IMMUNE MONITORING OF T AND B CELL RESPONSES

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What is Immune Monitoring?

Defining immune correlates of clinical responses,
understanding the specificity of anti-tumor immune responses,
understanding why treatments fail,
improving therapy from an informed perspective

Immune monitoring encompasses several fields

- **Immunology (defining myeloid and lymphoid compartments)**
- **Pathology (immune infiltrates, heterogeneity of antigen expression)**
- **Genomics and proteomics (correlates, predictive signatures)**
- **Imaging (follow up effectors *in vivo*)**

Focus on cellular and humoral immune responses

Importance of immune monitoring of T and B cell responses in cancer patients

Define the spontaneous immunogenicity of tumors

Prognostic or diagnostic potential of immune responses

**Predictive potential of immune responses to therapy:
correlates of clinical response**

**Follow changes in immunity to assess intended
and unintended effects of treatment – Compare trials to each other**

**“You won’t know how to vaccinate until you know how to immunize.
And you won’t know how to immunize until you know how to monitor.”
*Lloyd J. Old***

Overview of presentation

Spontaneous vs. immunotherapy generated T and B cell responses

Techniques for monitoring T and B cells

Quantification vs. quality

Ex vivo vs. *in vitro* sensitization

Periphery vs. *in situ*

Correlation of immune responses with clinical events

Example of immunomonitoring of a cancer vaccine trial with NY-ESO-1 OLP

Future directions

Selection of techniques available for monitoring T and B cells

Historically:
- T cell quantification by cytokine release in supernatant (ELISA)
- CTL (CD8) by ^{51}Chromium Release Test
- Th (CD4) proliferation by ^{3}H-Thymidine Incorporation Assay
- Ab (B cells) by ELISA

Single cell level immune monitoring

ELISPOT (B and T cells)

Flow cytometry – Time-of-Flight mass spectrometry – Cell sorting

Intracellular Staining of Cytokines – Phosphoflow – Tetramers

Advantages

- High sensitivity
- Quantitative
- May distinguish subpopulations
- Efforts to harmonize methods

Limitations

- Antigen may need to be identified
- Technically more challenging
- Can be expensive

Selection of techniques available for monitoring T and B cells

Comprehensive immune monitoring

Phenotyping of populations

Multiplex assays for cytokines

Immunogenomics of T and B cells

TCR and BCR sequencing

Seromics (protein array profiling of antibodies)

Immunohistochemistry and imaging of T and B cells (Immunoscore)

Advantages

- Suitable for immunotherapies where target antigen is not defined
- Discovery tool for broad correlations

Limitations

- Not necessarily cancer-specific
- Costly
- Complex to analyze - TMI

Quantitative vs. Qualitative Immune Monitoring

Is the immune response detectable? Relevant? Efficient?

Qualitative aspects measured

Specificity – Example: Distinguish his-tag specific responses from antigen-specific responses following protein vaccine

Avidity or titer (serial dilution of target antigen or epitope amount required for minimal reactivity) – Tumor recognition

Polyfunctionality (ability to produce multiple cytokines, various effector functions)

Polyclonality (epitope mapping within an antigen)

Surface markers related to function (memory, naïve, effector, central, periphery, tissue homing, activation [ICOS, 4-1BB, OX40], suppression [CTLA-4, PD1])

Immunohistochemistry and imaging of T and B cells (Immunoscore)

Phenotypic vs. functional analyses of T cells

Surface markers may inform on the type of cells but ultimate functional tests may be required

Cytotoxic T cell	
Surface phenotype	αβ TCR, CD3, CD8
Transcription factors	EOMES, T-bet, BLIMP1
Effector molecules secreted	Perforin, granzyme, IFNγ

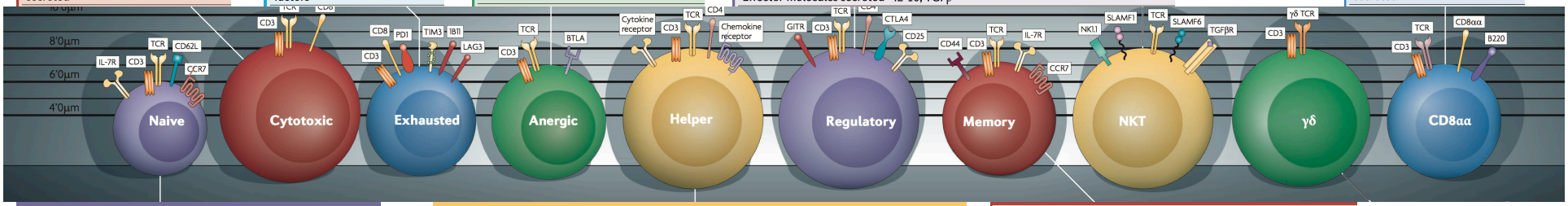
Exhausted T cell	
Surface phenotype	CD3, CD8, PD1, TIM3, 1B11, LAG3
Transcription factors	BLIMP1

Anergic T cell	
Surface phenotype	αβ TCR, CD3, BTLA
Effector factors	GRAIL, CBL-B, ITCH, NEDD4

Natural T_{Reg} cell	
Surface phenotype	αβ TCR, CD3, CD4, CD25, CTLA4, GITR
Transcription factors	FOXP3, STAT5, FOXO1, FOXO3
Effector molecules secreted	IL-10, TGFβ, IL-35

Inducible T_{Reg} cell	
Surface phenotype	αβ TCR, CD3, CD4, CD25, CTLA4, GITR
Transcription factors	FOXP3, FOXO1, FOXO3, STAT5, SMAD2, SMAD3, SMAD4
Effector molecules secreted	IL-10, TGFβ

CD8αα T cell	
Surface phenotype	αβ or γδ TCR, CD3, CD8αα, B220
Effector molecules secreted	IL-10, TGFβ



CD4⁺ αβ T cell	
Surface phenotype	αβ TCR, CD3, CD4, CCR7, CD62L^{hi}, IL-7R (CD127)
Transcription factors	THPOK

CD8⁺ αβ T cell	
Surface phenotype	αβ TCR, CD3, CD8, CCR7, CD62L^{hi}, IL-7R (CD127)
Transcription factors	RUNX3

T_H1 cell	
Surface phenotype	αβ TCR, CD3, CD4, IL-12R, IFNγR, CXCR3
Transcription factors	T-bet, STAT4, STAT1
Effector molecules secreted	IFNγ, IL-2, LTα

T_H2 cell	
Surface phenotype	αβ TCR, CD3, CD4, IL-4R, IL-33R, CCR4, IL-17RB, CRTH2
Transcription factors	GATA3, STAT6, DEC2, MAF
Effector molecules secreted	IL-4, IL-5, IL-13, IL-10

T_H9 cell	
Surface phenotype	αβ TCR, CD3, CD4
Transcription factors	PU.1
Effector molecules secreted	IL-9, IL-10

T_H22 cell	
αβ TCR, CD3, CD4, CCR10	
AHR	
IL-22	

T_H17 cell	
Surface phenotype	αβ TCR, CD3, CD4, IL-23R, CCR6, IL-1R, CD161 (human only)
Transcription factors	RORγt, STAT3, RORα
Effector molecules secreted	IL-17A, IL-17F, IL-21, IL-22, CCL20

T_{FH} cell	
Surface phenotype	αβ TCR, CD3, CD4, CXCR5, SLAM, OX40L, CD40L, ICOS, IL-21R, PD1
Transcription factors	BCL-6, STAT3
Effector molecules secreted	IL-21

Central memory T cell	
Surface phenotype	CCR7^{hi}, CD44, CD62L^{hi}, TCR, CD3, IL-7R (CD127), IL-15R
Transcription factors	BCL-6, BCL-6B, MBD2, BMI1
Effector molecules secreted	IL-2, CD40L Low levels IL-4, IFNγ, IL-17A

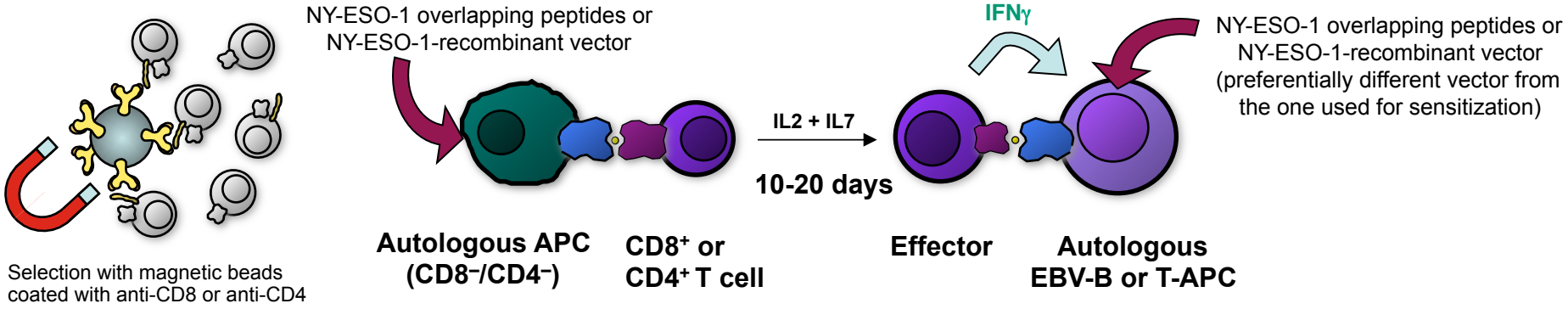
Effector memory T cell	
Surface phenotype	CD62L^{low}, CD44, TCR, CD3, IL-7R (CD127), IL-15R, CCR7^{low}
Transcription factors	BLIMP1
Effector molecules secreted	Rapid and high production of inflammatory cytokines

Tetramers, constitutive surface markers

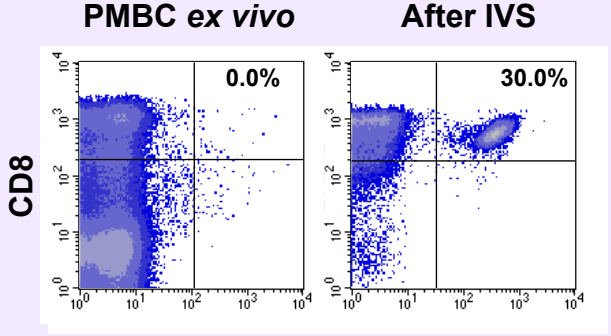
Cytokine secretion, Cytotoxicity, Upregulation of surface markers (ICOS, CD154)

Adapted from Dong C and Martinez GJ. Poster in Nature Reviews Immunol © 2010 (with Abcam)

Ex vivo vs. *in vitro* sensitization for CD8⁺ and CD4⁺ T cell responses: Example for NY-ESO-1 CD8⁺ T cell responses



