Strategies for Assessment of Immunotoxicology in Preclinical Drug Development

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SNBL is committed to freeing patients from suffering by supporting drug development and improving medical technology.
Preclinical Immunotoxicology

The study of evaluating adverse effects of the immune system following exposure to various agents (antibody conjugates, recombinant proteins, oligos and chemical therapeutics).
Immunotoxicology Testing Strategy

Employ assays to assess endpoints outlined in the ICH S8 Guidance

“Toxicity to the immune system encompasses a variety of adverse effects. These include suppression or enhancement of the immune response...”

1. Hematological changes such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/lymphocytosis.

2. Alterations in immune system organ weights and/or histology (e.g. changes in thymus, spleen, lymph nodes, and/or bone marrow.

3. Changes in serum globulins that occur without a plausible explanation, such as effects on the liver or kidney, can be an indication that there are changes in serum immunoglobulins.

4. Increased incidence of infections.

5. Increased occurrence of tumors can be viewed as a sign of immunosuppression in the absence of other plausible causes such as genotoxicity, hormonal effects, or liver enzyme induction.

6. Additional immunotoxicity testing...depending on the nature of immunological changes observed.
   - TDAR, immunophenotyping, NK cell activity etc.
Tools to Assess Immunotoxicology

Methods

Anatomical Pathology
Clinical Pathology
Flow Cytometry
Ligand-Binding Assays

Measured Endpoints

- Total and Differential Leukocyte Counts
- Acute phase protein levels
- Organ Weight & Cellularity
- Histopathology
- Complement, Ig Profiles
- Immunophenotyping
- T-dependent antibody response (TDAR)
- Anti-drug antibody response (ADA)
- Cytokine/chemokine profiles
- Complement activation
Flow Cytometry

Immunophenotyping of leukocyte lineages and subsets, functional profiling and cell enumeration.

- **Cell Lineage and Phenotyping**
  - T cell subsets, thymocytes, B cells, plasma cells, NK cells, monocytes, dendritic cells, granulocytes, stem cells, platelets
  - NK cell Degranulation
  - Activation Marker

- **Function**
  - Intracellular cytokine stains
  - Cytokine bead arrays
  - NK cell killing assay (non-radioactive)
  - Proliferation

- **Tissues**
  - Blood, spleen, lymph nodes, thymus, bone marrow
Flow Cytometry Technology

Input:
Fluorescently labeled cells

Output:
1) Light scatter
2) Fluorescence

Flow Cytometer

- 1) Light scatter
- 2) Fluorescence

Granulocytes
Monocytes
Lymphocytes
Common Flow Cytometry Panels for Immunophenotyping

<table>
<thead>
<tr>
<th>T/B/NK cells</th>
<th>T/B/NK cells/ Monocytes</th>
<th>T cell Activation</th>
<th>B cell Activation</th>
<th>T cell Cytokines</th>
<th>Hematopoietic stem cells</th>
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<tbody>
<tr>
<td>CD3</td>
<td>CD3</td>
<td>CD3</td>
<td>CD20</td>
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<td>CD4</td>
<td>CD4</td>
<td>IgD</td>
<td>CD4</td>
<td>CD34</td>
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<td>CD8</td>
<td>CD27</td>
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<td>7AAD</td>
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<td>CD16</td>
<td>CD25</td>
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<tr>
<td>CD20</td>
<td>CD69</td>
<td></td>
<td></td>
<td>IFN-γ</td>
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<td>IL-2</td>
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<td></td>
<td></td>
<td>TNF-α</td>
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**INNATE IMMUNITY (RAPID RESPONSE)**
- Natural Killer Cell
- Dendritic Cell
- Macrophage
- Neutrophil
- Eosinophil

**ADAPTIVE IMMUNITY (SLOW RESPONSE)**
- B Cell
- T Cell
  - γδ T Cell
  - CD4 T Cell
  - CD8 T Cell
- Natural Killer T Cell
- Antibodies
In vitro Assays to Assess Mechanisms of Immunotoxicity

Lymphocyte Functional Profiling: Intracellular cytokine staining

T Cell

Antigen + costimulation → Intracellular cytokine synthesis

Innate Immune Functional Prolifling: NK cell cytotoxicity

NK cell (effector) + Co-culture → Killed cells
Flow Cytometry Challenges in Preclinical Immunotoxicology

- Blood sampling volumes (in vitro analysis)
- Drug Mediated Immune Activation or Depletion?
- Measurement of rare populations (Hematopoietic stem cells)

Solutions

- Activation Marker Panels as an alternative
- Reference ranges
- Use of reagents to exclude debris (nucleic acid dye)
Peripheral Blood Immunophenotyping of Rare Populations – Viability Marker

Nucleic acid dye to exclude debris and quantify the rare population

Gating First on Viable cells

Gating on Rare Population

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Peripheral Blood Immunophenotyping – Reference Ranges

Establish reference ranges to determine normal levels

**Total Lymphocyte Counts by Age**

- **CD3+ (T cells)**
- **CD3-CD16+ (NK cells)**
- **CD3-CD20+ (B cells)**

**Lymphocyte Lineages at PND 360**

- 68%
- 26%
- 6%

*Total lymphocyte counts by age with reference to CD3+ (T cells), CD3-CD16+ (NK cells), and CD3-CD20+ (B cells).*

