The workshop objective

The objective was to consider state-of-the-art approaches to the identification of biomarkers and surrogate markers of tumor burden with the emphasis on assays in the blood, lymph nodes and within the tumor itself.
Surrogate end-points

Definition: end-points other than overall survival used to make conclusions or predictions about cancer progression/regression or responses to therapy

Disease related:
- Histologic markers: dysplasia, hyperplasia, CIS, tumor stages
- Serum markers: CEA, PSA, CA125, etc
- RR, TTP

Mechanistic (biomarkers):
- Immunologic
- Genetic
- Proteomics-based
- Molecular
- Functional
Areas of consideration

- Genomic analysis of cancer *
- RT-PCR for molecular markers of cancer
- Serum/plasma and tumor proteomics *
- Immune polymorphisms *
- High content screening by flow and imaging cytometry
- Immunohistochemistry and tissue microarrays
- Assessment of immune infiltrates and tumor necrosis
Genomics and proteomics in cancer

- Emphasis on high throughput screening/profiling followed by identification

- Recommendations re sample acquisition and banking:
  serial samples in order to get a dynamic view prospective collections linked to clinical trials
  standardized DNA, RNA amplification specimen processing/storage under GLP
  serum or plasma for proteomics??
RT-PCR for detection of circulating tumor cells (CTC)

- Objective is to get “molecular footprint” of cancer in blood, LN, BM
- Need to have a marker gene for each tumor type
- Need RT-PCR for sensitivity (1-10 CTC/10^6 lymphocytes)
- Sample processing (whole blood vs. PBMC)
- Immunomagnetic bead enrichment in epithelial cells
- Emphasis on CTC validation vs. disease stages, recurrence, prognosis and survival to confirm clinical usefulness
High-content screening by flow or imaging cytometry to follow changes in immune cells

- Intimate and unique relationship of cancer and the host immune system
- How does tumor affect phenotype/ functions of immune cells?
- If tumor induces detectable alterations in phenotype/functions of immune cells, could we use these as biomarkers or surrogate endpoints?
Defects in APM

Immature phenotype

Cytokine imbalance

Signaling defects

Death-inducing signals

Inhibitory factors

Activating signals

ROS

Inhibitory cytokines

PGE₂

Defects in APM

Immature phenotype

Cytokine imbalance

Signaling defects

Growth-promoting signals

Death-inducing signals

Inhibitory factors
What to measure, how and where?

- Tumor site vs. blood vs. LN
- Selection of the immune cell type which is altered in marker expression, signaling, migration, cytokine production, etc in a tumor-bearing host
- Choice of methods (screening vs. confirmatory) that are robust but simple to use in correlative studies to determine clinical usefulness of the selected cancer biomarker
Frequencies of CD8+tetramer+ T cells in PBMC and TIL of patients with head and neck cancer

gated on CD3 and CD8

**Patient # 1:**
Tetramer frequency

- **p53 tetramer**$_{149-159}$
- **p53 tetramer**$_{264-272}$

![Graphs showing PBMC and TIL tetramer frequencies for Patient # 1](image-url)
Fas-L expression on the tumor and TIL apoptosis

- Fas-L expression was seen on all tumors
  - high 17/28
  - Low 11/28

- High expression of Fas-L was associated with
  - Apoptosis in TIL
  - Reduced $\zeta$ expression in TIL

*Reichert et al, Clin. Cancer Res. 8: 3137, 2002*
Apoptotic CD8+ T cells in the nest of lymphocytes at the tumor site

Red = alive CD8+ T cells
Blue = dying CD8+ T cells
Circulating CD3+Fas+Annexin+ cells in patients with HNC and controls

- Patients: n=37

- Controls: n=37

p < .0001
Isolation and characteristics of biologically-active MV in the sera of patients with HNC

MV from patients’ sera contain FasL (42 kDa) and HLA class I molecules and induce Jurkat cell apoptosis.
Association of MV containing high, low or no FasL with disease in 27 SCCHN patients

<table>
<thead>
<tr>
<th>MV/FasL vs T stage</th>
<th>MV/FasL vs N stage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High FasL</td>
<td>N0 4</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>N1 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N3 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Low FasL</td>
<td>8</td>
<td>13</td>
</tr>
</tbody>
</table>

- MV/FasL vs T stage: T1 4, T2 0, T3 0, T4 10
- MV/FasL vs N stage: N0 4, N1 3, N2 6, N3 1

p = 0.0094

Sera of patients with stage IV disease and + nodes contain MV with the high level of FasL

p < 0.12
Annexin V binding to circulating CD8+ T lymphocytes

NC : normal controls
AD : patients with active disease
NED : patients with no evidence of disease

P<0.0001
Clinical significance of CD8+ T cell apoptosis in patients with cancer?

