IMMUNOTOXICOLOGY: THE WHY, WHAT, WHEN, AND HOW
ICH S8 (2005):
- “Evaluation of potential adverse effects of human pharmaceuticals on the immune system should be incorporated into standard drug development”
- “If the pharmacological properties of a test compound indicate it has the potential to affect immune function, additional immunotoxicity testing should be considered...weight of evidence (WOE) approach to decide if additional immunotoxicity studies are needed”
- “In addition, immunophenotyping of leukocyte populations, a non-functional assay, can be conducted to identify the specific cell population affected and might provide useful clinical biomarkers

ICH S9 (2009): “additional endpoints (such as immunophenotyping by flow cytometry) might be included in the study design”

Guidance for testing unintended immunosuppression or enhancement, not intended immunoenhancement caused by Immunomodulatory mAbs
WHY

The majority of immunotox testing is conducted on a case by case basis:

- The target of the drug is an immune system component (i.e., immunomodulatory drug).
  - Immuno-suppression
  - Activation (e.g., immuno-oncology)
    - Both could result in exaggerated pharmacology

- The mechanism of action of the drug suggests potential off target immune effects.
- Indications of immune dysfunction were observed in early development efficacy or toxicology studies.
- The intended patient population is immunocompromised.
WHY

MEDICAL DEVICES GUIDANCES

• It is recommended that all devices undergo some type of testing to see if they inappropriately activate the immune system (specifically, irritation/sensitization), however the extent of testing depends on the type and duration of patient contact.

• ISO 10993-10: irritation, sensitization
• ISO 10993-11: systemic toxicity (material-mediated pyrogenicity)
• ISO 10993-6: implantation
## WHY

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<th>Device Categories</th>
<th>Biological Effect</th>
<th>Initial Evaluation</th>
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<td>C. Permanent (&gt;30 days)</td>
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<td>Surface devices</td>
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<td>Mucosal membrane</td>
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<tr>
<td>Blood</td>
<td>Cytotoxicity, Sensitization</td>
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Standard immunotoxicology evaluation that is usually included in toxicity testing includes:

- **Clinical Pathology Assessment**
  - hematology
  - serum chemistry

- **Gross anatomy**
  - lymphoid organ weight

- **Histology—Microscopic Evaluation**
  - depletion of lymphocytes
  - expansion of lymphocytes
  - infiltration of immune cells
  - margination of neutrophils
WHY and WHAT

Immunotoxicology/Immunopharmacology assessments that may be included on animal studies based on target, previous history of literature, regulatory requests, clinical findings etc.

- Immunophenotyping
  - Identifying/characterizing white blood cell sub-populations in peripheral blood and lymphoid tissues (flow cytometry, IHC/IF)

- T-cell dependent antibody response
- Cytokine profiling
- Serum Immunoglobulin Isotyping
- Pharmacodynamic activity endpoints
- Anti-drug antibody analysis
- Innate immune function testing
- NK activity
- Lymphocyte activation
- Etc........
WHEN and WHY

When would these assessments be included in test article assessments?

- PK/PD studies
  - Often limited “n” size
- Early exploratory toxicity studies
  - Often limited “n” size
- Pivotal (FIH enabling) toxicity studies
- Chronic toxicity studies
  - Consider especially for biologics to assess continued PD or toxicity in presence of anti-drug antibodies
  - Assess increase in severity/incidence
- Separate investigative immunotoxicity studies
  - Usually based on findings in toxicity studies or in the clinic or by regulatory request
WHY And HOW: Immunophenotyping

- Identifying white blood cell sub-populations in peripheral blood and lymphoid tissues
- Flow cytometric methods are often used to identify leukocyte populations
- Antibodies (Abs) against specific “markers” or receptors on the leukocyte cell surfaces are used (or intracellular).
- Specificity of antibodies is instrumental
- For multiple Ab panels, each Ab is conjugated (labeled) with a unique fluorochrome.
- Lasers within the flow cytometer identify the fluorochrome by emission spectra, thus identifying the cell “marker”
- “Standard” T/B/NK panel to complex panels that identify specialized leukocytes and/or maturation state thereof e.g.:
  - T-reg, activated T-cells, immature B-cells, mature B-cells, central or memory effector cells etc.
- Immunophenotyping markers and Ab availability vary from species to species
- Panels identifying highly specialized cells are best analyzed using Ab panels with 7+ fluorophores
HOW: Immunophenotyping

- Incubate whole blood with cell marker specific antibodies
  - Wash
  - Lyse red blood cells
  - Wash
  - Fix samples with paraformaldehyde
  - Perform flow cytometric analyses

Sometimes not this simple........

