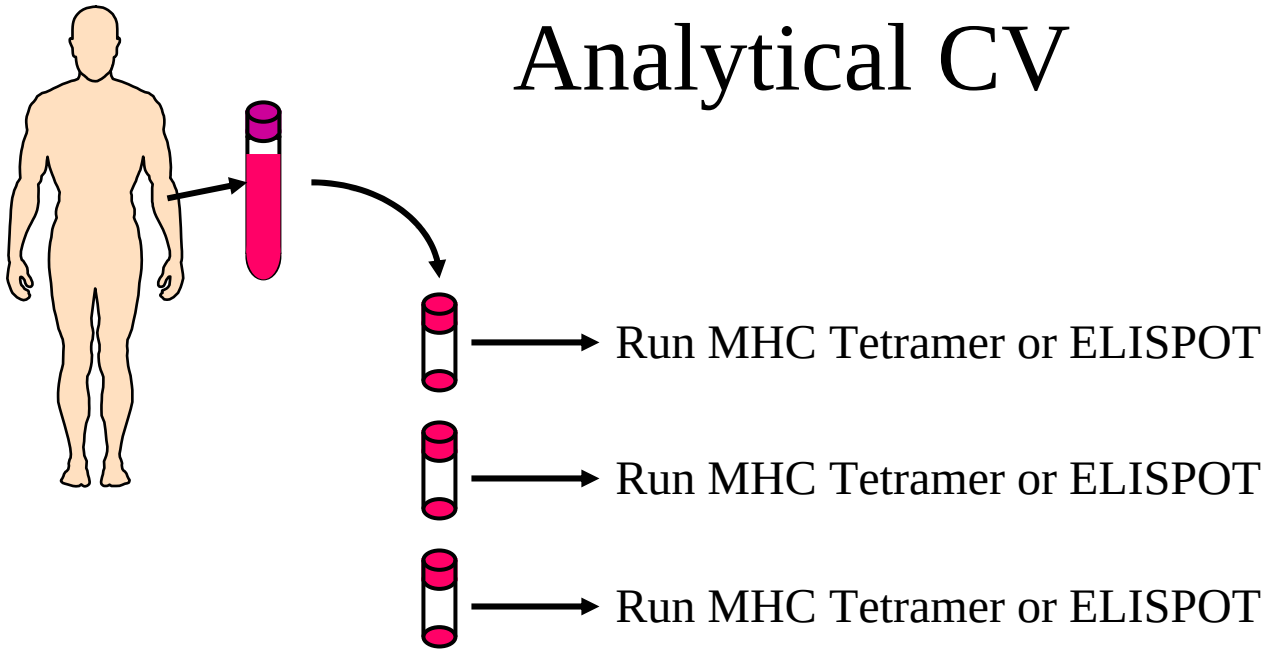
How can we define a “positive immunological response” taking into account the assay imprecision?

1. Minimize pre-analytical variability with assay optimization and SOPs.

2. Reference Change Value (RCV) = $\sqrt{2} \times Z \times \sqrt{aCV^2 + pCV^2}$

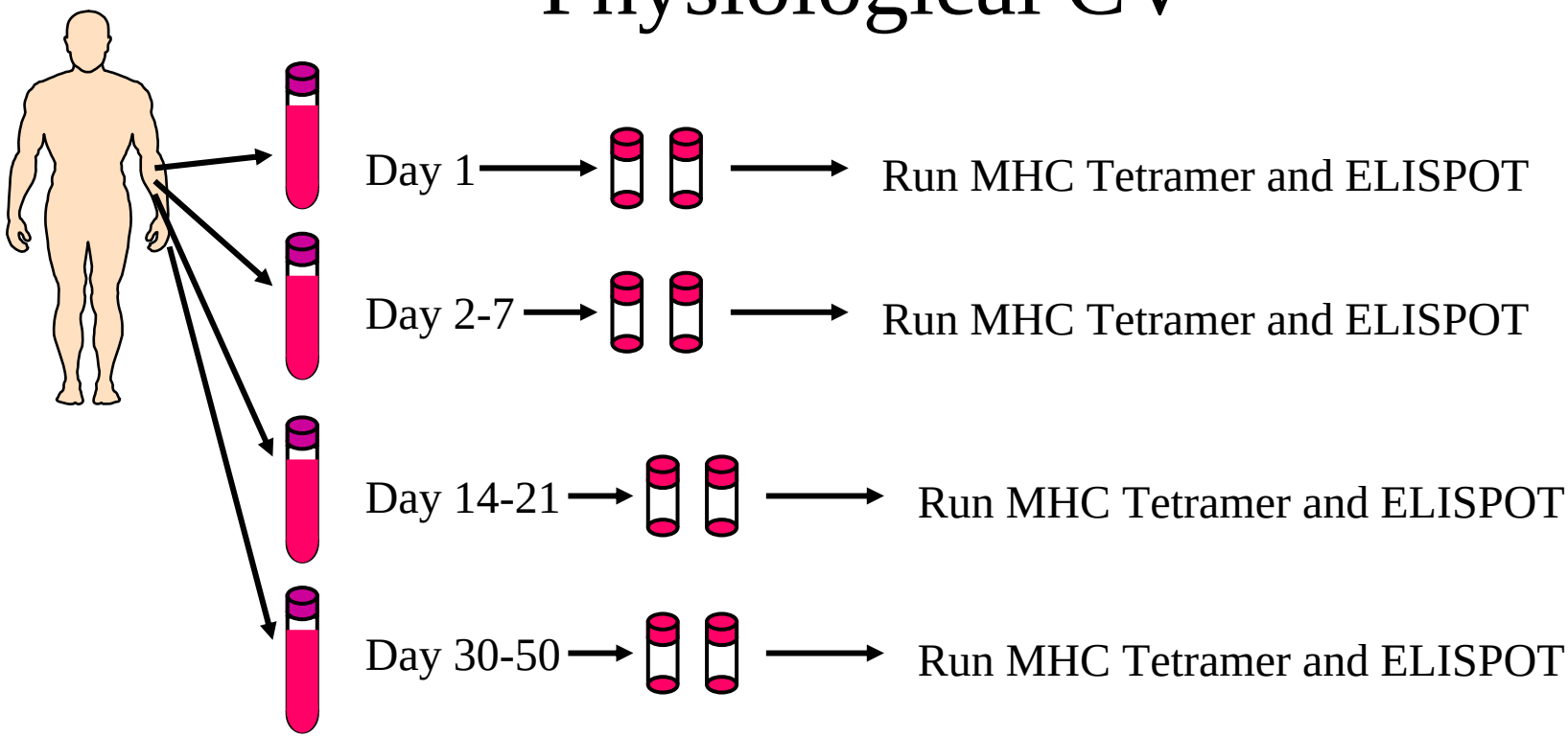
Z = Two-tailed Z score with 0.05% significance.
aCV = Analytical variation.
pCV = Physiological variation.

Analytical CV



- Goal: Determine the inherent assay variability running replicate samples.
- Method: Analyze replicate samples with values above the LLD from the same blood draw re-run at different time points.

