Fundamental Approaches to Immunotoxicity Assessment in Preclinical Safety Studies

Presented by:
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Overview

• Disclaimer: Not a comprehensive immunotoxicity discussion
  • Practical “weight-of-evidence” approach
  • When/how to apply additional testing
• Utilizing parameters for Standard Toxicity Studies (STS)
  • Hematology, pathology, etc.
• Lymphocyte Subset Analysis (Immunophenotyping)
• T-cell Dependent Antibody Response Testing (TDAR)
  • Biologic validation of ELISA methods
• Translating into humans
ICH S8 Guidance 2006

- Most commonly followed
- Focused on immunosuppression and enhanced activation
  - “Standard toxicology study (STS) endpoints sufficient to identify the majority of immunotoxic effects”
  - “Weight-of-evidence” and case-by-case

STS Endpoints

- Hematology – cytopenias, leukocytosis
- Gross, organ weight, and microscopic pathology of immune organs
  - ↓Organ weights, lymphoid depletion
- Serum biochemistry - ↓globulins
- Tumor and infection incidence
ICH S8 Guidance

• Should include
  • Statistical analysis
  • Dose/exposure relationship
  • Safety margin
  • Changes that occur as secondary effects (e.g. stress, anorexia)
  • Possible cellular or molecular targets/mechanisms
  • Reversibility

• Is there potential impact on the immune system?
• Immune tissues or cells
• Increased incidence of infections/tumors

YES?
ICH S8 Guidance – Additional Points

Assay characterization and validation

• Standard validation required
  • Inter/intra assay precision and accuracy
  • Limit of detection (LOD)
  • Linear range
    (range of quantitation)
  • Stability
  • Robustness
  • Incorporation of positive controls

Not applicable to all assay types

Spirit of “fit-for-purpose” – IMPORTANT!

Interpretation of stress-related changes

• “….evidence of stress should be compelling in order to justify not conducting additional immunotoxicity testing….”
• Do not over call stress!
FDA Guidance 2002

General Mention

• Use STS endpoints to determine if further testing warranted
• Same weight-of-evidence approach
• Examples, details, and references

Specific Mention

• PK studies indicate drug concentrates in immune tissues
• Suggests evaluation of developmental immunotox
  1. intended for pregnancy
  2. immunosuppression
• Inhalation and dermal studies
  • Sensitizing potential
• Adverse immunotoxicity vs. intended pharmacology
FDA Guidance 2002

5 adverse event categories

• Immunosuppression
  • Leukopenia, ↓organ weights, cell depletion, ↓globulins, infections
  • TDAR
    • Supports separate study of satellite animals

• Immunogenicity

• Hypersensitivity/allergic reactions
  • Specific examples of Type I, II, III, and IV
  • Extensive

• Autoimmunity
  • Examples, no standard methods

• Immunostimulation
  • STS and cytokines
ICH S8 and FDA Guidance

**Additional testing** – contingent upon results of STS parameters

- Functional and Non-functional
- TDAR (T-cell Dependent antibody response)
  - FDA - separate study or satellite animals
  - ICH S8 – include in STS
- Immunophenotyping of lymphocyte populations
- Natural Killer (NK) Cell Activity Assays – *In vitro*
- Host resistance assays (pathogens or tumor cells)
- Neutrophil/macrophage functional Assays
- Cell-mediated immunity
  - Hypersensitivity/DTH
Standard Toxicology Study (STS) Endpoints

Immunosuppression

- Cytopenias - (granulocytes and lymphocytes)
- Immune organ weight decreases
  - Lymph nodes, spleen, thymus
- Immune organ lymphoid depletion
  - Often correlates with circulating lymphocytes
  - Bacterial sepsis, abscesses, pneumonia

Enhanced immune activation

- Leukocytosis, neutrophilia, left shift
  - No microscopic correlates
- Acute phase response (fibrinogen, CRP, etc.)
- Microscopic inflammation not associated with organ toxicity
  - E.g. catheter sites, injection sites
When to do Immunotoxicity Testing?

Other

• Anaphylaxis/hypersensitivity reactions
• Suspect autoimmune
  • Hemolysis - ↓red cell mass, ↑TBIL, splenic EMH, ↑hemosiderin pigment
  • Thrombocytopenia (suspicious)
  • Vasculitis

1. Impact on immune tissues/cells
2. Increased infections
3. Mechanism of action
4. When they tell you to! (regulators)
Question – What first line Immunotoxicity assays do you incorporate into your preclinical studies?