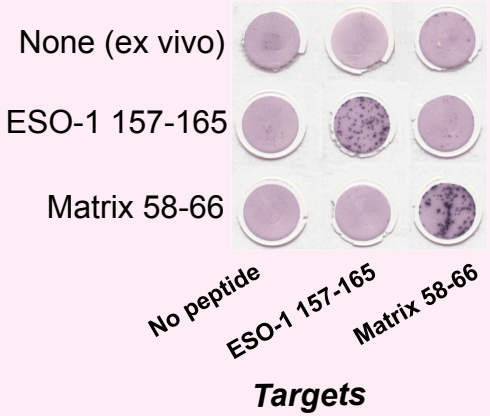
Tetramers in PBMC



HLA-A2 / NY-ESO-1 p157-165 Tetramer

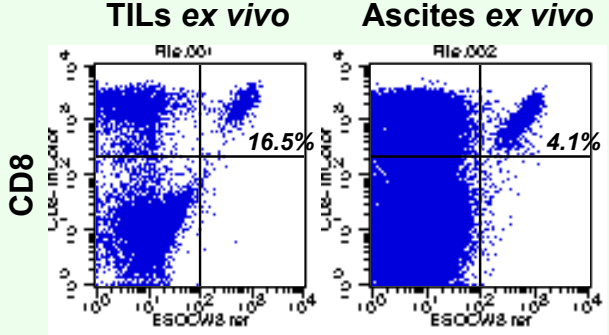
TCR RECOGNITION

ELISPOT



IFNγ RELEASE

Tetramers in Tumors



HLA-Cw3 / NY-ESO-1 p92-100 Tetramer

TCR RECOGNITION

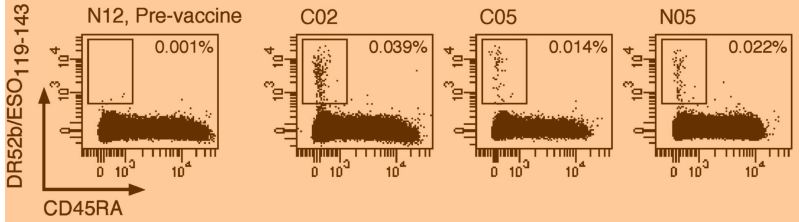
Difficult to detect *ex vivo* from PBMC unless strong viral epitope (CMV, EBV), analog peptide of differentiation antigens (gp100, Melan-A)

Gnjatic et al. Proc Natl Acad Sci U S A. 2000;97:10917-22

Ex vivo vs. *in vitro* sensitization for CD8$^+$ and CD4$^+$ T cell responses: Pros and Cons

Ex vivo monitoring

Advantages
- Quantitative
- Phenotype of antigen-specific cells unmodified by cell culture



Ayyoub et al. Proc Natl Acad Sci U S A. 2010;107:7437–42.

Limitations
- Requires many cells (>10^7 for a single tetramer staining)
- Difficult to perform multiple specificity controls
- Tetramers not always available
- Results can be questionable if too close to sensitivity threshold

In vitro sensitization

Advantages
- Fewer cells needed from precious clinical samples
- Clear yes/no detection without *de novo* induction of T cells
- Allows for multiple specificity controls and targets
- Independently assess CD8 and CD4

Limitations
- Semi-quantitative
- Cell culture may modify phenotype

Where to monitor? Periphery or tissue?

In situ monitoring

Advantages
- Most relevant: at tumor site
- Phenotype of antigen-specific cells unmodified by cell culture

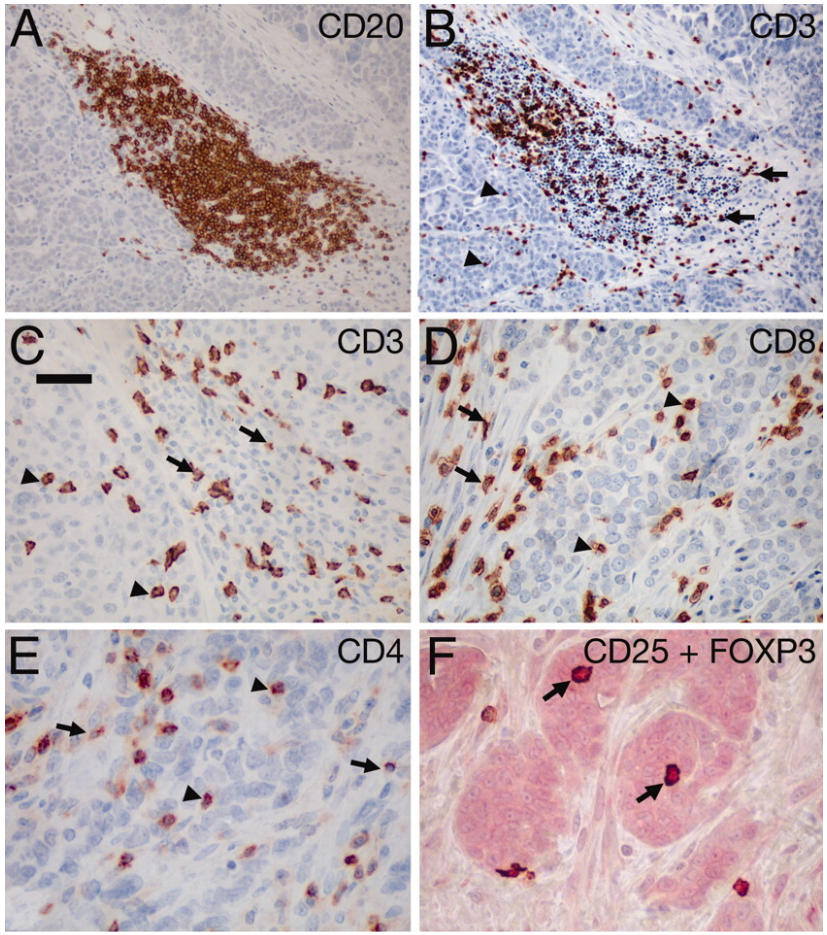
Limitations
- Generally not accessible
- Not enough cells to test
- Quality of tissue
- Heterogeneity and sampling bias

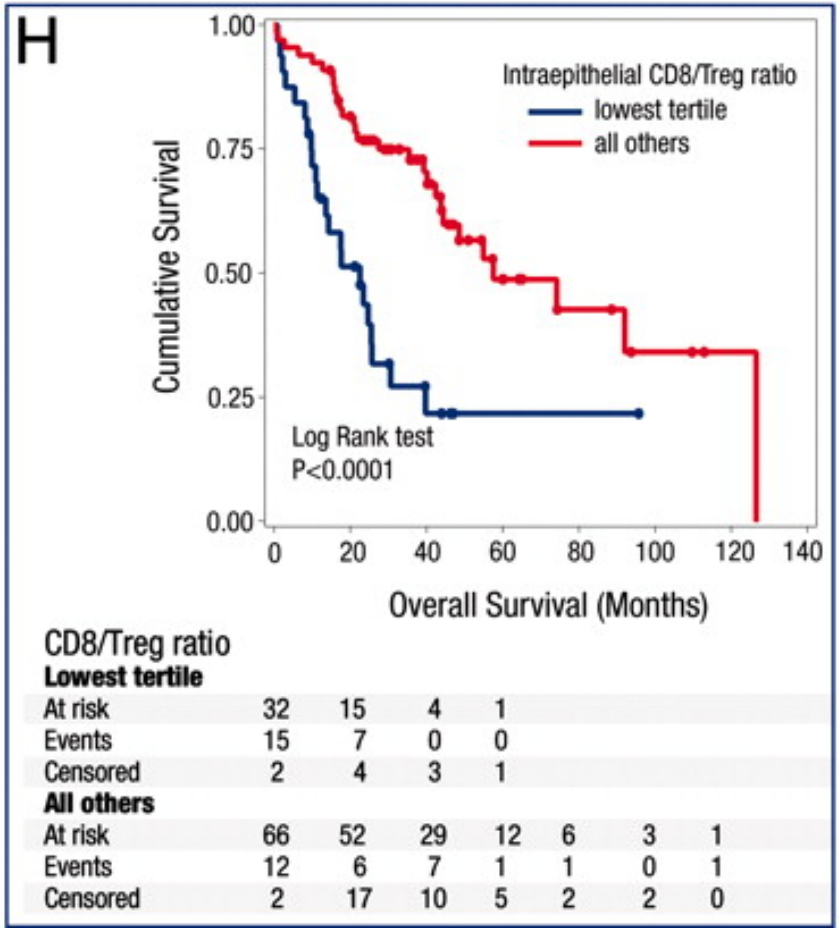
Peripheral blood

Advantages
- Systemic
- Easy access
- Sufficient amounts
- No biopsy needed

Limitations
- May not reflect tumor environment
- Potentially rarer precursors
- Representative or not?

Intraepithelial CD8$^+$ TILs and a high CD8$^+$/Treg ratio are associated with favorable prognosis in ovarian cancer

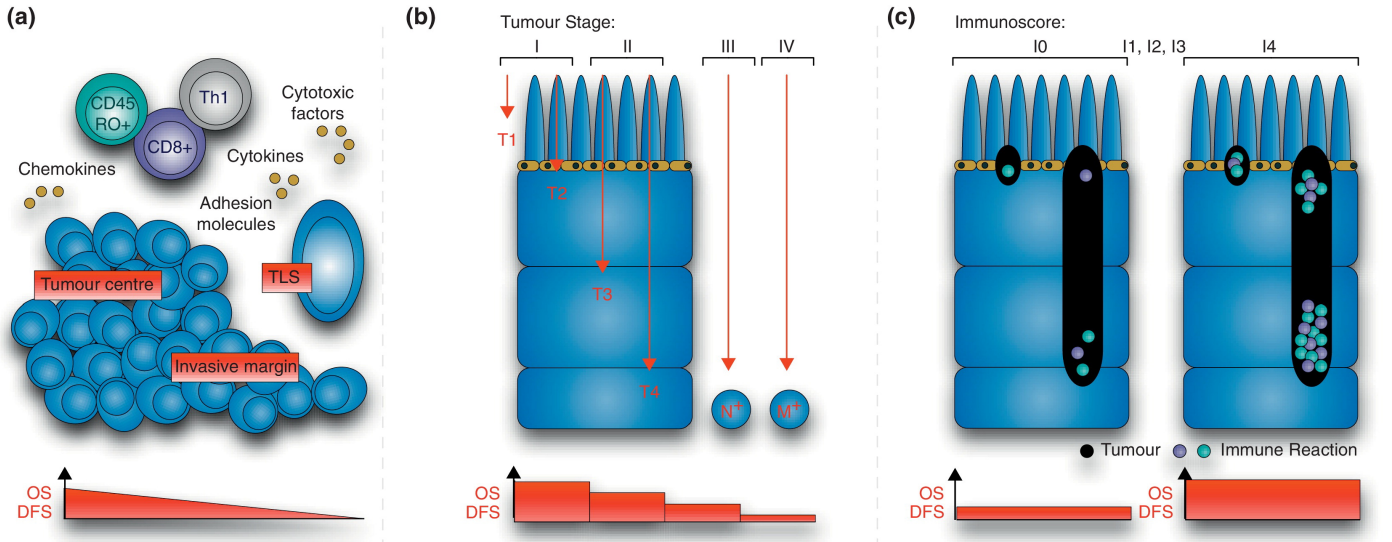




CD8/Treg ratio							
Lowest tertile							
At risk	32	15	4	1			
Events	15	7	0	0			
Censored	2	4	3	1			
All others							
At risk	66	52	29	12	6	3	1
Events	12	6	7	1	1	0	1
Censored	2	17	10	5	2	2	0

Proc Natl Acad Sci U S A. 2005 Dec 20;102(51):18538-43.