Physiological CV



- Goal: Determine the changes over time in baseline levels of circulating antigen-specific T cells.
- Method: Analyze samples from different blood draws taken within a period of 2 months without any treatment.
- 121 blood draws to 31 subjects; 90% (28 subjects) had the 4 scheduled blood draws.

Subject Eligibility

- Healthy Donors (10):
  - HLA-A*0201.
- Patients with Melanoma (21):
  - HLA-A*0201.
  - No prior therapy within 30 days.
  - No therapy during blood procurements (1-2 mo.).
  - Stage II-IV melanoma.
  - MART-1 positive by IHC or RT-PCR.
  - No concurrent immunosuppressive therapy.

Patients with Melanoma

No Evidence of Disease (NED)

Stage	Trial No.	Prior Therapy
II	107	MART1/DC
III	105	None
	111	IFN- α, MART1/DC, CP-675,206
IV	100	MART1/DC
	108	None
	109	MART1/DC, CP-675,206
	110	BCG, MART1/DC, CP-675,206
	113	MART1/DC
	115	IL-2, MART1/DC, CP-675,206

Alive with Disease (AWD)

Stage	Trial No.	Prior Therapy
III	102	None
	103	Chemotherapy, MART1/DC, CP-675,206
IV	101	None
	104	None
	106	MART1/DC, CP-675,206
	112	MART1/DC, CP-675,206
	114	CP-675,206
	116	CP-675,206
	117	Chemotherapy, MART1/DC
	118	Chemotherapy, IL-2, IFN-α, CP-675,206
	119	Chemotherapy, MART1/DC, CP-675,206
	120	None

In all cases, prior MART1/DC and CP-675,206 had been completed over 3 months from enrollment.

Tetramer Assay Optimization

- Tetramer source: BC > NIAID.
- Flow cytometer monitoring: Daily FlowSet fluorospheres.
- Gating: Initial gating out non-CD8 cells (BC approach) > Initial gating on lymphocytes by size (consensus workshop approach, Keilholz *et al*. JIT 02).
- Number of CD8+ events: 30-200,000.

MHC Tetramer
Low Limit of Detection (LLD)

Epitope	No. Samples	Mean	SD	LLD
Negative FLYSYFALV[1]	121	0.02	0.01	0.03
$AFP_{325\text{-}332}$[2]	119	0.05	0.03	0.11

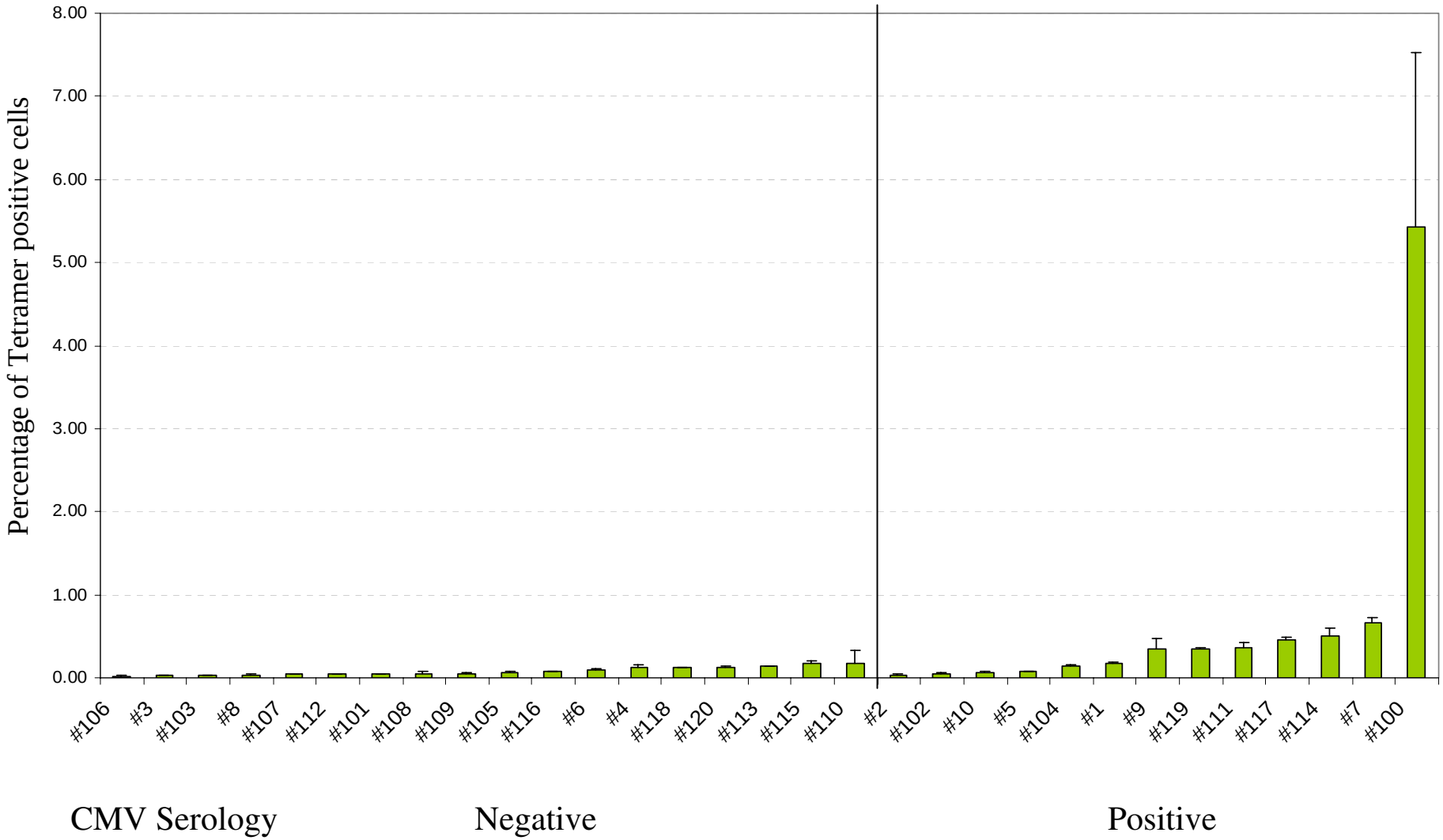
1. Ruppert, J., *et al*. Cell 1993;74:929-937.
2. Butterfield, L.H., *et al*. Journal of Immunology 2001; 166: 5300-5308.

Analytical CV
MHC Tetramer Assay

Epitope	No. Samples	Mean	SD	aCV
EBV $BMLF1_{259\text{-}267}$	26	3.19	0.26	8%
$CMVpp65_{495\text{-}503}$	10	0.37	0.04	10%
$MART\text{-}1_{26\text{-}35}$	66	5.29	0.44	8%

5-8 replicate aliquots of the same cryopreserved samples.
Results over the LLD for the assay.