- Discriminates patients with cancer from healthy controls
- Higher in patients with AD vs. those with NED, but not a significant discriminator
- Together with signaling defects (ζ chain) and CD95 expression on T cells, apoptosis correlated with the nodal involvement
- Potentially, could evolve into a marker of tumor aggressiveness or predictor of survival
Characteristics that are often altered in circulating T cells

- T-cell absolute numbers
- T-cell subset changes (naïve, memory)
- Expansion of Tregs (CD4+CD25^{high})
- Decreased ζ chain expression
- Increased apoptosis (CD95+, Annexin V+)
- Cytokine profiles
- Memory T-cell functions
- Tumor-specific T-cell responses
## Absolute # vs. % (means +/- SD)

<table>
<thead>
<tr>
<th>Absolute #</th>
<th>CD3+</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 148</td>
<td>Patients</td>
<td>1081 +/- 601</td>
<td>670 +/- 412</td>
</tr>
<tr>
<td>N = 58</td>
<td>Normal Controls</td>
<td>1512 +/- 494</td>
<td>1005 +/- 360</td>
</tr>
<tr>
<td>p value</td>
<td>&lt; .0001</td>
<td>&lt; .0001</td>
<td>.0012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percentage</th>
<th>CD3+</th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 148</td>
<td>Patients</td>
<td>71 +/- 11</td>
<td>44 +/- 11</td>
</tr>
<tr>
<td>N = 58</td>
<td>Normal Controls</td>
<td>70 +/- 9</td>
<td>47 +/- 9</td>
</tr>
<tr>
<td>p value</td>
<td>.6374</td>
<td>.1141</td>
<td>.0917</td>
</tr>
</tbody>
</table>
NED patients studied < 2 and > 2 years after surgery

CD4/CD8 = 1.78
Decreased expression of $\zeta$ in Annexin+CD3+T cells in the peripheral circulation of patients with melanoma

The % of cells positive for $\zeta$ vs. MFI for $\zeta$ in CD3+ T lymphocytes of the patients and normal controls
Characterization of Activation/Differentiation Status of Tetramer Stained Cells (MART 1)

Gated on tetramer MART 1\(^+\) CD8\(^+\) cells

Healthy Donor

Melanoma Patient

CD45RA

CCR7

EM

TD

Healthy Donor

Melanoma Patient
Expansion of CD8^+GP100_{209-217}^+ T cells and change of differentiation status in this subset seen in one melanoma patient treated with multi-epitope vaccine.
Multi-color flow cytometry for phosphorylated STAT1 levels in activated immune cells

- Sensitive, quantitative, fast, uses few cells; measures early events

1. Surface staining with anti-CD3 Ab
2. Cell permeabilization
3. Intracytoplasmic staining with Ab to phosphorylated STAT1
Embarassing wealth of riches

- Emphasis on Ag-specific responses and multicolor high content screening

**T-cell**

- Cytokine expression by RT-PCR
- Secreted cytokines in fluid phase (CBA/Luminex)
- Cell mediated lysis by flow cytometry; CTL function, CD107
- T-cell activation and proliferation (CFSE) by flow cytometry
- Intracellular cytokines by flow cytometry (ICS)
- Multimer binding by flow cytometry
- Secreted cytokines by capture ELISA
- Cytokine secreting single cells by ELISPOT

**Bulk assays**

**Single-cell assays**
Immunohistochemistry/Tissue microarrays

Immune infiltrates into tumor

- IHC/TMA useful clinically in estimating prognosis or responses to therapy
- In research, IHC/TMA is considered crucial for the identification and mapping of new biomarkers
- Tissue quality and epitope preservation
- Prospective collection in clinical trials
- Advancements strategies: multicolor labeling, confocal imaging, morphometry
- Need for standardization
- Data mining
A retrospective study of tumor biopsies

- 132 primary OSCC (Follow up > 5 Years)
- Immunohistochemistry for detection of DC and the ζ chain
  - Antibodies to S100, p55-protein, CD3 and CD247
  - Morphometrical analysis (cell number/HPF)
- Parameters evaluated:
  - Tumor size, TNM staging categories, grading, survival, recurrence
- Statistical analysis:
  - Proportional hazards regression
  - Multivariate survival analysis
  - Kaplan-Meier survival estimation
TIL in patients with HNC had variably but significantly decreased expression of TCR-associated ζ chain.
DC Counts in tissue

- S100
- p55

Bar chart showing:
- 0-10/HPF
- 11-20/HPF
- >20/HPF
Multivariate Analysis
(Kaplan-Meier, S100 und zeta)

Reichert et al, Cancer 91: 2136-2147, 2001

Overall survival by S100 and Zeta

<table>
<thead>
<tr>
<th>S100</th>
<th>Zeta</th>
<th>#alive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 or 1</td>
<td>3/22</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2/5</td>
</tr>
<tr>
<td>2 or 3</td>
<td>0 or 1</td>
<td>9/20</td>
</tr>
<tr>
<td>2 or 3</td>
<td>2</td>
<td>56/85</td>
</tr>
</tbody>
</table>