Immunoscore: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome

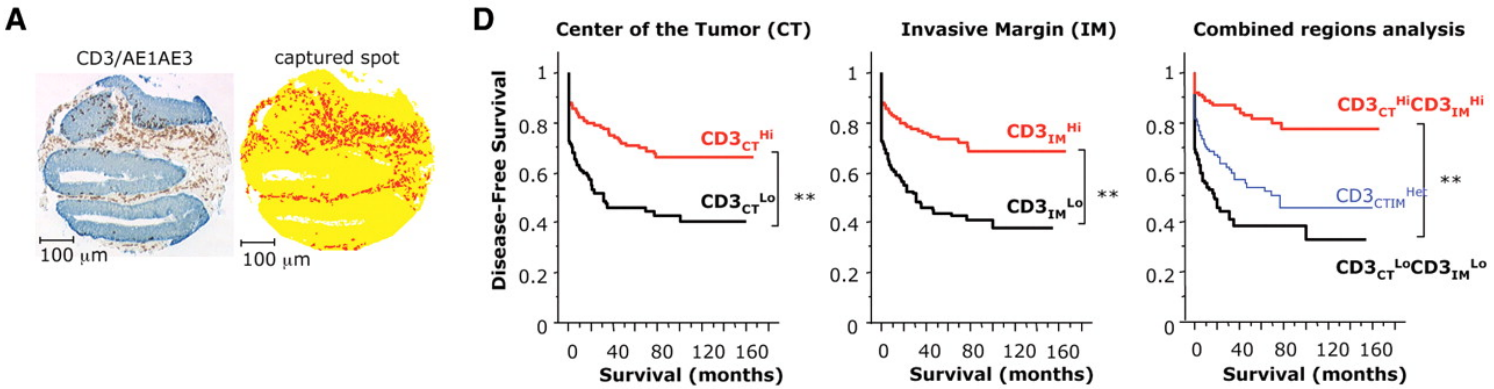


Immune Contexture	Key Parameters
Type	CTLs (CD3+CD8+) Memory T cells (CD3+CD45RO+)
Location	Tumour centre (CT) Invasive margin (IM) Presence and quality of TLS
Density	Continuous
Functional orientation	Th1 cell-associated factors Cytotoxic factors Chemokines, cytokines Adhesion molecules

TNM Staging	Key Parameters		
Tumour (T)	Longitudinal extent of tumour burden		
Lymph node (N)	Presence of cancer cells in draining and regional lymph nodes		
Metastases (M)	Evidence of distant site metastases		
Cox analysis (ref. 23)	DFS HR/P-value	OS HR/P-value	DSS HR/P-value
	1.38/0.09 ns	1.18/0.29 ns	1.43/0.10 ns

Immuno-score	Key Parameters		
Type	CTLs (CD3+CD8+) Memory T cells (CD45RO+)		
Location	Tumour centre (CT) Invasive margin (IM)		
Density	Predefined cut points		
Cox analysis (ref. 23)	DFS HR/P-value	OS HR/P-value	DSS HR/P-value
	0.64/<0.0001	0.71/<0.0001	0.63/<0.0001

Adapted from Angell et al.
Curr Opin Immunol. 2013;25:261



Adapted from Galon et al.
Science 2006;313:1960-4

Why monitor when patients with measurable immunity still have cancer?

Majority of trials fail to show correlation between immune responses and clinical responses

Humoral and cellular immunity may be insufficient or happen too late

Escape mechanisms of the tumor from immunosurveillance

Influence of heterogeneity of antigen expression

Active mechanisms of immunosuppression, especially at the tumor site

Co-inhibitory molecules, regulatory T cells

Maybe correlation with immune responses will become more evident with immunotherapeutic drugs able to provide better clinical benefit

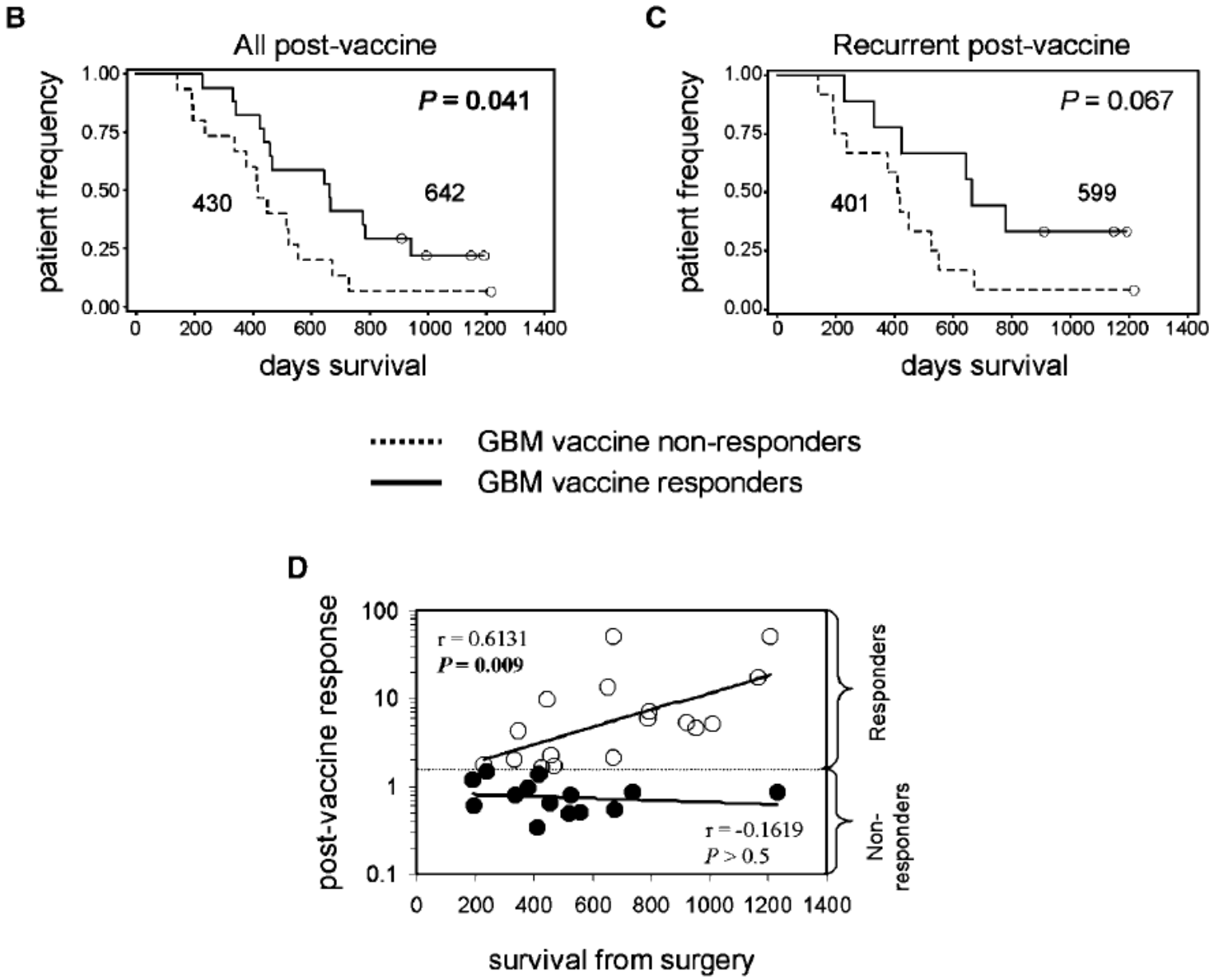
Correlations between immune responses and clinical outcome

Ogi C & Aruga A. Oncoimmunology. 2013;2:e26012

Table 3. Evaluation of immune response and clinical outcome after therapeutic cancer vaccines by log-rank test using the Kaplan-Meier model

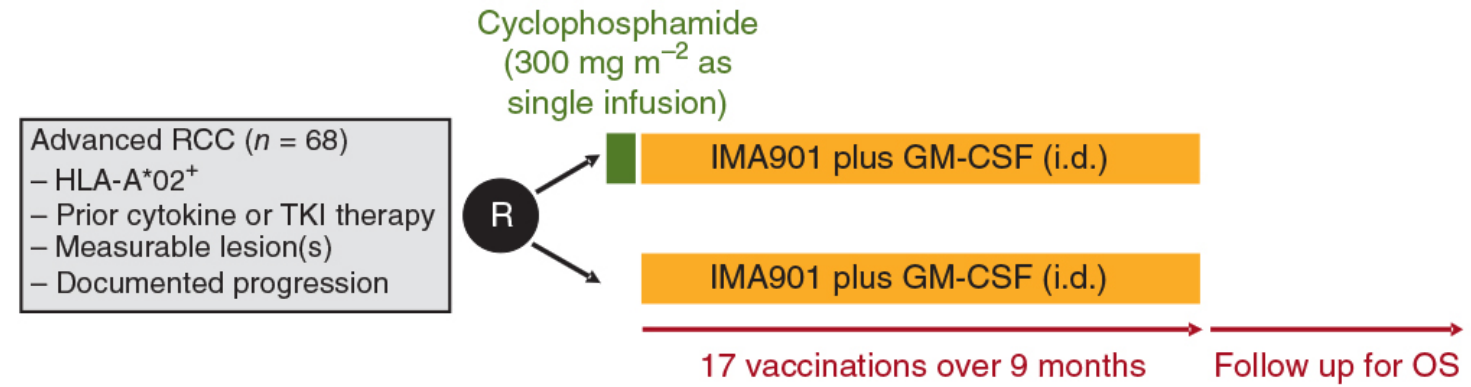
Product	Cancer	Phase	Evaluation results	Positive Correlation	Reference
Provenge®	Prostate cancer	P I/II	TTP correlated with development of an immune response to prostatic acid phosphatase (PAP) and with the dose of dendritic cells received.	Y	29
		P III (IMPACT)	An antibody titer of more than 400 against PA2024 or PAP after baseline lived longer than did those who had an antibody titer of 400 or less ($p < 0.001$ and $p = 0.08$, respectively). No survival difference could be detected between patients in the sipuleucel-T group who had T-cell proliferation response to PA2024 or PAP and those who did not.	Y	10
Canvaxin®	Melanoma (Stage IV)	P II	5-y OS rate was 75% for patients who had an elevated level of anti-TA90 IgM and a strong DTH response, 36% for patients who had either an elevated IgM response or a strong DTH response, and only 8% if neither response was strong ($p < 0.001$)	Y	30
	Melanoma (Stage II)	P II	Anti-TA90 IgM levels $\geq$ 1:800 were significantly correlated with improved 5-y DFS and improved 5-y OS.	Y	30
	Melanoma (Stage IIIa and IV)	After P II	Survival correlated significantly with delayed cutaneous hypersensitiity ($p = 0.0066$) and antibody response ($p = 0.0117$).	Y	31
Specifid™	Non-Hodgkin's lymphoma	P II (after rituximab)	There was no correlation observed between the development of anti-Id immune response and the achievement of an objective response or duration of EFS.	N	33
BEC2	Small cell lung cancer	P III	The survival of responders was better than that of non-responders, although this did not reach statistical significance (median survival, 19.2 v 13.9 mo for responders v non-responders; $p = 0.0851$).	Y	21