CMVpp65$_{495\text{-}503}$ Tetramer Staining



Physiological CV
CMVpp65$_{495\text{-}503}$ Tetramer Assay

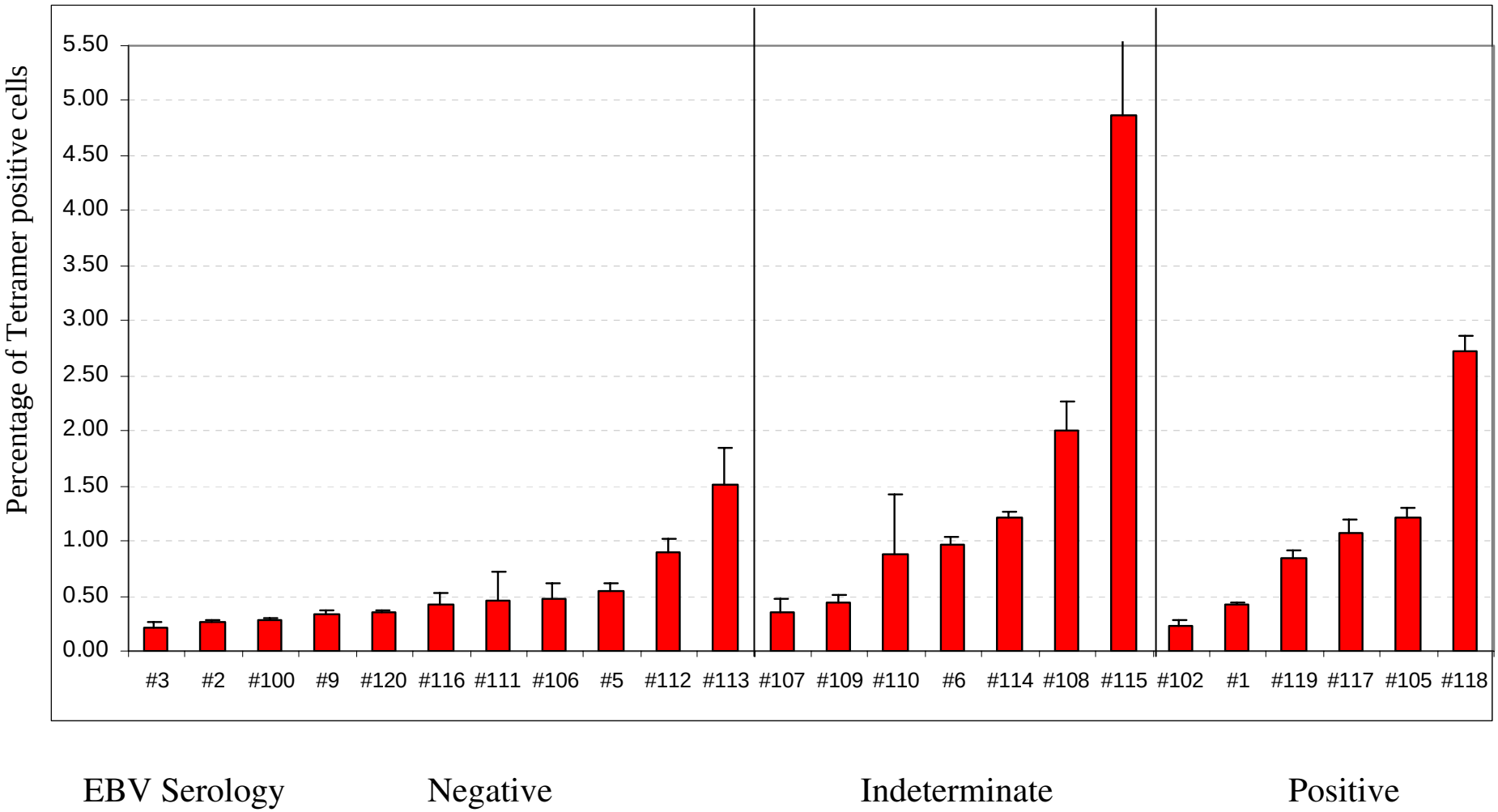
All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Seronegative	73	0.08	0.03		41%
Seropositive	47	0.87	0.07	0.012	8%

Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Seronegative	69	0.08	0.03	41%
Seropositive	47	0.87	0.07	8%

EBV BMLF1$_{259\text{-}267}$Tetramer Staining



Physiological CV
EBV $BMLF1_{259-267}$ Tetramer Assay

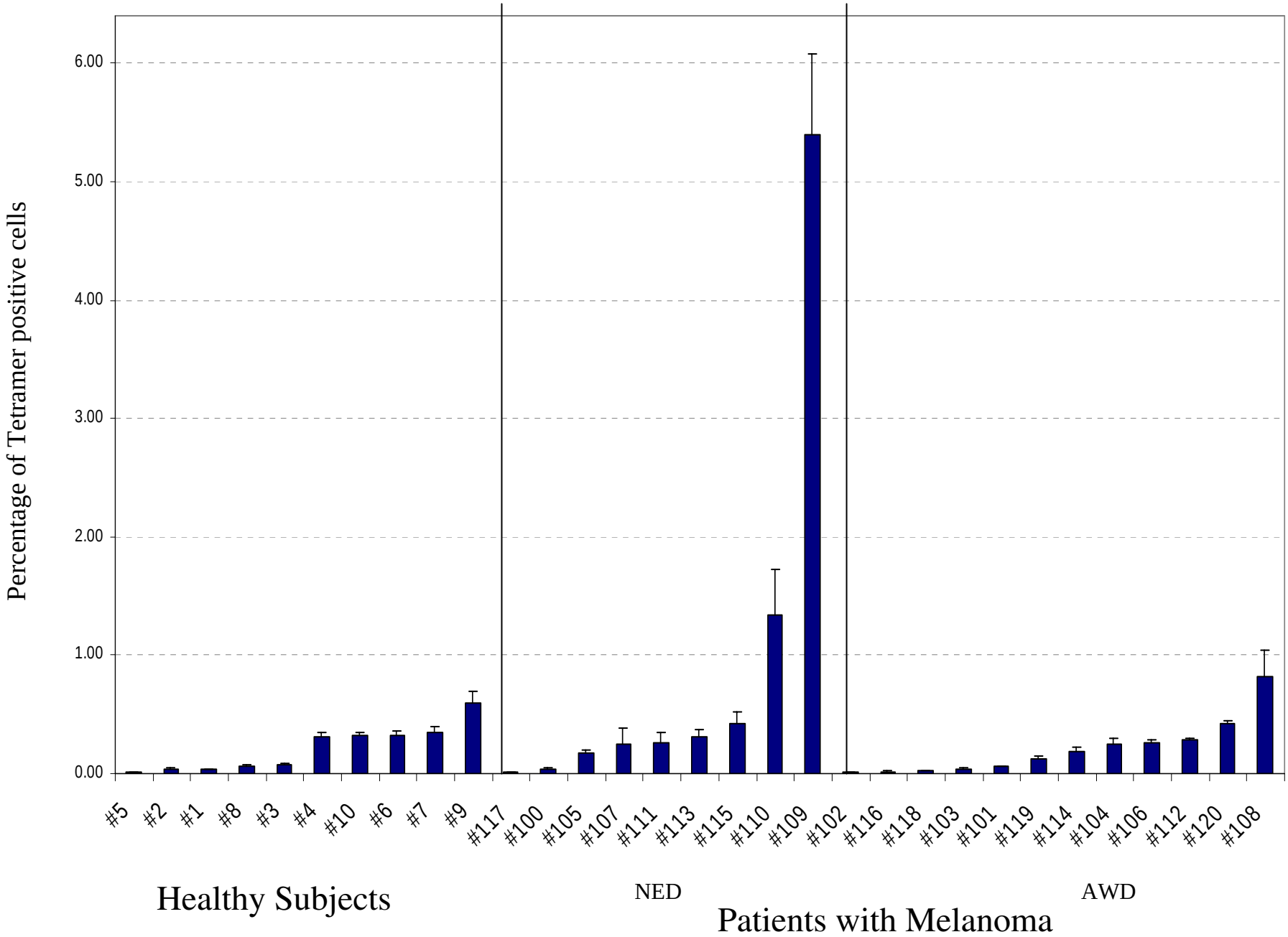
All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Seronegative	43	0.52	0.18		36%
Indeterminate	27	1.53	0.52	0.004	34%
Seropositive	24	1.08	0.14	0.005	13%