Reichert et al, Cancer 91: 2136-2147, 2001
Multivariate Analysis
(Kaplan-Meier, S100 and TU-Stage)

[from: Reichert et al., Cancer 2001;91:2136-47]
Cytokine balance in disease

- Therapeutic goal: shift the balance

**TH1-dominant diseases**
- Autoimmunity, GVHD

**TH2-dominant diseases**
- Allergy, HIV, Cancer

- TH1
- TH2

- IL-2
- IFN-γ

- IL-4
- IL-5
- IL-10
Multi-Analyte Soluble Bead Array Technology

Capture bead

Analyte

Microsphere color identifies analyte

Analyte: body fluid supernatant
Volume: 50 μL
How The Bio-Plex protein array system works

- Up to 100 microspheres are in a bead set. Each is color-coded and conjugated with a MAb specific for a unique protein analyte.

- A flow-based instrument with 2 lasers and associated optics measures biochemical reactions that occur on the surface of the colored microspheres.

- A high-speed digital signal processor efficiently manages the fluorescent output.
Cytokines Chemokines & More

- **Human**
  - TNF-α, IFN-γ, TGF-β1, IL-1Ra, IL-2 sR
  - IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-13, IL-15, IL-18
  - G-CSF, M-CSF, GM-CSF, EGF, FGF-7, SCF, MIG, VEGF, HGF
  - FLT-3, MCP-1, MIP-1α, MIP-1β, Rantes, IP-10, LIF

### Inflammation
- G-CSF
- IL-6
- IL-8
- TNF-α

### Th-1/Th-2
- IFN-γ
- IL-4
- IL-5
- TNF-α
- IL-2
- IL-7
- IL-12
- GM-CSF
- IL-13
- IL-18

### Autoimmune
- IL-1b
- IL-6
- TNF-α
- IL-12

### Hematopoiesis
- G-CSF
- IFN-γ
- IL-1β
- IL-6
- GM-CSF
Conclusions: high throughput assay platforms are here!

- **Technology is rapidly evolving**: 17-color flow, cytometric bead arrays, confocal immuno-microscopy, microfluidics, immunoassay-based microarrays, immuno-PCR. All aimed at a high throughput, small sample volumes, rapid detection.

- **Profiling**: changes in several biomarkers.

- **Potential future benefits**: identification of individual markers or profiles of immunologic markers which will serve as surrogate endpoints useful in predicting survival, clinical responses to therapy or in immunodiagnosis (e.g., screening general populations).

- **Biomarker validation**: many promising biomarkers but few formally validated; we need more cost-effective validation, based on solid mechanistic insights and clinical correlative studies.
Advantages of a central laboratory operated as a GLP facility

- QA and QC in place assuring quality and reliability of monitoring
- State-of-the-art technologies
- Assay development, standardization and validation
- Decreased cost of immune monitoring which is essential for biotherapy protocols
- Result interpretation in conjunction with statisticians aware of immune-based analyses
- Banking of samples which are accompanied by clinical outcome data for future research
Acknowledgements

- Immunologic Monitoring and Cellular Products Laboratory (IMCPL)
- Many postdoctoral fellows
- My clinical colleagues:
  Jonas T. Johnson, MD
  Robert L. Ferris, MD, PhD
  John M. Kirkwood, MD
  Michael T. Lotze, MD
## Development timelines and cost

### Table 1: Typical vaccine development timeframes and costs

<table>
<thead>
<tr>
<th>Phase</th>
<th>Years to Market</th>
<th>Probability of reaching market (%)</th>
<th>Cost at Stage ($m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research (Preclinical)</td>
<td>11</td>
<td>10</td>
<td>400</td>
</tr>
<tr>
<td>Development</td>
<td>8</td>
<td>20</td>
<td>350</td>
</tr>
<tr>
<td>Phase I</td>
<td>6</td>
<td>20-30</td>
<td>280</td>
</tr>
<tr>
<td>Phase II</td>
<td>5</td>
<td>30-50</td>
<td>200</td>
</tr>
<tr>
<td>Phase III</td>
<td>3</td>
<td>50-90</td>
<td>10</td>
</tr>
<tr>
<td>BLA Filed</td>
<td>1</td>
<td>90-95</td>
<td>5</td>
</tr>
<tr>
<td>Approval</td>
<td>0</td>
<td>99</td>
<td>0</td>
</tr>
</tbody>
</table>

Source: Jarvis (2002)
Rational use of surrogate endpoints

- Mechanistic surrogate endpoint
- Disease-related surrogate endpoint
- Time-to-progression surrogate endpoint
- Overall survival endpoint