- Depending on Abs, the blood might need to be washed prior to Ab incubation.
- If primary antibody is not directly conjugated with fluorochrome, indirect immunostaining is needed, i.e., addition of fluorochrome-conjugated secondary antibody
- If intracellular target, cells will need to be fixed and permeabilized.
Results: Immunophenotyping

- No finding at the low dose for total lymphocytes (standard hematology) or B-cell absolute numbers (data not shown)

- Specialized Immuno-phenotyping with B-cell and IgM specific antibodies: drug-related effects at the low dose; thus re-defining the NOEL

Rat peripheral blood phenotyping with a T-cell (CD3), B-cell (CD45RA), anti-IgM
Marginal zone depletion in splenic white pulp was observed by routine H&E histopathology staining.
Splenic tissue from rats treated with a compound associated with lymphoid depletion was immunostained with anti-B-cell (red), anti-IgM (blue) and anti-IgG (green) Ab.

Images are 3x3 tiles of 9 congruent fields at 40x magnification.

Specialized immunostaining demonstrates changes at lower doses.
**WHY: Functional Assessments**

**T-cell dependent antibody response (TDAR)**

- Common assessment for both environmental and pharmaceutical immunotoxicity testing, which is used to address the question: 
  
  Can a suitable antibody response be mounted in the presence of the test article (or toxicant)?

- TDARs require intact and functional T-cells, B-cells and antigen presenting cells (e.g. macrophage, dendritic, and B cells)
WHAT: TDAR

Adapted from: Loureiro et al. Biomolecules 2015: 5(3), 1783-1809
Basic Procedure

- Expose animal to test article
- Immunize animal with immunogen (KLH, SRBC, HepB surface antigen, Ova etc...)
- Collect blood, separate plasma or sera,
- Analyze for antibodies specific for the immunogen

- Typical schedule of blood draw:
  - Days 4-7 post-immunization (IgM)
  - Days 14, 21, 28 post-immunization (IgG)

- Typically the analysis platform is ELISA
HOW: TDAR Method

- Immunogen-specific Ig
- Enzymatic Detection Conjugate
- Secondary Ab
- Substrate
- Chromogenic Rxn

Immunogen
T-cell depletion and decrease in IgM expression on B-cells was associated with a measurable suppression in the ability to elicit an antibody response to KLH.

For this program, effects on the immune system were not intended for the therapeutic outcome, but were not a complete surprise due to the target’s involvement in molecular signaling pathways resulting in lymphocyte development and homeostasis.
WHY and WHEN: Functional Assessments

Innate Immune Cell Function Assessment

☑ Can a suitable innate immune response be mounted in the presence of the test article (or toxicant)?

When:
☑ In vitro assessments conducted to characterize MOA or potential toxicities
☑ On study ex vivo: depends on MOA, may be proactively included on study to assess potential toxicity or as PD activity
☑ Opportunistic infections observed in toxicity studies

What:
☑ Phagocytosis, Respiratory Burst, Chemotaxis, Cell killing activity etc.
  ☑ Macrophages, Monocytes, NK cells etc.
WHAT, WHY, HOW: Phagocytosis

- Phagocytosis is the engulfment of foreign material.
- Mechanism for clearance of foreign material and dead cells.
- Use of fluorescent-labeled particles or bacteria allows for easily monitored phagocytic capacity.
HOW: Phagocytosis

1. Labeling – FITC-e.coli

2. Quenching

3. Lysing and Fixation

4. Washing and DNA Staining

5. Flow cytometric analysis
RESULTS: Phagocytosis

Median (n= 5 to 6) % inhibition of the per day averaged control group mean fluorescence intensity
Data were normalized with pretest mean fluorescent values prior to calculation of % inhibition.
WHAT, WHY, HOW: Respiratory Burst

- Sudden release of superoxide radicals
- Kill intracellular bacteria
- Use of fluorescent-labeled substrates of respiratory burst allows for monitoring
HOW: Respiratory Burst

1. Activation
   - 2 x 100 µl whole blood at 0°C
   - Incubate precisely for 10 min. at 37°C
   - +20 µl FMLP at 0°C
   - +20 µl bacteria at 0°C
   - Vortex!

2. Oxidation
   - + 20 µl substrate solution at 37°C
   - Incubate precisely for 10 min. at 37°C
   - Vortex!

3. Lysing and Fixation
   - + 2 ml lysing solution at room temp. (RT)
   - Centrifuge 5 min at 250 g at 4°C and decant
   - Incubate 20 min at RT
   - Vortex!