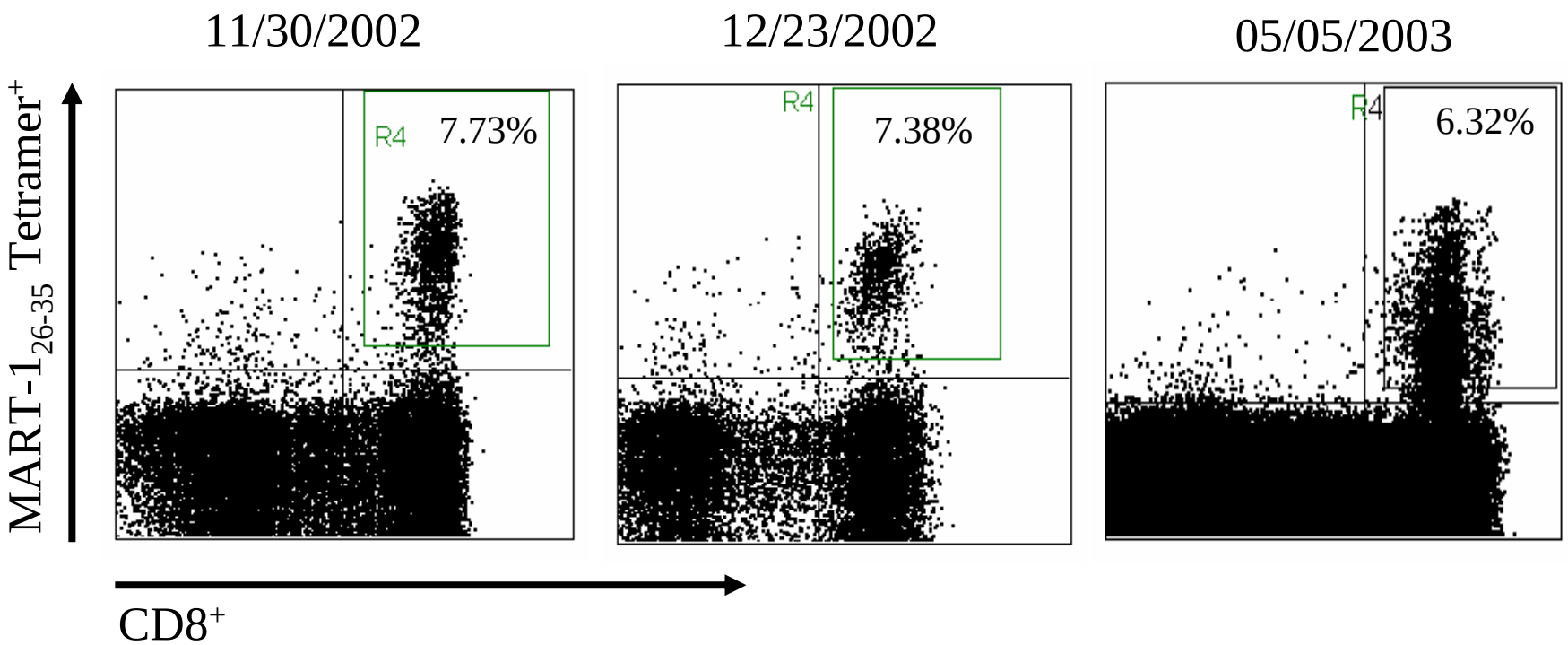
Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Seronegative	43	0.52	0.18	36%
Indeterminate or Seropositive	51	1.32	0.35	26%

MART-1_{26-35} Tetramer Staining



Subject #109: Circulating MART-1_{26-35}-Specific CD8+ T Cells Over 6 Months



Physiological CV
MART-1_{26-35} Tetramer Assay

All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Healthy Donors	39	0.21	0.05		24
NED	33	1.00	0.33	0.01	33
Active Melanoma	47	0.13	0.03	0.33	20

Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Healthy Donors	35	0.23	0.05	24
NED	33	1.01	0.32	32
Active Melanoma	31	0.19	0.04	19

IFN-γ ELISPOT Assay Optimization

- APC: K562/A*0201 > T2 > JY.
- APC/PBMC Ratio: 1:10 > 1:2.
- Peptide-pulsing Titration: 10 μg > 50 μg.
- No. PBMC per Well: $1 \times 10^5 > 2 \times 10^5$.
- PBMC Restimulation: In plate > In culture flask.

- Spot-forming Cells: CD8 > NK > CD4.
- A*0201 phenotyping of K562/A*0201 cells: > 96%.
- Mycoplasma contamination of K562/A*0201 cells: Negative

IFN-γ ELISPOT
Low Limit of Detection (LLD)

Epitope	No. Samples	Mean	SD	LLD
Negative FLYSYFALV[1]	411	3	1	6
$AFP_{325-332}$[2]	359	6	1	8