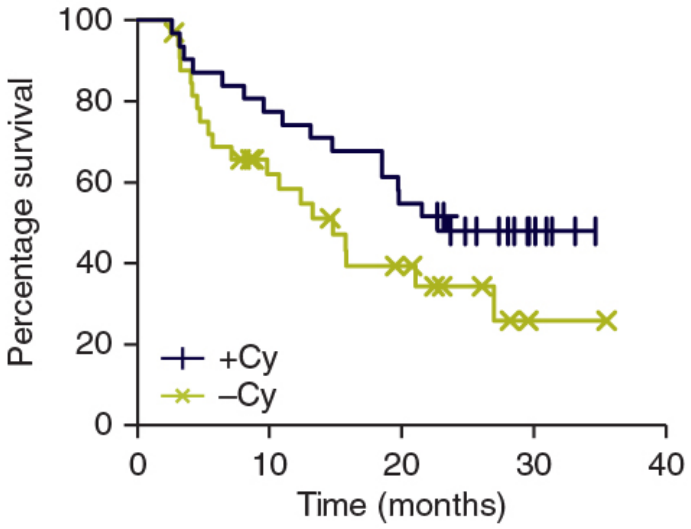
Association with survival in DC + autologous lysate vaccine in glyoblastoma patients (GBM)



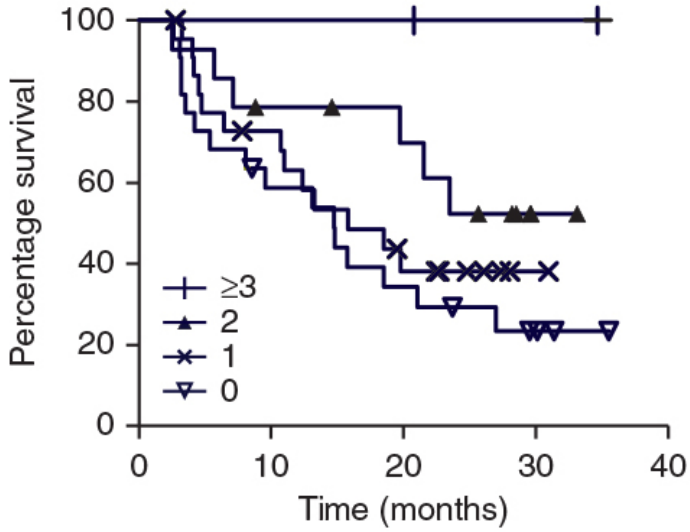
Wheeler CJ et al. Cancer Res 2008; 68:5955

Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival





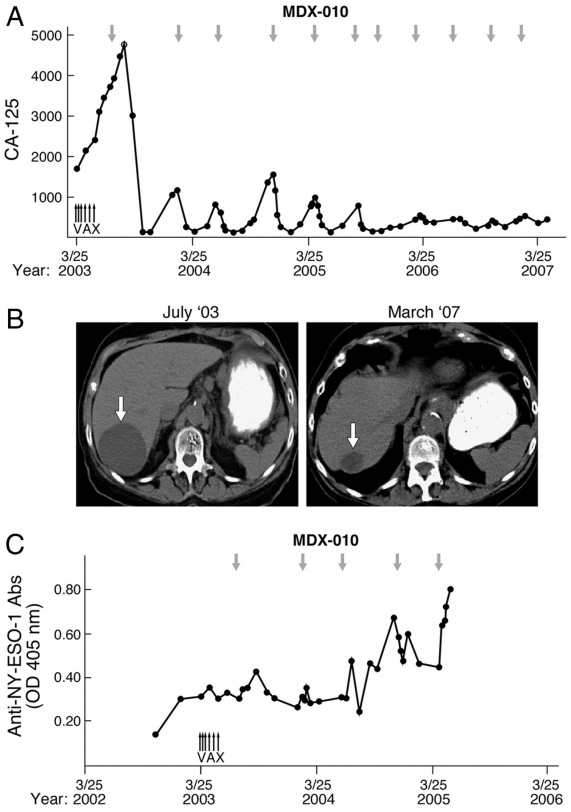
Overall survival of subjects treated with (*n* = 31) or without (*n* = 33) cyclophosphamide



Overall survival of subjects with no detectable immune responses (*n* = 22), immune responses to one tumor-associated peptide (TUMAP) (*n* = 23), two TUMAPs (*n* = 14), or at least three TUMAPs (*n* = 2)

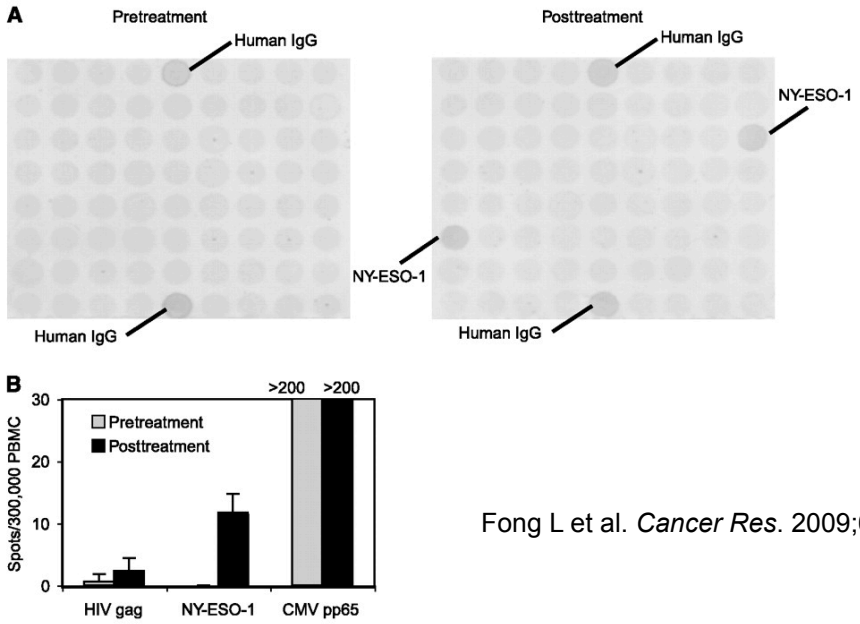
Sporadic evidence of changes in NY-ESO-1 serum antibody with clinical course following anti-CTLA-4 or other therapies

Ovarian carcinoma combination immunotherapy with CTLA-4 blockade and irradiated autologous tumor cells engineered to secrete GM-CSF (GVAX)



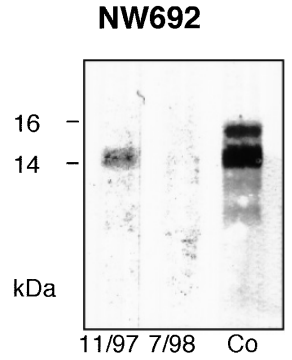
Hodi FS et al. *PNAS* 2008;105:3005-3010.

Prostate cancer combination immunotherapy with CTLA-4 blockade and GM-CSF



Fong L et al. *Cancer Res*. 2009;69:609-615.

Bladder cancer who underwent curative resection of a NY-ESO-1 expressing primary tumor (Western blot)



Jäger E et al. *Int. J. Cancer.* 1999;84:506-510

Correlation of NY-ESO-1 antibody with clinical course following anti-CTLA-4 treatment with ipilimumab

In collaboration with Jedd Wolchok and Jim Allison MSKCC/Ludwig Center and with Ruth Halaban and Mario Sznol, Yale University - Melanoma sera

Patients with NY-ESO-1 antibodies before CTLA-4 treatment

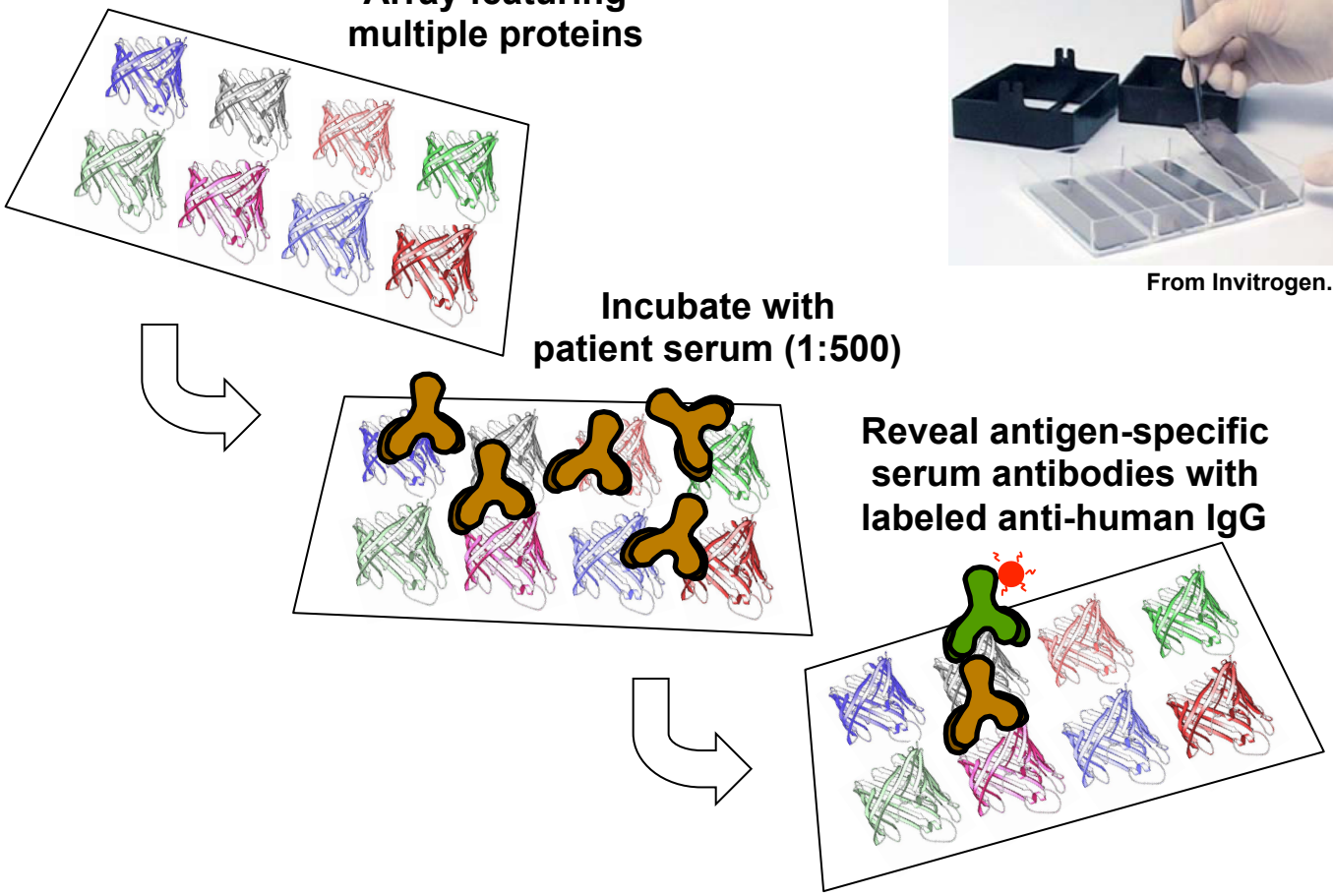
Status at wk 24	# patients (%)	NY-ESO-1 SERONEGATIVE # (%)	NY-ESO-1 SEROPOSITIVE # (%)
CR	4 (2.9%)	3	1
PR	14 (10.0%)	10	4
SD	30 (21.4%)	23	7
Clinical Benefit	**48** (34.3%)	**36** (30.5%)	**12** (54.6%)
No Clinical Benefit	**92** (65.7%)	**82** (69.5%)	**10** (45.4%)
Total	140 (100%)	118	22