A. Standard lymphoid organ histopathology, weights, and hematology
B. Immunophenotyping
C. T-cell dependent antibody response (TDAR)
D. Cytokine and/or acute phase protein evaluation
E. In vitro cell activity assays (e.g. NK cell activity)
F. 2 or more of the above
**Immunosuppression vs. Stress**

**Hematology**
- Lymphocytes most commonly affected
  - Stress not always dose dependent
  - Look for effects on neuts/eos

**Pathology**
- Immune organ effects
  - Thymus most sensitive
- Increases adrenal gland weights
  - Hypertrophy of zona fasicularis

**Other**
- Hyperglycemia
- Corticosteroid evaluations not fruitful?

<table>
<thead>
<tr>
<th></th>
<th>Epinephrine (Minutes)</th>
<th>Corticosteroid (Hours)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>↑↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Platelets</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RBC</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Everds NE et. al. (2013) *Tox Path*
Stress vs. Immunosuppression – Other Factors

Stress
- Often associated with overt toxicity
- ↓ Food consumption/body weight клинические наблюдения
- “Tends” to be less consistent/dose dependent
- Thymus most sensitive to stress

Immunosuppression
- Lymphoid effects reaching lower than other toxicity signals
- Likely to be direct effect if no thymic changes

Sometimes have both…..
- Immunotoxicity → stress → ↓ food consumption → ↓marrow and lymphoid cellularity

Guidance specifically addresses (ICH and FDA)
Markers of Enhanced Immune Activation

Acute Phase Proteins

• Non-specific markers of inflammatory cascade/process
• Most produced by liver in response to cytokine activation (IL-1, IL-6, etc.)
  • Hours to days
• Must use appropriate species specific markers
  • Fibrinogen (most)
  • C-reactive protein (NHP and canines)
  • A-2 macroglobulin (A2M), A-1 acid glycoprotein (AGP) (rats)
  • Haptoglobin and serum amyloid A (mice and swine)

Globulins

• Total and IgG, IgM, and IgE
  • Anaphylaxis
• Validated methods!
Markers of Enhanced Immune Activation

Cytokines

- Involved in cell-cell messaging
  - Many cells secrete – lymphocytes, macrophages, dendritic/APCs
- Minutes to hours – compound specific
- What good are they?
  - Elucidate mechanisms (pro and anti-inflammatory markers)
  - Cause or effect of inflammation?
  - Predictive - early signs
- Luminex/multiplex panels
  - Methods not standardized – assays generally not as tight as APPs
  - Validated methods!
- \textit{In vivo VS in vitro}
  - \textit{In vitro} - most common, recommended for mechanistic studies
  - \textit{In vivo} – may not be representative – TGN 1412
APPs vs. Cytokines (rats)

Honjo T et al. (2010) Lab Animals
# NHP Lymphocyte Immunophenotyping Panel

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Antigen Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>CD45</td>
</tr>
<tr>
<td>T-cells</td>
<td>CD45, CD3</td>
</tr>
<tr>
<td>T(_{\text{helper}}) Cells</td>
<td>CD45, CD3, CD4</td>
</tr>
<tr>
<td>T(_{\text{cytotoxic}}) Cells</td>
<td>CD45, CD3, CD8</td>
</tr>
<tr>
<td>B-cells</td>
<td>CD45, CD20</td>
</tr>
<tr>
<td>NK Cells</td>
<td>CD45, CD159a</td>
</tr>
<tr>
<td>Regulatory T Cells</td>
<td>CD4, CD25, Foxp3</td>
</tr>
</tbody>
</table>

*Couple with hematology*
TDAR Testing

T-cell Dependent Antibody Response

- Immune function assessment
  - Immunosuppression
- Ability to mount antibody response to standardized antigen challenge
  - Keyhole Limpet Hemocyanin (KLH)
  - Sheep Red Blood Cells (SRBC)
  - Tetanus Toxoid
- Coordinated activity of macrophages, T-helper cells, and B-cells
- Antigen-specific IgM followed by IgG responses
- Supplements hematology and lymphoid organ assessment
- Further studies required regarding mechanisms of dysfunction
- FDA vs EPA requirements
Classic TDAR Response