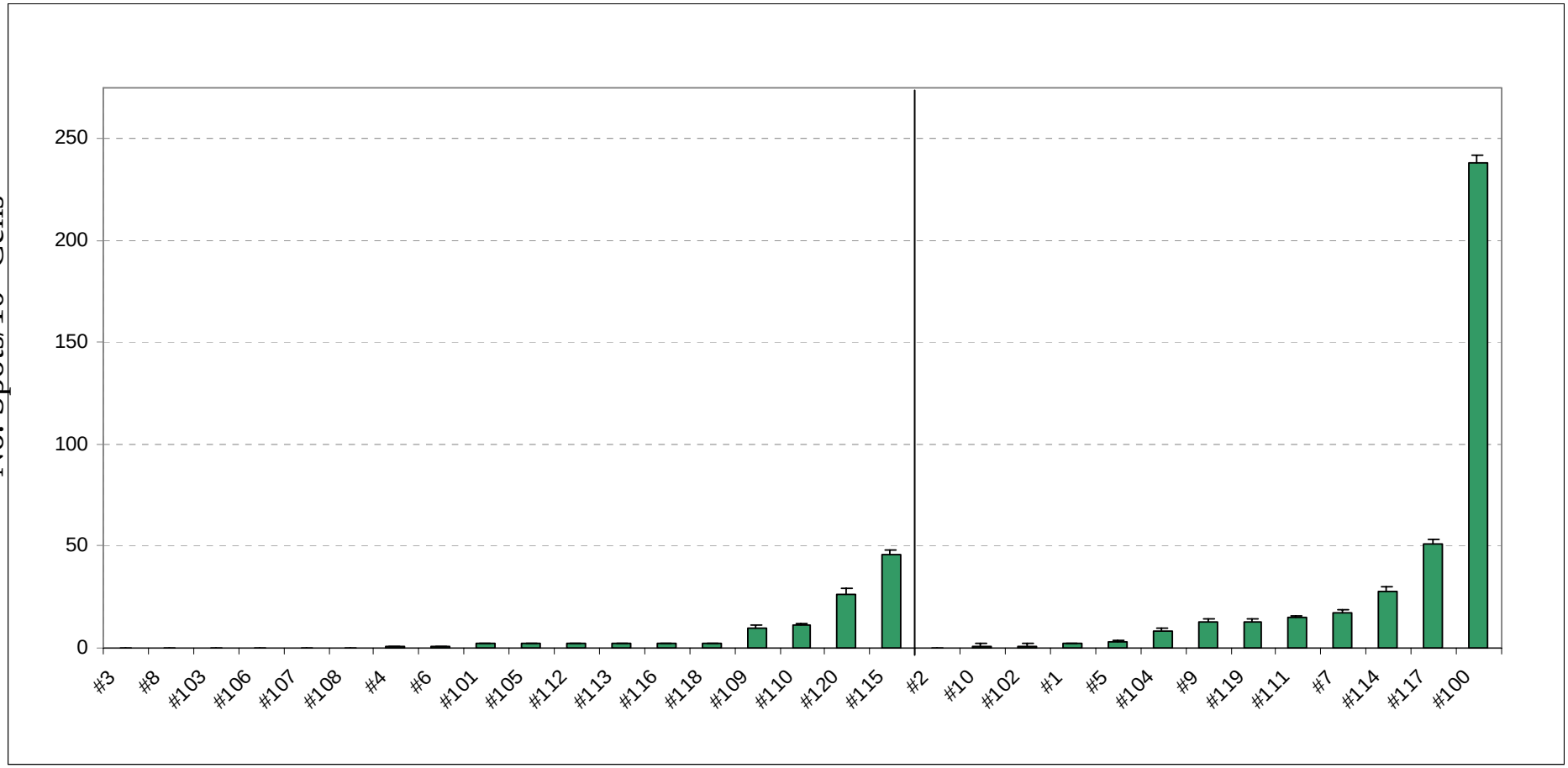
1. Ruppert, J., *et al*. Cell 1993;74:929-937.
2. Butterfield, L.H., *et al*. Journal of Immunology 2001; 166: 5300-5308.

Analytical CV
IFN-γ ELISPOT Assay

Epitope	No. Samples	Mean	SD	aCV
EBV $BMLF1_{259-267}$	24	77	12	16%
$CMVpp65_{495-503}$	-	-	-	-
$MART\text{-}1_{27-35}$	61	27	18	27%
$MART\text{-}1_{26-35}$	50	176	39	22%

7-9 replicate aliquots of the same cryopreserved samples.
Only samples over the LLD for the assay.

CMVpp65$_{495\text{-}503}$ IFN-γ ELISPOT



CMV Serology Negative Positive

Physiological CV
CMVpp65$_{495\text{-}503}$ ELISPOT Assay

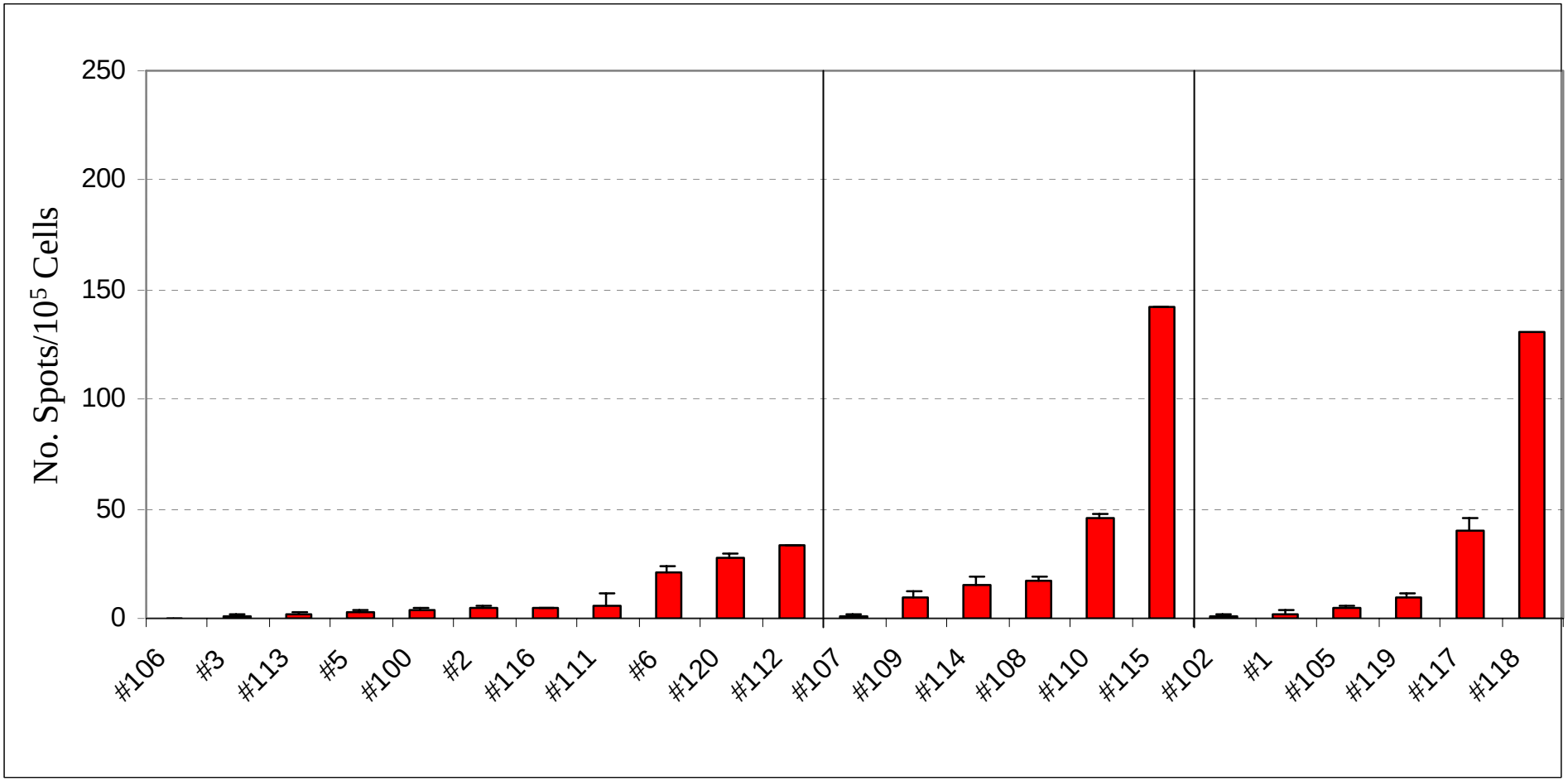
All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Seronegative	219	4	2		37%
Seropositive	132	32	3	< 0.0001	11%

Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Seronegative	46	16	4	28%
Seropositive	77	49	5	10%

EBV $BMLF1_{259-267}$ IFN-γ ELISPOT



EBV Serology Negative Indeterminate Positive

Physiological CV
EBV $BMLF1_{259\text{-}267}$ ELISPOT Assay

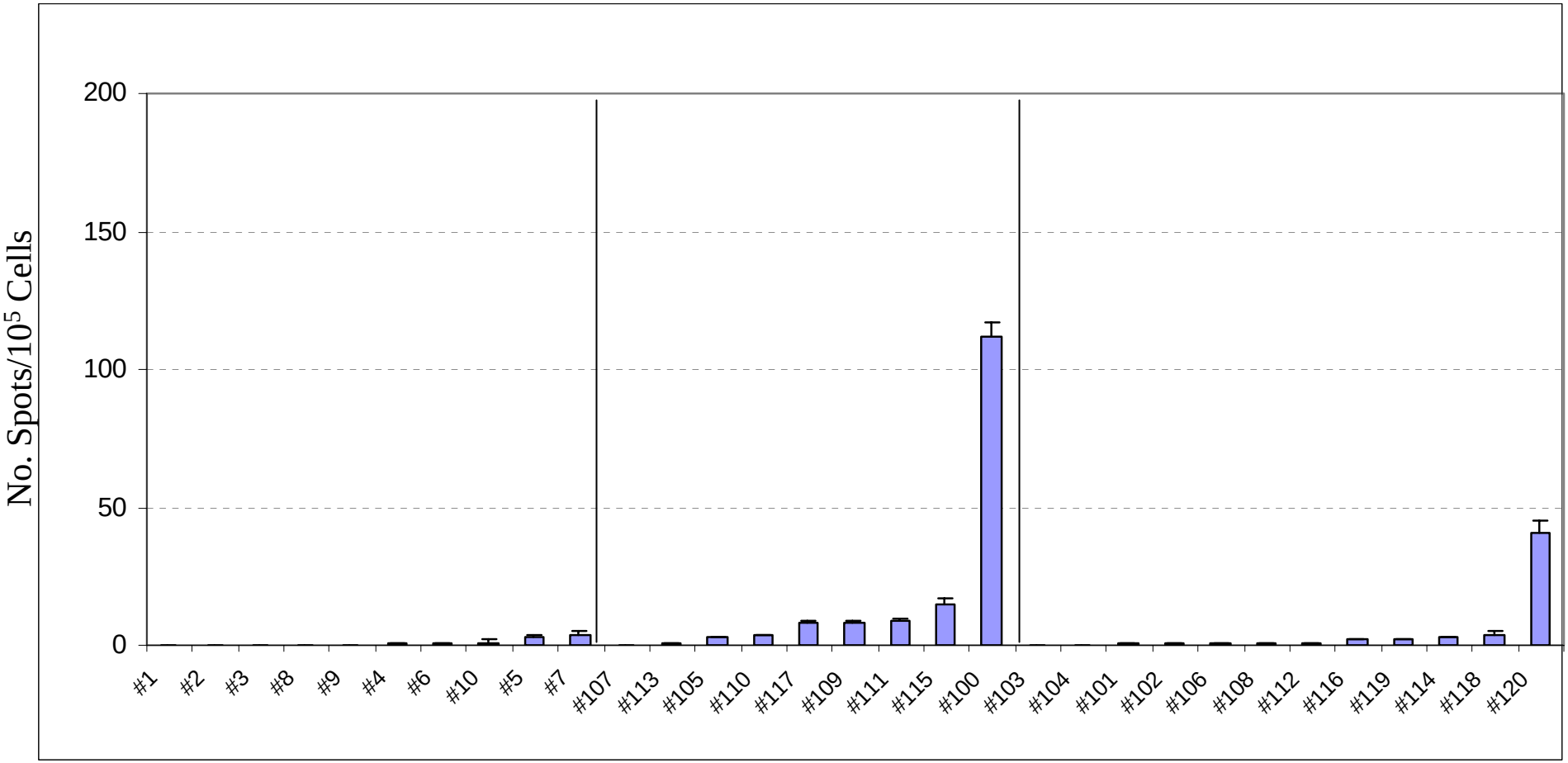
All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Seronegative	147	10	3		33%
Indeterminate	73	34	5	0.015	16%
Seropositive	75	32	5	< 0.0001	17%

Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Seronegative	49	23	7	29%
Indeterminate or Seropositive	103	47	8	18%

MART-1_{27-35} IFN-γ ELISPOT



Physiological CV MART- 1_{27-35} ELISPOT Assay

All samples:

Clinical Group	No. Samples	Mean	SD	P Value	pCV
Healthy Donors	118	1	1		62%
NED	108	17	2	0.0045	14%
Active Melanoma	138	6	2	0.0015	32%
MART-1_{26-35} Active Melanoma	53	13	4		34%

Samples over the LLD for the assay:

Clinical Group	No. Samples	Mean	SD	pCV
Healthy Donors	-	-	-	-
NED	46	37	5	12%
Active Melanoma	22	28	7	24%
MART-1_{26-35} Active Melanoma	32	18	6	31%

Reference Change Value (RCV)

- After minimization of pre-analytical variability, the assay imprecision has:
  - Random error following a Gaussian distribution.
  - Components of assay variability: aCV and pCV.

- RCV: Allows to define which change from baseline to post immune intervention would be beyond the assay variability.

Reference Change Value (RCV) for Tetramer Assay

Epitope	No. Samples aCV	aCV	No. Samples pCV	pCV	Z score	RCV
EBV $BMLF1_{259-267}$	26	8	24	13	2.09	36%
$CMVpp65_{495-503}$	10	10	27	9	2.10	30%
$MART\text{-}1_{26-35}$ NED	66	8	34	33	2.01	75%
$MART\text{-}1_{26-35}$ Active Melanoma	66	8	48	20	2.01	47%

$$RCV = \sqrt{2} \times Z \times \sqrt{aCV^2 + pCV^2}$$

Reference Change Value (RCV) for ELISPOT Assay

Epitope	No. Samples aCV	aCV	No. Samples pCV	pCV	Z score	RCV
EBV $BMLF1_{259-267}$	24	16	72	17	2.01	55%
$CMVpp65_{495-503}$	-	-	114	11	-	-
$MART\text{-}1_{27-35}$ NED	61	27	33	9	2.01	68%
$MART\text{-}1_{27-35}$ Active Melanoma	61	27	28	24	2.01	83%
$MART\text{-}1_{26-35}$ Active Melanoma	50	22	34	31	2.02	90%