According to Immune-related response criteria:
Clinical Benefit
CR: Complete Response
PR: Partial Response
SD: Stable Disease
No Clinical Benefit
POD: Progression of Disease (includes MR: mixed response)
DOD: Dead of Disease

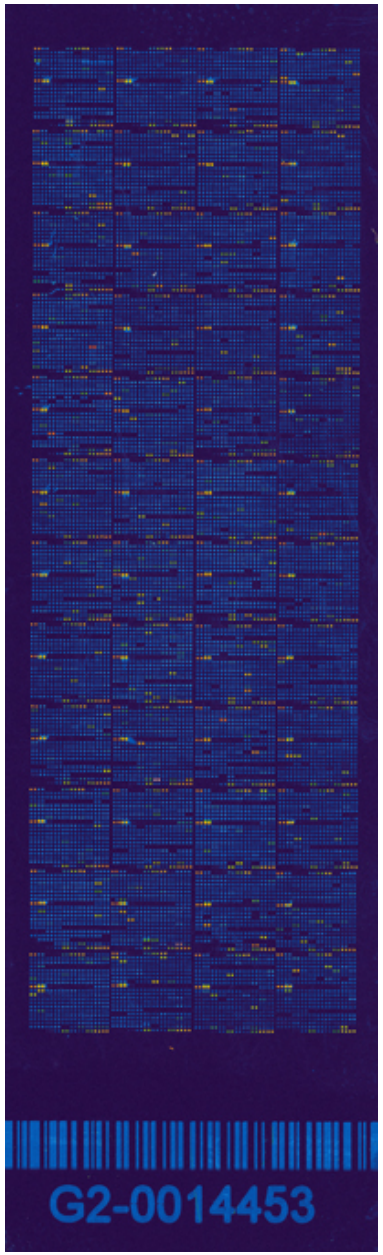
Fisher's exact test (two-tailed):
P value 0.0481
RR=1.8(1.1-2.9)

Yuan, Gnjatic et al. Proc Natl Acad Sci U S A. 2011;108:16723

Seromics: Methodology for antibody profiling with protein microarrays



From Invitrogen.com

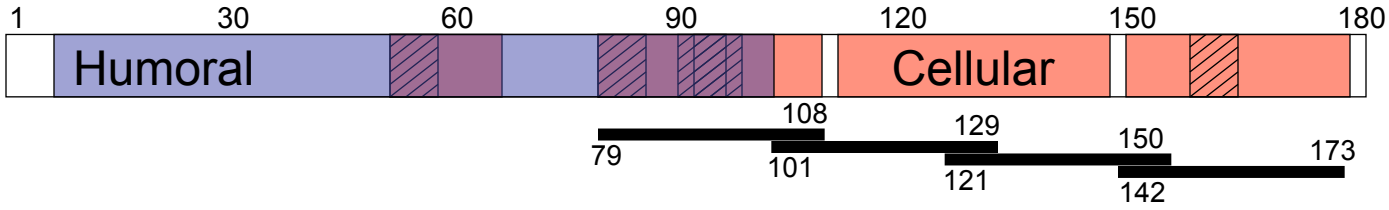


Arrays may contain >9000 proteins mostly full-length baculovirus-produced GST-fusion proteins randomly selected, both known and predicted sequences

Phase I study LUD2006-001 / MSK07-152: Immunization Schedule

(PI: Paul Sabbatini, Clinical trial NCT00616941)

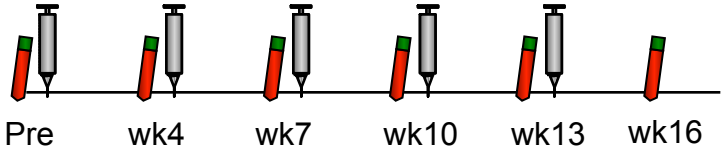
Epithelial ovarian cancer patients in 2nd or 3rd complete remission (NY-ESO-1 expression optional)



1mg NY-ESO-1 Overlapping Long Peptides (OLP4) (250µg of each peptide)
with or without 0.5-1ml Montanide and 1.4mg Poly-ICLC

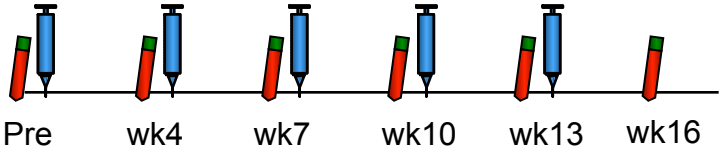
Cohort 1
4 patients

OLP4 without adjuvant - s.c.



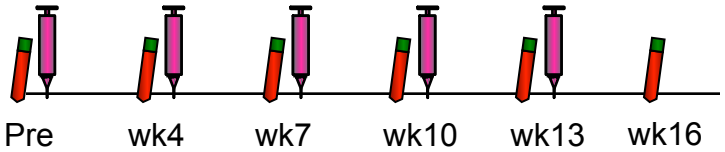
Cohort 2
13 patients

OLP4 with **Montanide**™ - s.c. (total 1ml)



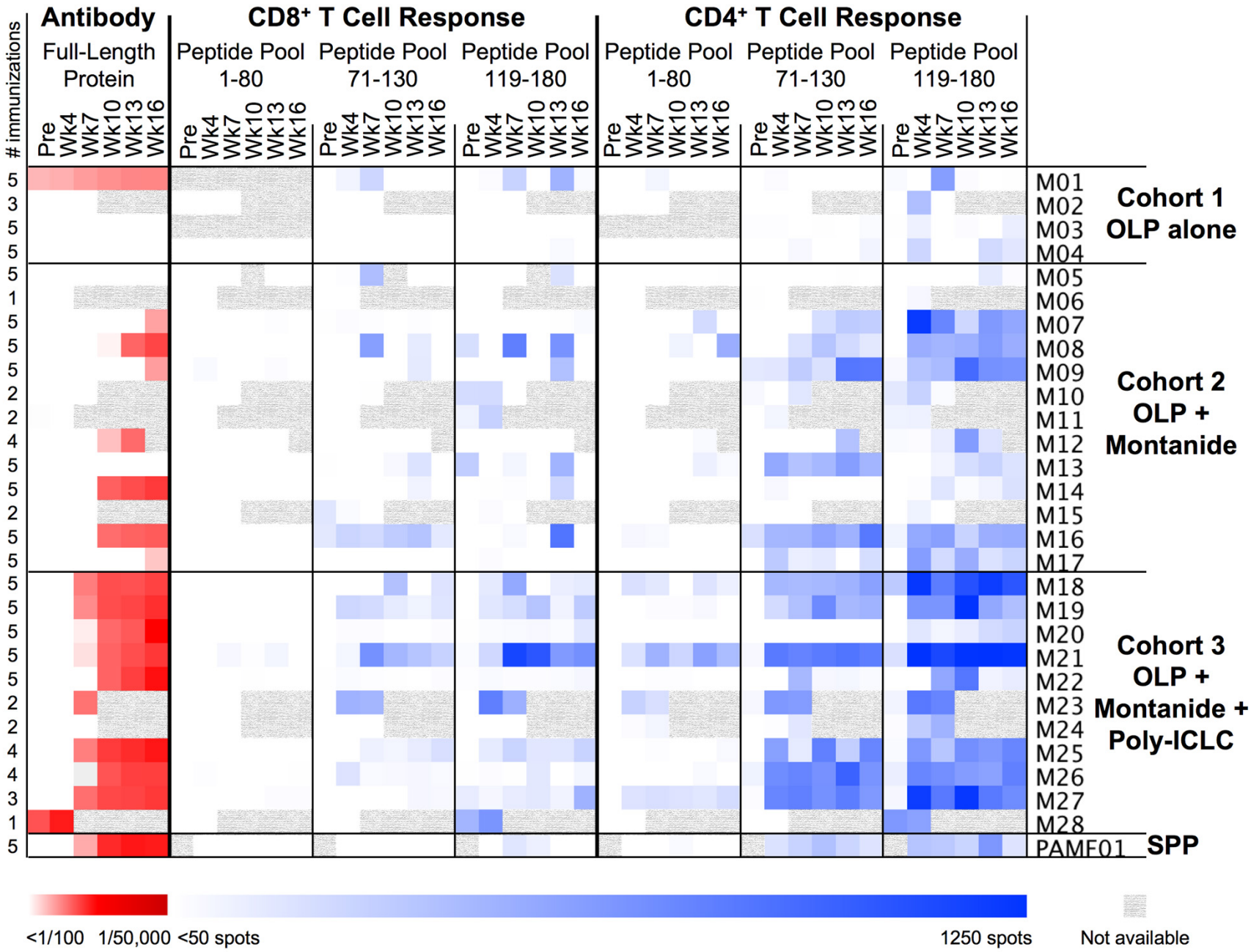
Cohort 3
11 patients
(+1 SPP)

OLP4 with **Montanide**™ and **Poly-ICLC** (Hiltonol™) - s.c. (total 2ml)



Blood collection
(Plasma and PBMC)