* *n = 17-74*
Summary of Flow Cytometry in Immunotoxicology

- Measurement of **numbers** of each immune cell subsets after drug administration.
- Measurement of immune cell **activation status** after drug administration.
- **Specific mechanisms** of immunotoxicity
  - Innate immune dysfunction (NK mediated cytotoxicity)
  - Lymphocyte dysfunction (proliferation and cytokine production)
Ligand-Binding Assays for Assessment of Immunotoxicity

Adaptive Immune Response:
- T-dependent Antibody Responses (TDAR)
- Anti-Drug Antibodies (ADA)
- Cytokines/Chemokines

Innate Immune Response:
- Complement Activation
- Cytokines/Chemokines
Enzyme linked Immunosorbent Assay (ELISA)

Detection of antibodies (TDAR, ADA) and biomarkers
T cell-dependent Antibody Response (TDAR) by Monitoring Antibodies to KLH by ELISA

T cell function can be assessed by antigen-specific antibody production and class switching.

King C, Nat. Rev. Immun., 2009
T cell-dependent Antibody Response (TDAR) by Monitoring Antibodies to KLH by ELISA

Detection of anti-keyhole limpet cyanin (KLH) primary and secondary antibody responses

![Graph showing primary and secondary response to KLH](image)

- **Primary Response**
- **Secondary Response**

KLH injection at time 0 and at time 7.

Anti-KLH Ab (µg/mL) vs. Time (days):

- IgM
- IgG

**n = 15**
Monitoring Complement-Mediated Immunotoxicity by ELISA

Innate immune activation and systemic inflammation can be assessed by monitoring complement activation products after drug treatment.

Alternative Pathway Activation: Factor B (Bb) ELISA

Total Complement Activation: C3a and C5a ELISA

JR Dunkelberger et al. Cell Research 2010
Monitoring Complement-Mediated Immunotoxicity by ELISA

Control

Drug Treatment

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Cytokines and Chemokines as Inflammatory Biomarkers

Inflammation can be assessed by monitoring cytokine/chemokine levels before, during, and after drug treatment.

Strategies for Multiplexed Biomarker Analysis - Luminex

Luminex Multiplex Technology

Theoretically up to 100 different analytes per well
Strategies for Multiplexed Biomarker Analysis - Mesoscale

MSD Multiplex Technology

Up to 10 different analytes per well.
Monitoring Inflammatory Cytokine/Chemokine Levels by Luminex and Mesoscale

### Plasma IFN-γ Levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>IFN-γ Concentration (pg/mL)</th>
<th>Luminex</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Tx</td>
<td>10</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>LPS</td>
<td>80</td>
<td>Blue</td>
<td>Red</td>
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<tr>
<td>PMA</td>
<td>120</td>
<td>Blue</td>
<td>Red</td>
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</table>

**6 Hour Whole Blood Stimulation Condition**

### Plasma IL-12 p70 Levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-12p70 Concentration (pg/mL)</th>
<th>Luminex</th>
<th>MSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Tx</td>
<td>1</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>LPS</td>
<td>20</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>PMA</td>
<td>300</td>
<td>Blue</td>
<td>Red</td>
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</table>

**6 Hour Whole Blood Stimulation Condition**

### Comparison Table

<table>
<thead>
<tr>
<th>Luminex</th>
<th>MSD</th>
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<tbody>
<tr>
<td>&gt; 10 analytes</td>
<td>≤ 10 analytes</td>
</tr>
<tr>
<td>Mix and match capability (beads)</td>
<td>Pre-printed plates</td>
</tr>
<tr>
<td>2-3 fold dynamic range</td>
<td>4-fold or greater dynamic range</td>
</tr>
<tr>
<td>Less sample volume</td>
<td>Greater sample volume</td>
</tr>
<tr>
<td>Less sensitive</td>
<td>More sensitivity</td>
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</tbody>
</table>

**Luminex MSD**

- **Mix and match capability**: Luminex offers a mix and match capability with beads, whereas MSD uses pre-printed plates.
- **Dynamic range**: Luminex provides a 2-3 fold dynamic range, whereas MSD offers a 4-fold or greater dynamic range.
- **Sample volume**: Luminex is more sensitive to less sample volume, whereas MSD is more sensitive to greater sample volume.
- **Sensitivity**: Luminex is less sensitive compared to MSD, which has more sensitivity.
Ligand Binding Assay Challenges in Immunotoxicity Assessment

- Kinetics of the *in vivo* response after drug administration
  - Antibody/complement products/
- Matrix effects vs. Sensitivity
  - Spike and Recovery experiments indicate different MRD for each analyte

<table>
<thead>
<tr>
<th>Matrix Dilution</th>
<th>% Recovery</th>
<th>MIP-1β</th>
<th>IFN-γ</th>
<th>G-CSF</th>
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<tbody>
<tr>
<td>1:2</td>
<td>41.821</td>
<td>79.565</td>
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<td>1:4</td>
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<tr>
<td>1:10</td>
<td>71.394</td>
<td>87.180</td>
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</table>

- Kinetic analysis and multiplex panel design/reagent selection is key
Summary of Ligand Binding Assays to Assess Immunotoxicity

- Indirect measurements of immune activation after drug administration
- Determine B cell function through TDAR
- Measure immunogenicity of drugs via ADAs
- Complement activation by measuring split products
- Detect general inflammation with cytokines/chemokines
Questions?