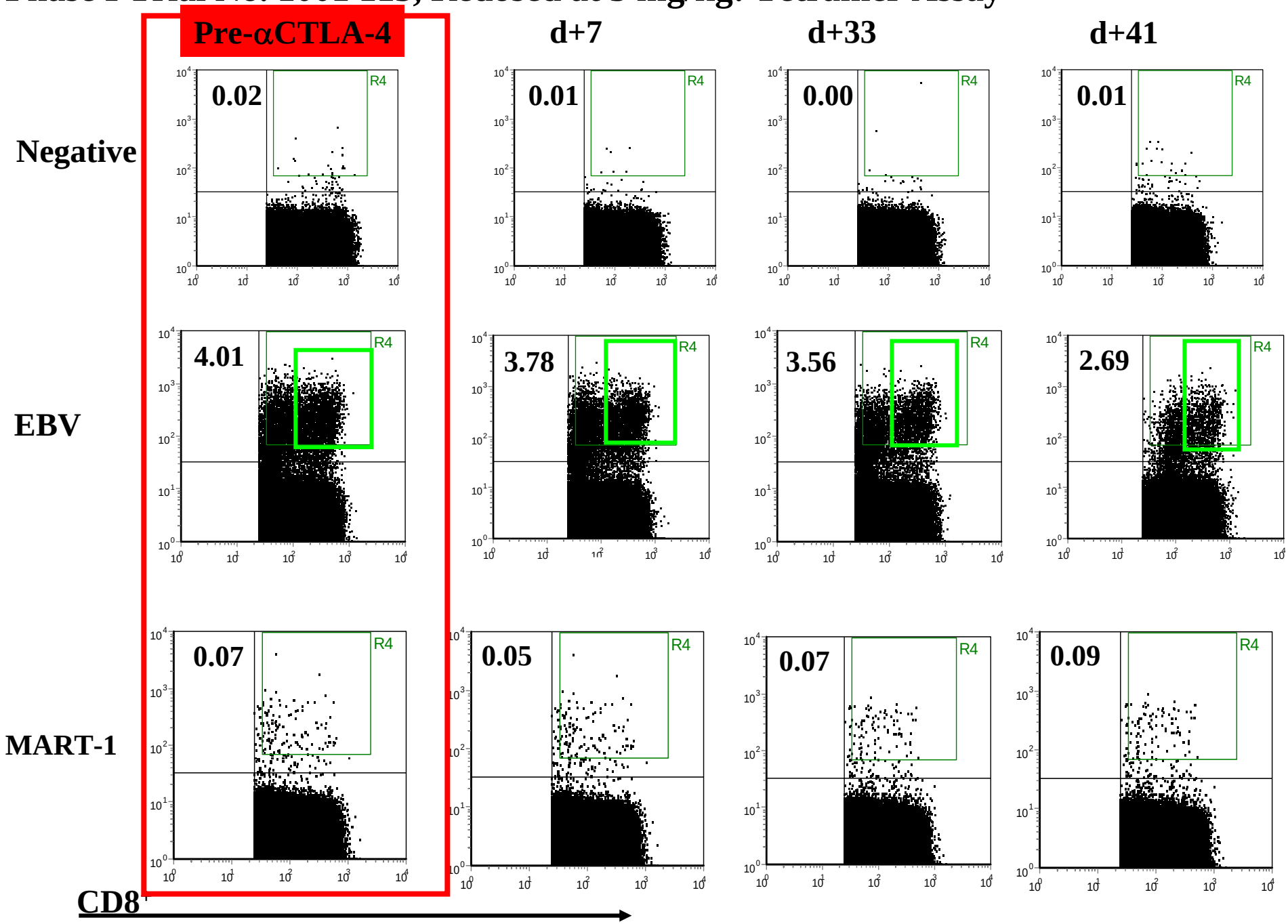
$$RCV = \sqrt{2} \times Z \times \sqrt{aCV^2 + pCV^2}$$

Exploratory Study of Antigen-Specific Immune Responses in the First-in-Human CP-675,206 Study (anti-CTLA4 mAb)

- 3 HLA-A2.1+ patients agreed to donate extra blood for immune monitoring at redosing.

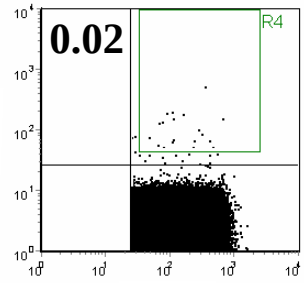
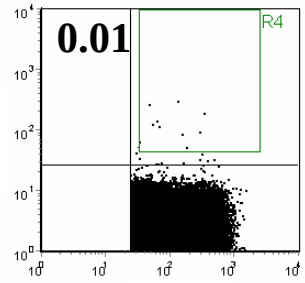
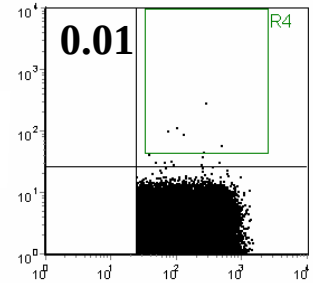
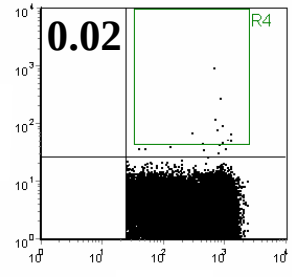
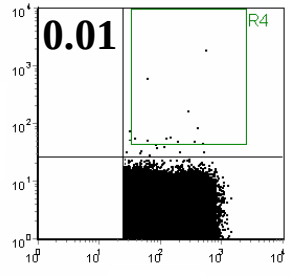
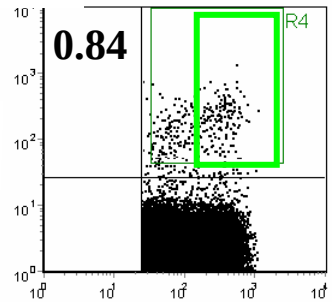
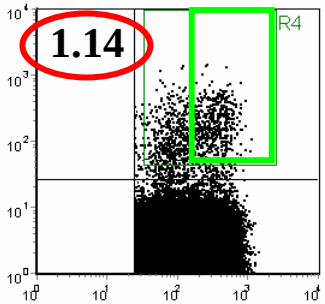
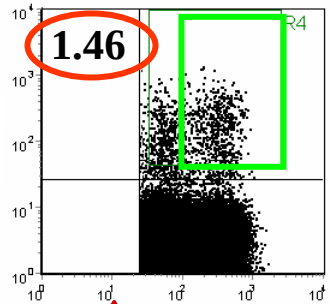
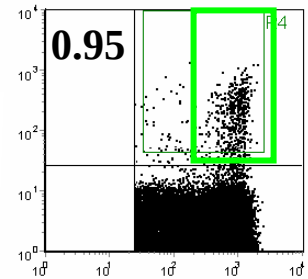
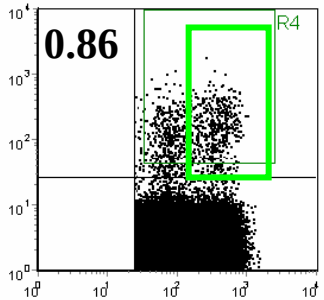
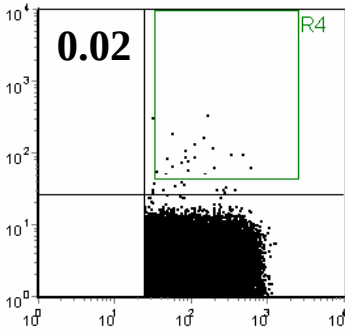
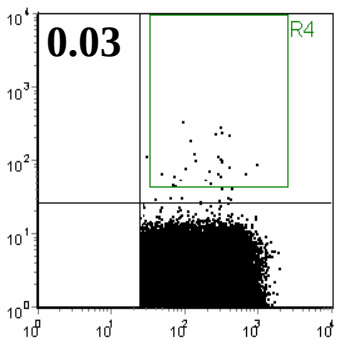
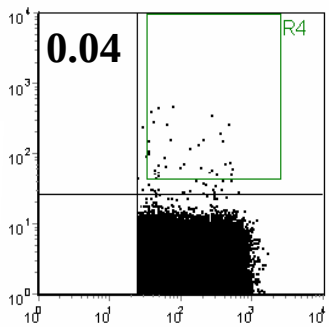
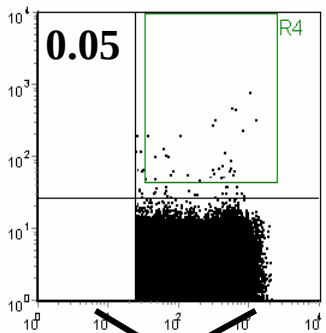
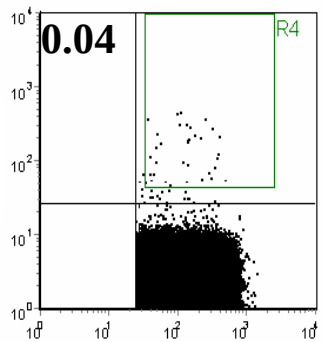
- CP-675,206 redosing for the following reasons:
  - 2 previously enrolled in a lower dose cohort.
  - 1 for clinical benefit.