- IgM precedes IgG
- Isotype switching
- Peak Response
  - IgM – 7-14d
  - IgG – 14-21d
- Use to time sampling
- IgM will wane
- IgG may persist
Classic TDAR IgM Response

- IgM Response
- Day 1, Day 7, Day 14, Day 21, Day 28
- Control
- Test Article

Immunize
TDAR Testing in NHP Overview

Retrospective review of 30 studies in NHP

• No gender differences
• No country of origin differences - NHP
• Most used KLH (87%), TT (34%), SRBC (12%)
• Substantial inter/intra-animal variability
• \( \leq 4 \) animals/group only identifies large differences
  • Combine sexes for more power
• Some differences in magnitudes and timing of responses based on source (rat)

Lebrec et. al. (2011) J Immunotoxicol
Lebrec et. al. (2013) J Immunotoxicol
TDAR Testing Guidelines

General Considerations

• All animals can be immunized
  • Separate cohorts not typical
  • Immunization does not significantly impact other endpoints (generally)

• Wide individual variation
  • Individual immune response
  • Analytical methods
  • Minimum 4-6 animals/sex/group recommended – combine sexes for statistics

• Immunization protocol and analysis should be consistent
  • Antigen source
  • Injection site – SQ, IV, IM, footpad
  • Analytical methods – lab to lab comparisons difficult
  • Prior viral exposure – false positive reported
TDAR Testing

When to immunize?

• Compound dependent
  • Sufficient time to impact test system – not only exposure
    • NOT Day1
  • 28 Day Studies – Day 7 or 14
  • 13 Week Studies – Day 21 or 28

When to draw samples for antibody levels?

• 2–4 times following immunization
• 7–14 days following immunization at 7 day intervals

Do I need a positive control group?

• Not required
Recovery groups and secondary responses?

- Compound dependent
  - Must have knowledge of and account for multiple variables
    - Half life/exposure – days to months
    - 30-45+ days for antibody response to subside
    - Test system resolution
      - Lymphoid repopulation etc.
  - Then re-immunize (secondary response)
    - Faster, more robust, longer
    - Altered dynamics (IgG>IgM)

- 13 Week + studies usually required
Biological Validation of ELISA Methods

• Cynomolgus monkeys
  • Control and positive control groups
  • 6/group/sex N=36

• Challenged KLH Day 21 and 71
  • Primary and secondary responses
  • 100 days

• Positive controls group (represents test compound)
  • Cyclophosphamide beginning Day 1

• Correlated with
  • Hematology
  • Immunophenotyping - lymphocytes
  • Histopathology – lymphoid organs
Lymphocyte Counts – Pooled Sexes

![Graph showing lymphocyte counts over time for different conditions.](image)

- **CYP**: Green line representing Control.
- **KLH/CYP**: Blue line representing KLH/CYP.
- **KLH**: Purple line representing KLH.

Day markers: 13, 28, 36, 46, 78, 85, 92, 100.
Immunophenotyping Results

Graphs showing the immunophenotyping results over time for different conditions: Control, KLH/CYP, and KLH.
Pathology – Organ Weights

Test Article-related Organ Weight Changes - Terminal
Male and Female (Percent change relative to control)

<table>
<thead>
<tr>
<th>Group:</th>
<th>KLH/CYP</th>
<th>KLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Number Examined</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Spleen (g)</td>
<td>↓21.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↓10.52</td>
</tr>
<tr>
<td>Thymus (g)</td>
<td>↓62.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↓61.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from Antigen 1 Vehicle; (p<0.05)

↑ - Increased
↓ - Decreased
M – Male
F – Female
## Microscopic Pathology

### Test Article-related Microscopic Observations – Terminal

#### Pooled Lymph Nodes

(iliac, popliteal, inguinal, and mandibular)

<table>
<thead>
<tr>
<th>Group:</th>
<th>Control</th>
<th>KLH/CYP</th>
<th>KLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M F</td>
<td>M F</td>
<td>M F</td>
</tr>
<tr>
<td>Number Examined</td>
<td>47 47</td>
<td>46 43</td>
<td>47 48</td>
</tr>
<tr>
<td>Lymph nodes (pooled)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depletion, lymphoid, generalized (minimal to mild)</td>
<td>0 0</td>
<td>7 6</td>
<td>0 3</td>
</tr>
<tr>
<td>Depletion, lymphoid, germinal center</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-minimal</td>
<td>0 0</td>
<td>0 0</td>
<td>1 0</td>
</tr>
<tr>
<td>Hyperplasia, lymphoid, germinal center</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-minimal</td>
<td>0 0</td>
<td>0 0</td>
<td>5 2</td>
</tr>
</tbody>
</table>

M – Male
F – Female
Anti-KLH IgM and IgG Responses

Aulbach et. al. (2013) ACT poster
Effects of Reduced Leukocytes

When do reductions actually adversely impact immune function?