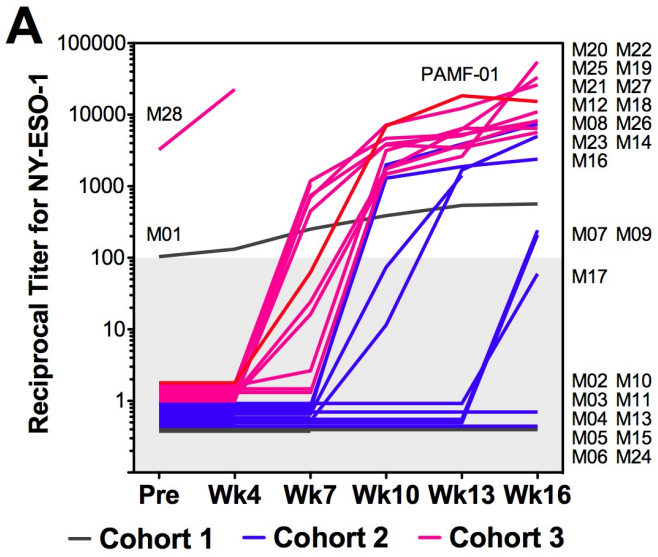
Summary of immune responses in OLP vaccination (Clin Cancer Res. 2012;18:6497-508)

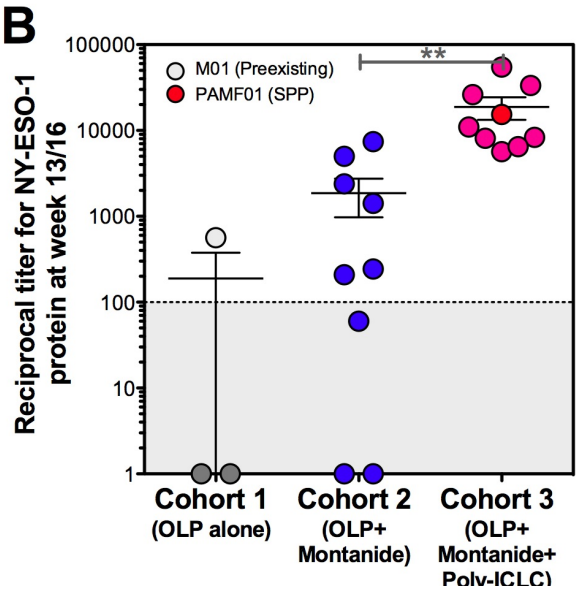


Antibody and CD4 T cell responses to NY-ESO-1 Overlapping Long Peptides vaccination

Ab responses
ELISA

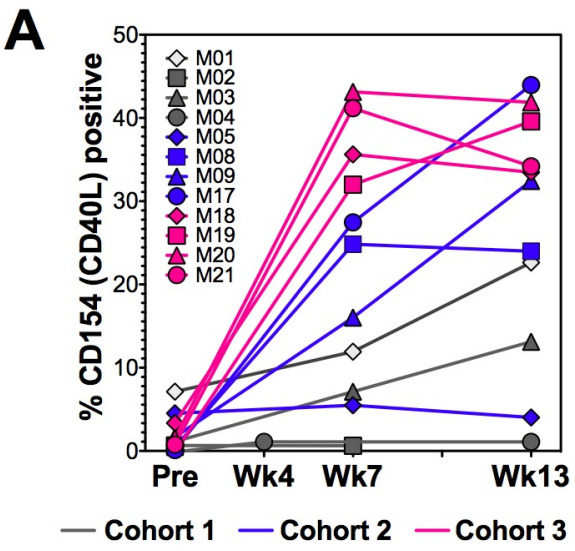
Titers extrapolated based on serial dilutions of plasma

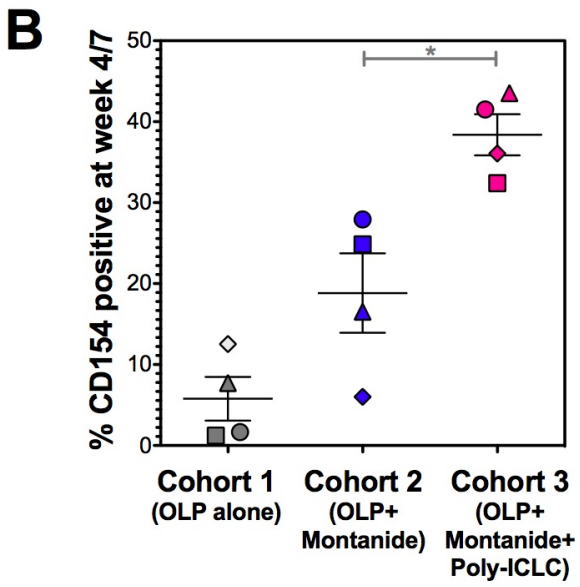




CD4 T cell responses
CD154 (CD40L)-based upregulation assay by flow cytometry

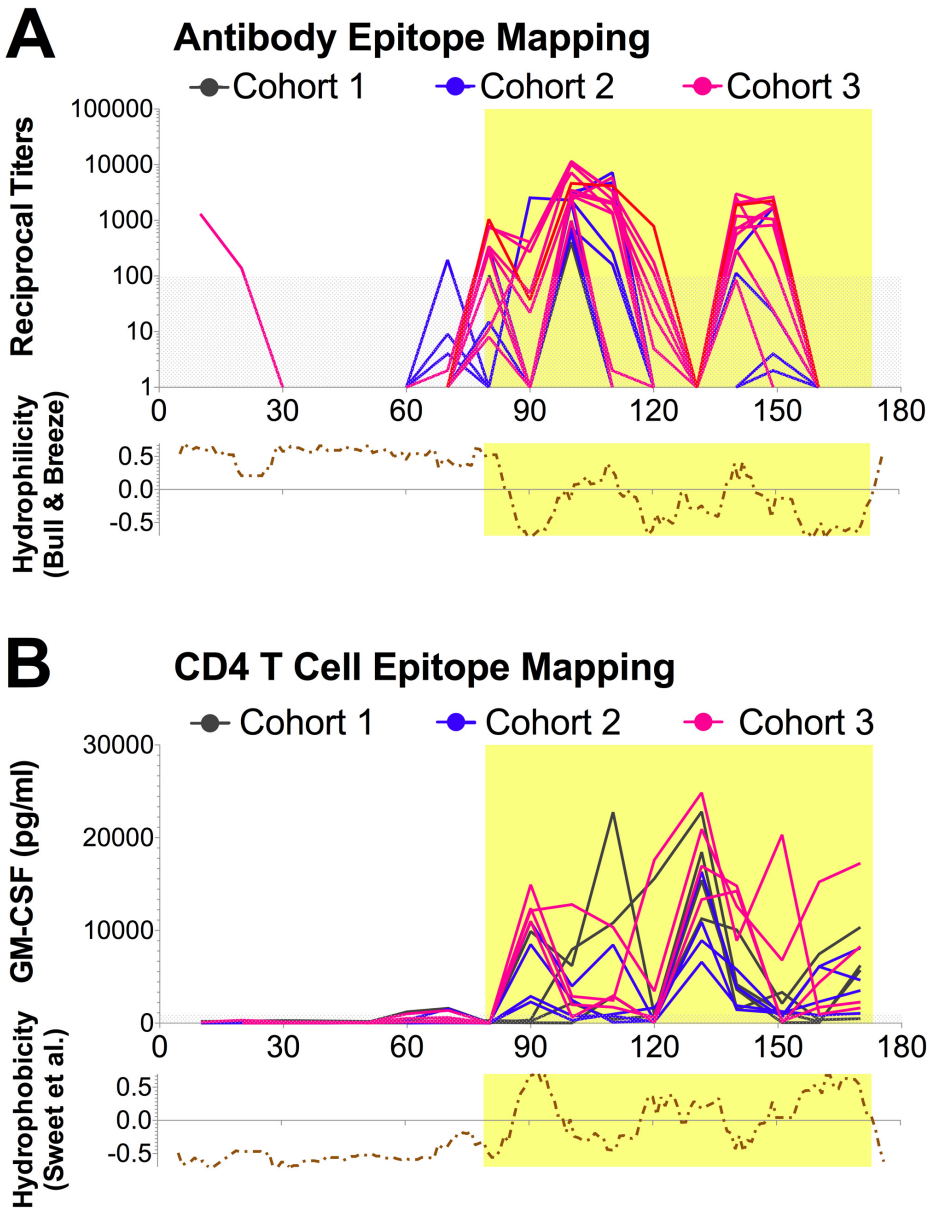
Detection of NY-ESO-1–specific CD4⁺ T cells in patients vaccinated with NY-ESO-1 overlapping peptides by CD40L expression 20 days after in vitro sensitization





Sabbatini, Tsuji et al. Clin Cancer Res. 2012;18:6497-508

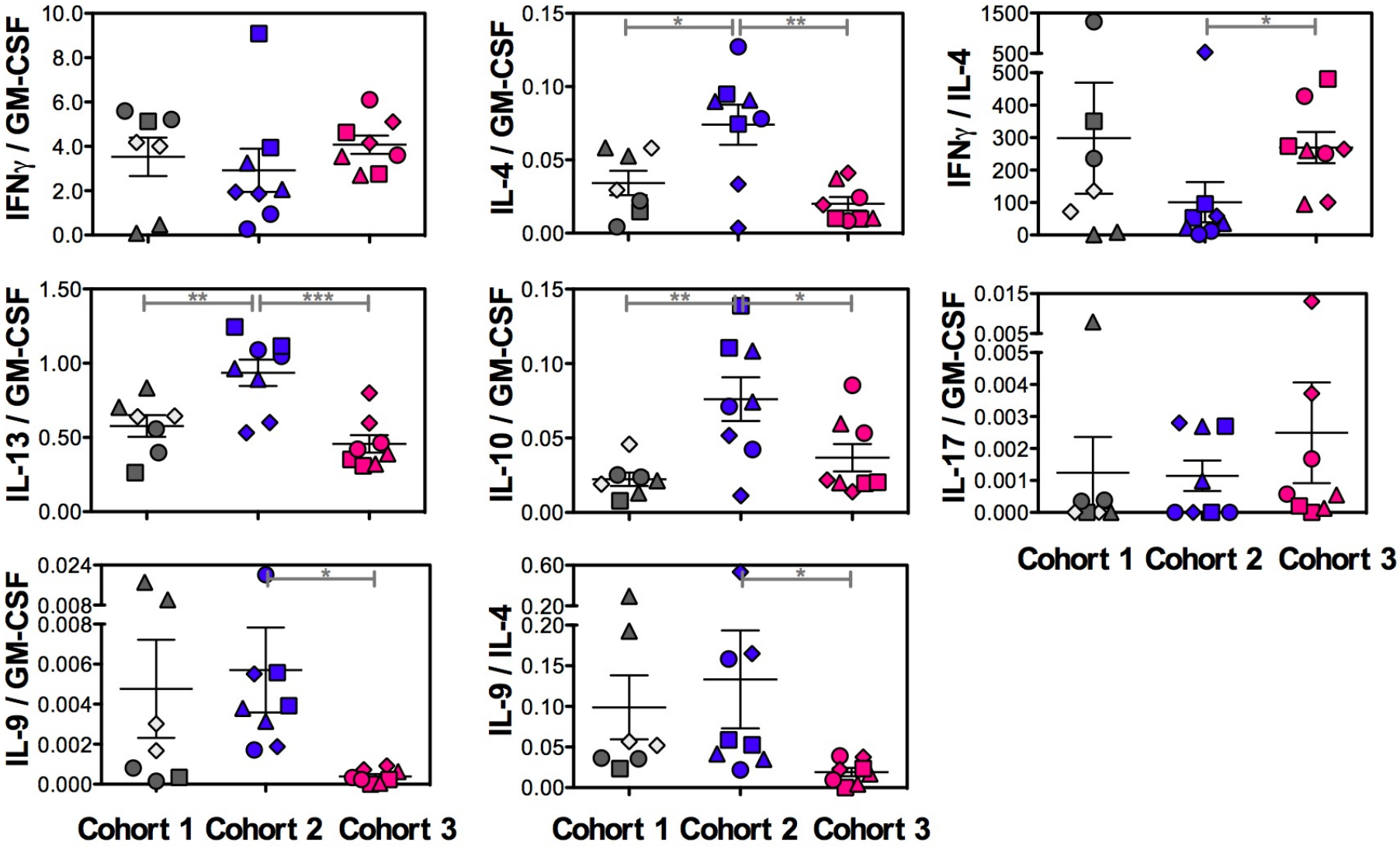
Mapping of epitopes recognized by antibody and CD4+ T cells after vaccination with OLP