Phase I Trial No. 1001 113, Redosed at 3 mg/kg: Tetramer Assay



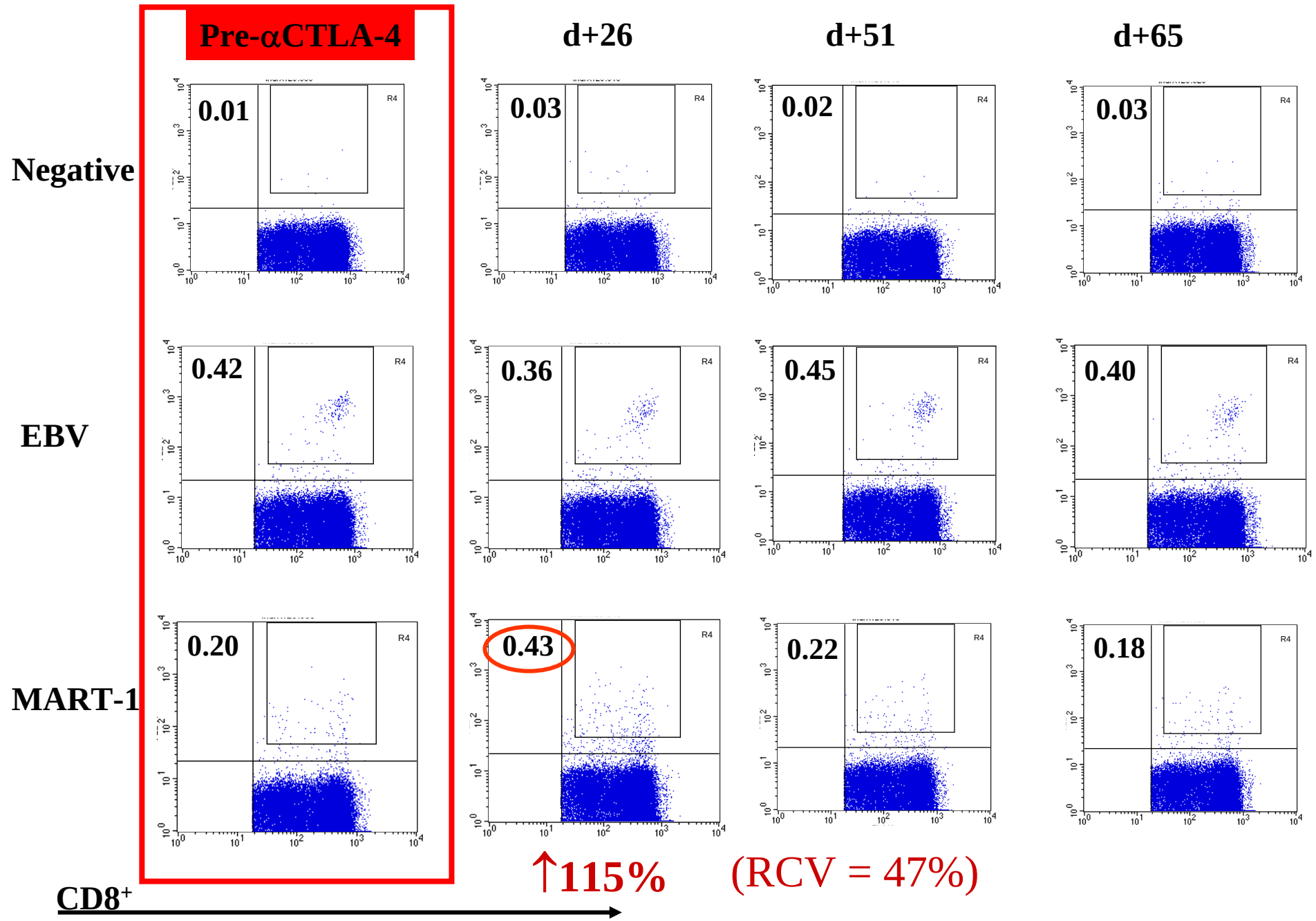
Phase I Trial No. 1001 102, Redosed at 6 mg/kg: Tetramer Assay

Pre-αCTLA-4

	d+2	d+27	d+41	d+80
Negative					
EBV					
		↑36%	↑74%	(RCV = 63%)	
MART-1$_{26-35}$					
			~~↑250%~~	(RCV = 47%)	

CD8$^+$ →

Phase I Trial No. 1001 107, Redosed at 6 mg/kg: Tetramer Assay



Conclusions

- A 50% increase in circulating MART-1-specific T cells using MHC tetramers is beyond the assay variability and should be considered a positive response to immunotherapy.
- An 80% increase in IFN-γ-producing MART-1-specific T cells using ELISPOT is beyond the assay variability and should be considered a positive response to immunotherapy.
- In a preliminary analysis, CTLA4 blockade with the monoclonal antibody CP-675,206 can increase the frequency of circulating antigen-specific T cells.

Acknowledgements

- UCLA:
  - Begonya Comin-Anduix, PhD
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