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

<table>
<thead>
<tr>
<th>Qualitative aspects measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity – Example: Distinguish his-tag specific responses from antigen-specific responses following protein vaccine</td>
</tr>
<tr>
<td>Avidity or titer (serial dilution of target antigen or epitope amount required for minimal reactivity) – Tumor recognition</td>
</tr>
<tr>
<td>Polyfunctionality (ability to produce multiple cytokines, various effector functions)</td>
</tr>
<tr>
<td>Polyclonality (epitope mapping within an antigen)</td>
</tr>
<tr>
<td>Surface markers related to function (memory, naïve, effector, central, periphery, tissue homing, activation [ICOS, 4-1BB, OX40], suppression [CTLA-4, PD1])</td>
</tr>
<tr>
<td>Immunohistochemistry and imaging of T and B cells (Immunoscore)</td>
</tr>
</tbody>
</table>
Phenotypic vs. functional analyses of T cells

Surface markers may inform on the type of cells but ultimate functional tests may be required:
- Cytokine secretion
- Cytotoxicity
- Upregulation of surface markers (e.g., 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

**Selection with magnetic beads** coated with anti-CD8 or anti-CD4

Gnjatic et al.

**Tetramers in PBMC**

**PMBC ex vivo**

<table>
<thead>
<tr>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A2 / NY-ESO-1 p157-165 Tetramer</td>
</tr>
</tbody>
</table>

**After IVS**

<table>
<thead>
<tr>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR RECOGNITION</td>
</tr>
</tbody>
</table>

**ELISPOT**

**Sensitization**

None (ex vivo)

- ESO-1 157-165
- Matrix 58-66

**Targets**

**IFNγ RELEASE**

**Tetramers in Tumors**

**TILs ex vivo**

<table>
<thead>
<tr>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-Cw3 / NY-ESO-1 p92-100 Tetramer</td>
</tr>
</tbody>
</table>

**Ascites ex vivo**

<table>
<thead>
<tr>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCR RECOGNITION</td>
</tr>
</tbody>
</table>

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

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

**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

<table>
<thead>
<tr>
<th><strong>Where to monitor? Periphery or tissue?</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In situ monitoring</strong></td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
</tr>
<tr>
<td>• Most relevant: at tumor site</td>
</tr>
<tr>
<td>• Phenotype of antigen-specific cells unmodified by cell culture</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
</tr>
<tr>
<td>• Generally not accessible</td>
</tr>
<tr>
<td>• Not enough cells to test</td>
</tr>
<tr>
<td>• Quality of tissue</td>
</tr>
<tr>
<td>• Heterogeneity and sampling bias</td>
</tr>
<tr>
<td><strong>Peripheral blood</strong></td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
</tr>
<tr>
<td>• Systemic</td>
</tr>
<tr>
<td>• Easy access</td>
</tr>
<tr>
<td>• Sufficient amounts</td>
</tr>
<tr>
<td>• No biopsy needed</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
</tr>
<tr>
<td>• May not reflect tumor environment</td>
</tr>
<tr>
<td>• Potentially rarer precursors</td>
</tr>
<tr>
<td>• Representative or not?</td>
</tr>
</tbody>
</table>
Intraepithelial CD8+ TILs and a high CD8+/Treg ratio are associated with favorable prognosis in ovarian cancer
Immunoscore: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome


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


<table>
<thead>
<tr>
<th>Product</th>
<th>Cancer</th>
<th>Phase</th>
<th>Evaluation results</th>
<th>Positive Correlation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provenge*</td>
<td>Prostate cancer</td>
<td>P I/II</td>
<td>TTP correlated with development of an immune response to prostatic acid phosphatase (PAP) and with the dose of dendritic cells received.</td>
<td>Y</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P III</td>
<td>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 &lt; 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.</td>
<td>Y</td>
<td>10</td>
</tr>
<tr>
<td>Canvaxin*</td>
<td>Melanoma (Stage IV)</td>
<td>P II</td>
<td>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 &lt; 0.001)</td>
<td>Y</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Melanoma (Stage II)</td>
<td>P II</td>
<td>Anti-TA90 IgM levels ≥ 1:800 were significantly correlated with improved 5-y DFS and improved 5-y OS.</td>
<td>Y</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Melanoma (Stage IIIa and IV)</td>
<td>After P II</td>
<td>Survival correlated significantly with delayed cutaneous hypersensitivity (p = 0.0066) and antibody response (p = 0.0117).</td>
<td>Y</td>
<td>31</td>
</tr>
<tr>
<td>Specifid™</td>
<td>Non-Hodgkin's lymphoma</td>
<td>P II (after rituximab)</td>
<td>There was no correlation observed between the development of anti-Igd immune response and the achievement of an objective response or duration of EFS.</td>
<td>N</td>
<td>33</td>
</tr>
<tr>
<td>BEC2</td>
<td>Small cell lung cancer</td>
<td>P III</td>
<td>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).</td>
<td>Y</td>
<td>21</td>
</tr>
</tbody>
</table>
Association with survival in DC + autologous lysate vaccine in glioblastoma patients (GBM)

B

All post-vaccine

$P = 0.041$

C

Recurrent post-vaccine

$P = 0.067$

D

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)

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

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


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

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

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)

Seromics: Methodology for antibody profiling with protein microarrays

Array featuring multiple proteins

Incubate with patient serum (1:500)

Reveal antigen-specific serum antibodies with labeled anti-human IgG

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.

Blood collection (Plasma and PBMC)

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)
## Summary of immune responses in OLP vaccination

*(Clin Cancer Res. 2012;18:6497-508)*

### Table: Summary of immune responses

<table>
<thead>
<tr>
<th>Antibody</th>
<th><strong>CD8⁺ T Cell Response</strong></th>
<th><strong>CD4⁺ T Cell Response</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Full-Length Protein</td>
<td>Peptide Pool 1-80</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
<td>WK4</td>
</tr>
<tr>
<td># immunizations</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>M01</td>
<td>M02</td>
</tr>
<tr>
<td>Cohort 1</td>
<td>OLP alone</td>
<td></td>
</tr>
<tr>
<td>Cohort 2</td>
<td>OLP + Montanide</td>
<td></td>
</tr>
<tr>
<td>Cohort 3</td>
<td>OLP + Montanide + Poly-ICLC</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th><strong>CD8⁺ T Cell Response</strong></th>
<th><strong>CD4⁺ T Cell Response</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide Pool 1-80</td>
<td>Peptide Pool 71-130</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
<td>WK4</td>
</tr>
<tr>
<td></td>
<td>1250 spots</td>
<td>Not available</td>
</tr>
</tbody>
</table>

### Notes:
- <1/100: < 1/100 spots
- 1/50,000: < 50 spots
- Not available
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

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

**A** Antibody Epitope Mapping

- Cohort 1
- Cohort 2
- Cohort 3

**B** CD4 T Cell Epitope Mapping

- Cohort 1
- Cohort 2
- Cohort 3

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

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

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

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

After presensitization with NY-ESO-1 OLP:
- M10
- M11
- M23
- M28

With fowlpox-NP:
- M10
- M11
- M23
- M28

NY-ESO-1-specific CD8⁺ T cell line M25-Wk10
- vv-ESO
- vv-NP

Peptide Concentration for presensitization:
- 6,000 nM
- 100 nM


vv: recombinant vaccinia virus
Measuring Tregs: Effect of depleting CD4+CD25+ T cells from CD4+ T responses against NY-ESO-1

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

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Ab</th>
<th>CD8</th>
<th>CD4</th>
<th>Integrated Ab, CD4 and CD8 responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: OLP alone</td>
<td>1/4</td>
<td>1/4</td>
<td>4/4</td>
<td>1/4</td>
</tr>
</tbody>
</table>

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

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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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