Sabbatini, Tsuji et al. Clin Cancer Res. 2012;18:6497-508

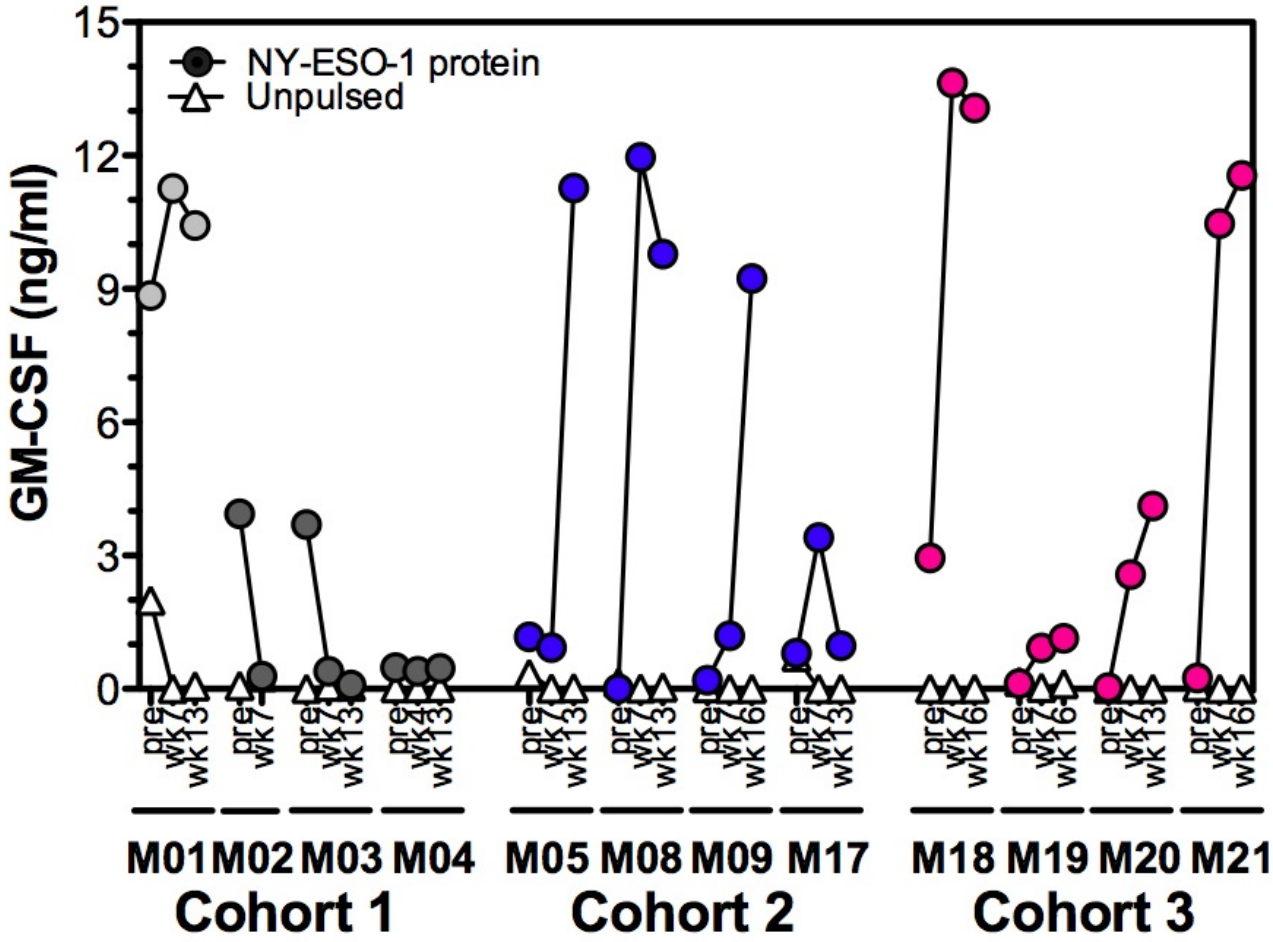
Tsuji et al. Cancer Immunology Research, 2013;1:340-350

Change of Th1/Th2 balance of NY-ESO-1-specific CD4+ T cells by vaccination with OLP with or without montanide and/or poly IC at week 13/16



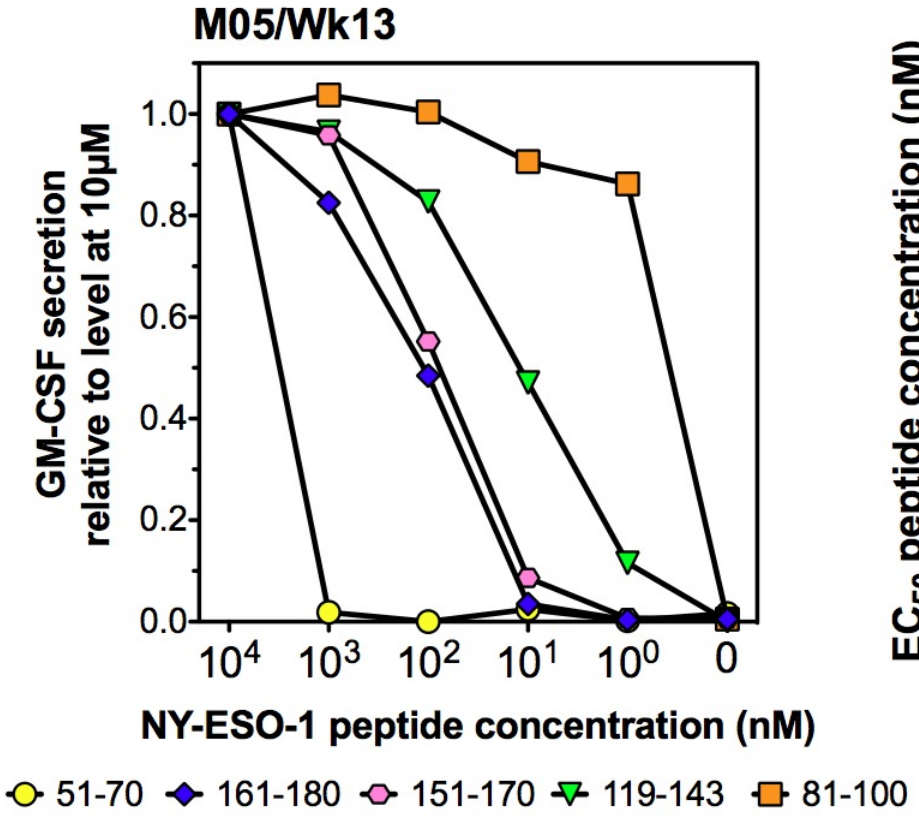
Tsuji et al. Cancer Immunology Research, 2013;1:340-350

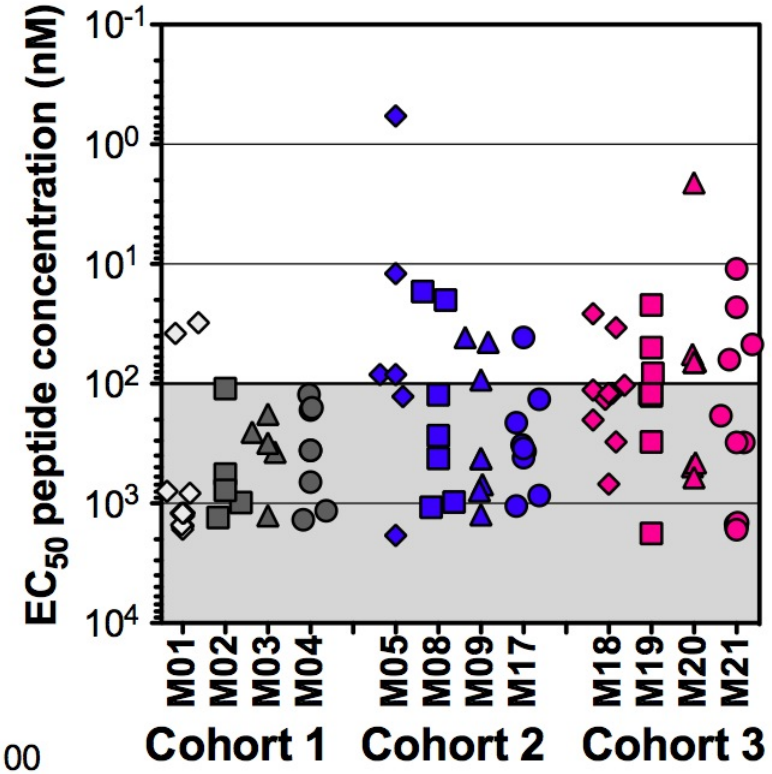
Recognition of naturally-processed NY-ESO-1 protein by CD4+ T cell lines from samples before and after vaccination with OLP with or without montanide and/or poly IC