4. Washing and DNA Staining
   - + 3 ml washing solution at 0°C
   - + 200 µl DNA staining solution at 0°C
   - Centrifuge 5 min at 250 g at 4°C and decant
   - Vortex!

5. Flow Cytometric Analysis
   - Live gate on leukocyte DNA during acquisition
   - Red fluorescence (DNA)
   - Gate on granulocytes
   - Gated granulocytes 99% positive cells Mean fluorescence in channel 208
   - Green fluorescence (R123)
RESULTS: Respiratory Burst

^Median (n= 5 to 6) % inhibition of the per day averaged control group mean fluorescence intensity
Data were normalized with pretest mean fluorescent values prior to calculation of % inhibition.
WHAT, WHY, HOW: NK cell activity

- NK cells have cytocidal activity that may be specific or non-specific in nature.
- Kill cells by perforin and granzyme release
- ADCC
- Important in tumor surveillance
- Therapeutic target to increase immune killing of tumor cells
- Function can be assessed by a variety of methods: granzyme or perforin release, CD107 expression, target cell killing etc.
HOW: NK cell activity flow based method

- Label target cells (e.g. K562 or YAC-1) with a fluorescent dye (e.g. DiO).
- Isolate PBMCs (effector cells).
- Add appropriate volume of effector cells to obtain desired effector to target cell ratio (E:T ratio) and, if appropriate, add IL-2.
- Incubate at 37°C, 5% CO2 for 18-24 hours.
- After incubation, place samples on ice and add live/dead stain (e.g. To-Pro, PI or 7-AAD)
- Analyze by flow.
RESULTS: NK cell activity flow based method

Target cells that have lost cell membrane integrity due to either spontaneous cell death or release of cytotoxic factors such as perforins.
RESULTS: NK cell activity flow based method
Other Itox Assessments............

- ANA (anti-nuclear antibody) assessment- potentially indicates auto-immunity
- Chemical mediators of hypersensitivity reactions: complement, histamine etc.
- Cytokine expression profiling on toxicity studies or In Vitro Cytokine Release assay in Human blood or PBMCs
  - ELISA based
  - Luminex (multiplex bead array assay), theoretically can analyze up to 100 analytes in less then 25 uL of serum
- Isotyping of Immunoglobulins
  - ELISA based
  - Multiplex (bead array or ECL- MSD assay) provides time saving options for isotyping.
Host Resistance Models

If the aforementioned assessments demonstrate immunotoxicity…

- Is there an affect on overall host resistance to infection?

- What types of models exist?
  - Bacterial, viral, fungi etc.

- What factors play into the decision to utilize a host resistance model in drug development?
  - Indication, patient population etc.
Immunogenicity: ADA

- Immunogenicity to a drug (anti-drug antibodies [ADA]) can alter the PK of a drug and/or efficacy.

- ADAs can result in toxicity including cytopenia, acute hypersensitivity reactions and/or immune–complex deposition.

- Factors that increase the potential of immunogenic response to a drug or environmental toxicant include...
  - Composition of drug: biologics- protein therapeutics
  - Hapten formation

- Typically ADA are measured by an immuno-assay such as a standard ELISA or an electrochemiluminescent (ECL) immuno-bridging assay.
Immunogenicity: ADA

**Standard ELISA**
- O.D. at 450 nm
- TMB
- Colored product
- Anti-human IgG-HRP
- ADA
- ADA
- Drug*

- Sample volume: 15-100 μL
- Wash steps: 3-4
- Duration: 4-24 hours
- Sensitivity: 100s ng/mL
- Drug tolerance: acceptable

**ECL Immuno-bridge**
- Emit light at 620 nm
- ADA
- SULFO-TAG-drug
- Biotin-drug
- Streptavidin

- Sample volume: 5-25 μL
- Wash steps: 1
- Duration: > 24 hours
- Sensitivity: 10s ng/mL
- Drug tolerance > ELISA

*In these examples the drug is a monoclonal antibody.*
Summary

**Why:** Immunotoxicology testing is required for new investigational drugs and chemicals.

**When:** Immunotoxicology testing is performed on standard toxicity studies as well as separate immunotoxicology studies on a case-by-case basis.

**What:** From standard hematology to host resistance models, lymphocyte populations to cell function assays, there are many assessments used to characterize the immunotoxicological/immunopharmacological potential of a test article. The assessments chosen are done so on a case-by-case basis; there is no set directive.

**How:** Methods using high-end technologies (MSD, flow cytometry, luminex, confocal microscopy) to standard ELISA-based methods