- Humans (>40% ↓ lymphocytes; >75% ↓ in granulocytes)

Adversity subjective

Rely on clinical evidence – infections etc.

No consistent guidance for animal studies

- Neutrophils <1000 cells/µL

Hannet I et. al. (1992) *Immunol Today*
Effects of Reduced Lymphocytes on TDAR

% Change in Cyclophosphamide Treated Relative Controls
7 days post Immunization

<table>
<thead>
<tr>
<th></th>
<th>Day 28</th>
<th>Day 78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>-74%(^a)</td>
<td>-70%(^a)</td>
</tr>
<tr>
<td>T Cells</td>
<td>-77%(^a)</td>
<td>-78%(^a)</td>
</tr>
<tr>
<td>CD4+</td>
<td>-70%(^a)</td>
<td>-73%(^a)</td>
</tr>
<tr>
<td>CD8+</td>
<td>-87%(^a)</td>
<td>-84%(^a)</td>
</tr>
<tr>
<td>B Cells</td>
<td>-62%</td>
<td>-27%</td>
</tr>
<tr>
<td>NK Cells</td>
<td>-92%(^a)</td>
<td>-87%(^a)</td>
</tr>
<tr>
<td>KLH IgM</td>
<td>-72%(^a)</td>
<td>-46%(^a)</td>
</tr>
<tr>
<td>KLH IgG</td>
<td>-84%(^b)</td>
<td>-60%(^a)</td>
</tr>
</tbody>
</table>

\(^a\) significant at (p<0.01)
\(^b\) significant at (p<0.05)
NHP Conclusions KLH

- Primary (D21) and secondary (D71) immunizations resulted in statistically significant increases in Anti-KLH IgM and IgG within 7-14 days post immunization
- Intermittent cyclophosphamide (CYP) dosing resulted in significant reductions in total lymphocytes and most lymphocyte subtypes as detected by flow cytometry
- Animals dosed with CYP had significant decreases in Anti-KLH IgM and IgG relative to immunized control animals indicating
- Detection of a compound-related reduction in immune function by these methods
Translating into Man

- Basic structure of immune systems similar
  - Lymphoid tissues, leukocytes, innate, acquired, humoral
- Species-specific variants
  - Antibody responses
  - Antigenic markers
- NHP often the only relevant species based on antibody cross reactivity with human target proteins
  - Share significant genetic homology
  - Immunoassay cross reactivity
- ICH S6 acknowledges antibody induction in animals not predictive of antibody formation in man
Translating into Man - Examples

Similarities

• Innate immunity – dendritic cell subsets in rhesus monkeys
  • myeloid (CD11c+/CD123\textsuperscript{neg}) and plasmacytoid (CD11c-/CD123\textsuperscript{+})
  • cytokine responses similar
  • DC TLR expression same as human; different from mice

Differences

• TGN 1412
  • CD28 superagonist – expressed on human but not NHP T-cells
  • Led to “cytokine storm” in 6 human volunteers – near fatal
  • Recommend \textit{in vitro} human studies in cases with mechanistic relations

Messaoudi I et. al. (2011) \textit{Antioxid Redox Signal}
Stebbings R et. al. (2007) \textit{J Immunol}
Conclusions

• Guidance supports weight-of-evidence case-by-case strategy for inclusion of immunotoxicity testing
• Considerations for species, stress, related mechanisms, pharmacology dictate a case-by-case approach
• STS endpoints drive
  • Lymphoid organ effects
  • Leukocyte effects
  • Inflammatory biomarkers
  • Infection incidence
• First Tier
  • TDAR (T-cell Dependent Antibody Response)
  • Lymphocyte Immunophenotyping
  • Acute phase protein and cytokines
• Validated and well-characterized methods
  • Immunization protocols
  • Ligand-binding assays
References