Tsuji et al. Cancer Immunology Research, 2013;1:340-350

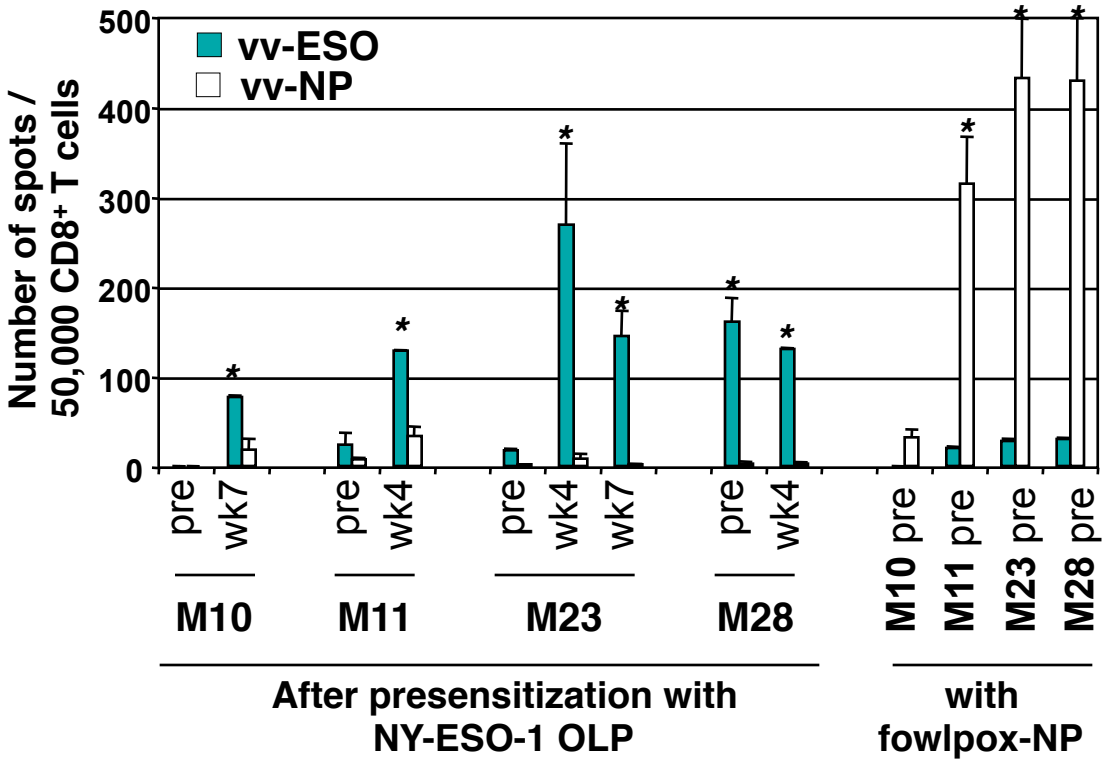
Analyzing the avidity of CD4+ T cell lines for the recognition of individual peptides

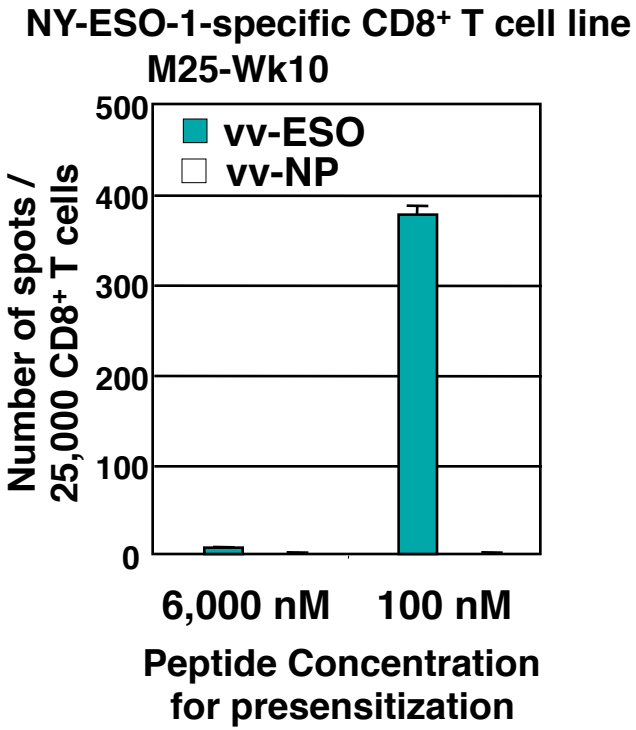




Tsuji et al. Cancer Immunology Research, 2013;1:340-350

Analyzing the quality of CD8+ T cell lines for the recognition of naturally processed NY-ESO-1

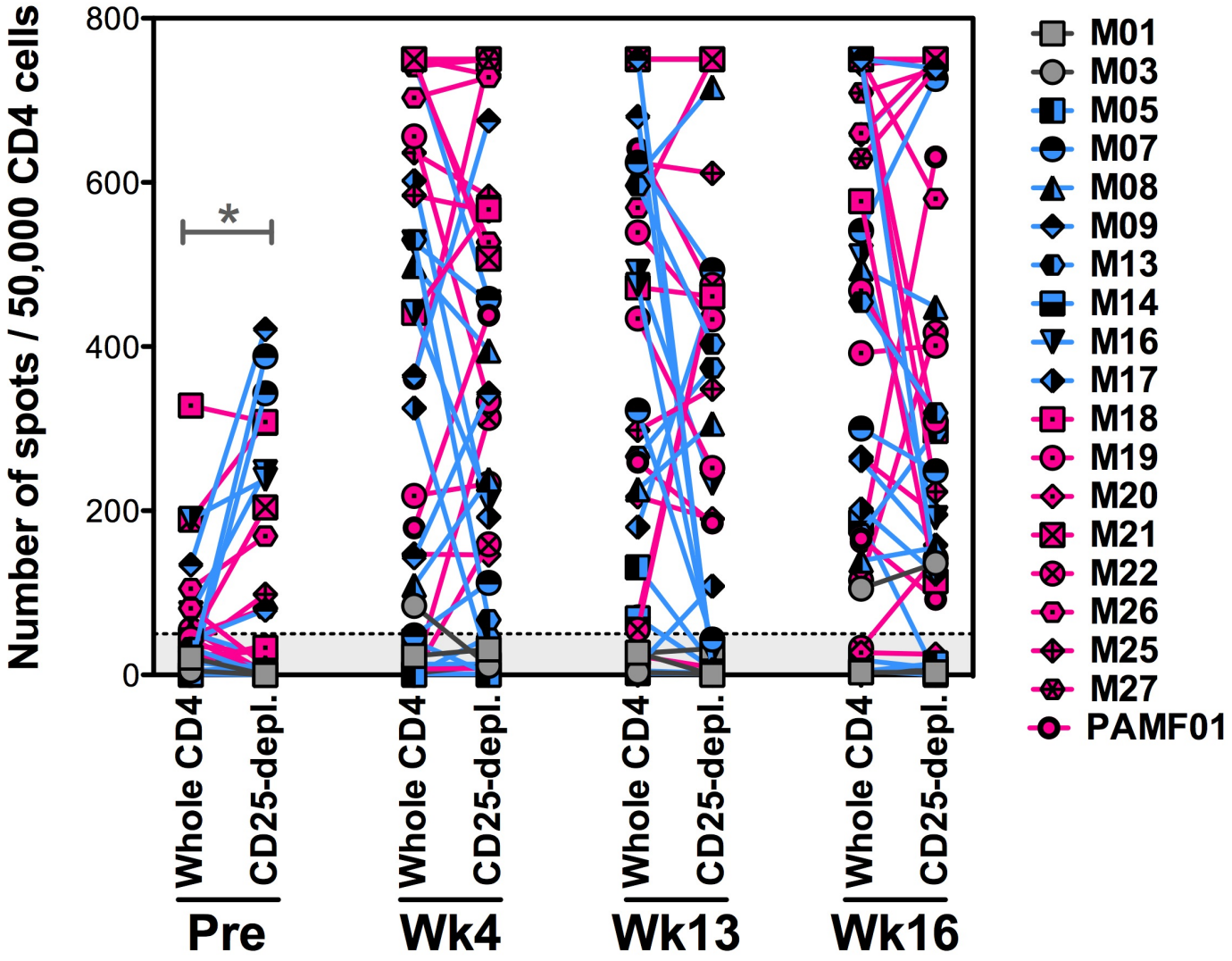




Sabbatini, Tsuji et al. Clin Cancer Res. 2012;18:6497-508

vv: recombinant vaccinia virus

Measuring Tregs:
Effect of depleting $CD4^+CD25^+$ T cells from $CD4^+$ T responses against NY-ESO-1

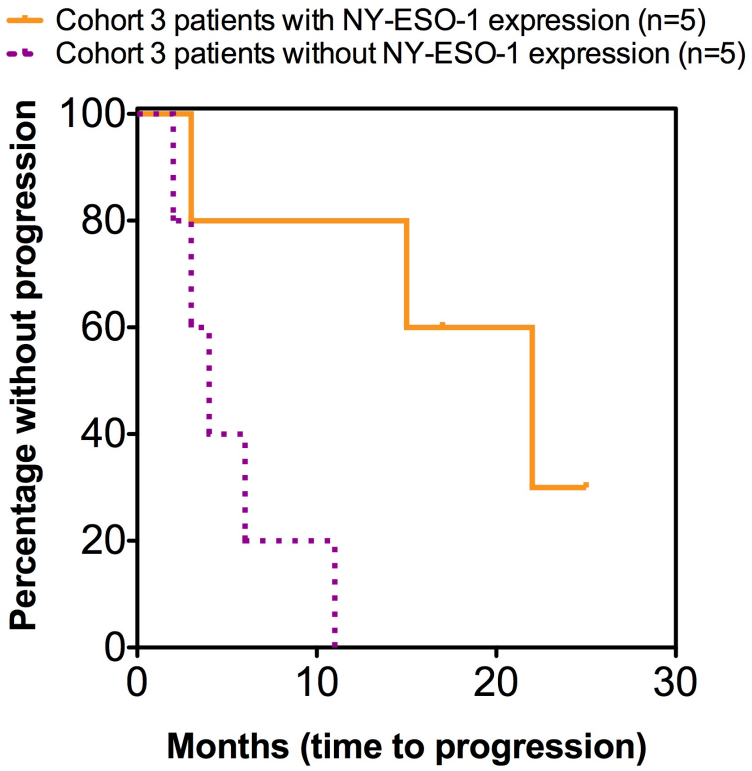


Sabbatini, Tsuji et al. Clin Cancer Res. 2012;18:6497-508

Comparative summary of cohorts from NY-ESO-1 overlapping peptide vaccine

Cohort	Ab	CD8	CD4	Integrated Ab, CD4 and CD8 responses
1: OLP alone	1/4	1/4	4/4	1/4
2: OLP+Montanide	6/13	9/13	12/13	4/13
3: OLP+Montanide+Poly-ICLC	10/11	10/11	11/11	10/11

Delayed time-to-progression in Cohort 3 patients with NY-ESO-1 tumor expression



Sabbatini, Tsuji et al. Clin Cancer Res. 2012;18:6497-508

Lessons learned and take home message – Key points and impact on field

Large array of methodologies available to study immune cells at the single cell level or in a comprehensive systemic manner

Immune monitoring of T and B cells can guide and inform future immunotherapy designs

Importance of defining parameters for optimal understanding of immunotherapy: *In situ* vs. periphery, *ex vivo* vs. *in vitro* sensitization, quality of responses

Limitations: Despite new tools such as HLA class II tetramer, challenging to study suppressive mechanisms in the antigen-specific setting

With more clinical benefit achieved by immunotherapy, expectation that immunological correlates will become important for prediction

Future directions

Microbiome

Single-cell genomics

Integration with systems biology and bioinformatics

Plasticity, ontogeny of immune cells – Variability over time

In situ specificity (tetramers for IHC, microdissection and functional analyses)

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