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Fundamental Approaches to Immunotoxicity Assessment in Preclinical Safety Studies

Presented by:

Adam Aulbach, DVM, DACVP
Director of Clinical Pathology, MPI Research
adam.aulbach@mpiresearch.com
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Overview

- Disclaimer: Not a comprehensive immunotoxicity discussion
  - Practical “weight-of-evidence” approach
  - When/how to apply additional testing
- Regulatory Guidance Overview – ICH S8 (2006), FDA (2002)
- 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

1st line

- 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?

	Epinephrine (Minutes)	Corticosteroid (Hours)	Overall
WBC	↑↑	↑	↑
Neutrophils	↑↑	↑	↑
Lymphocytes	↑	↓	↓
Eosinophils	-	↓	↓
Platelets	↑	-	-
RBC	↑	-	-

Everds NE et. al. (2013) *Tox Path*

Stress *vs.* Immunosuppression – Other Factors

Stress

- Often associated with overt toxicity
- ↓ Food consumption/body weight/clinical observations
- "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

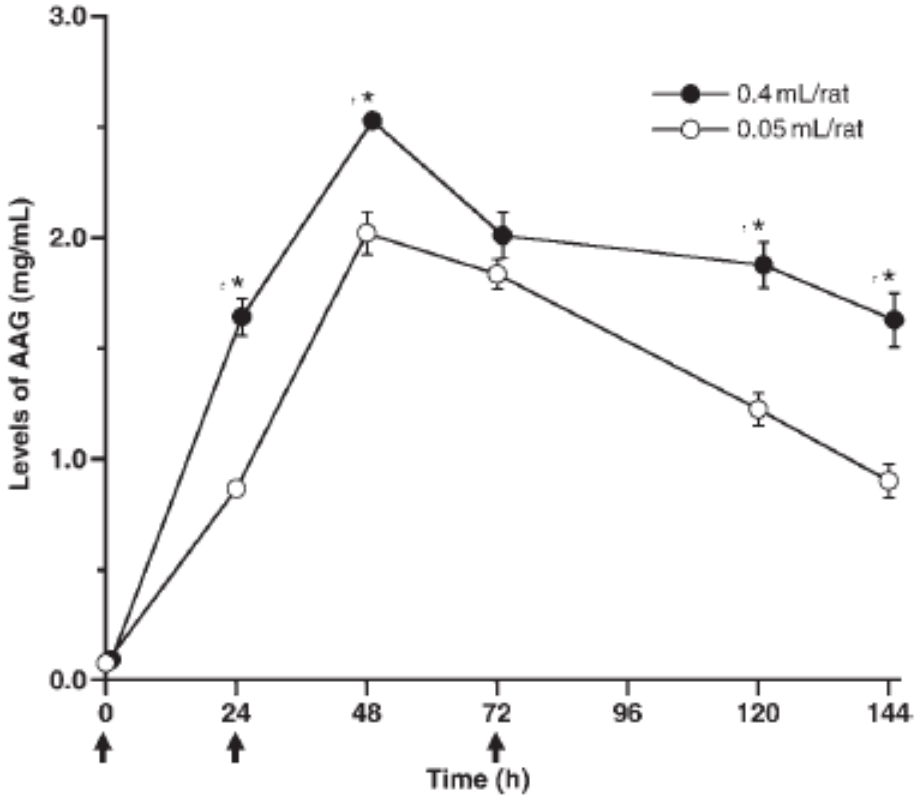
- Total and IgG, IgM, and IgE
  - Anaphylaxis
- Validated methods!

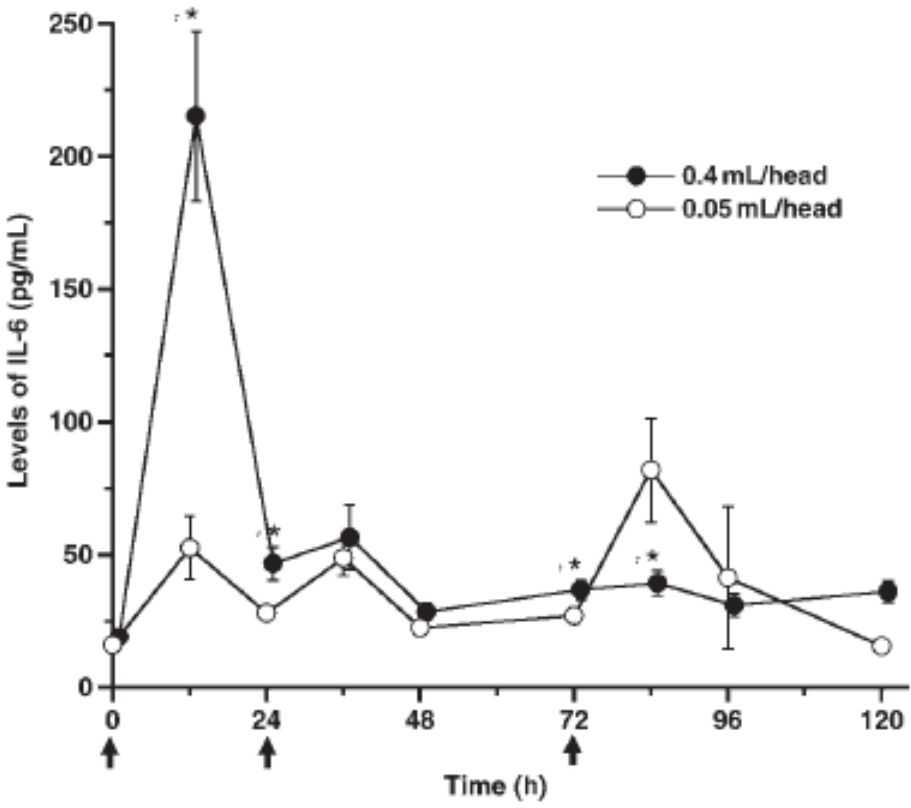
Markers of Enhanced Immune Activation

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!
- *In vivo* VS *in vitro*
  - *In vitro* - most common, recommended for mechanistic studies
  - *In vivo* – may not be representative – TGN 1412

APPs *vs.* Cytokines (rats)





Honjo T et. al. (2010) *Lab Animals*

NHP Lymphocyte Immunophenotyping Panel

Immunophenotype	Antigen Markers
Lymphocytes	CD45
T-cells	CD45, CD3
T_{helper} Cells	CD45, CD3, CD4
$T_{cytotoxic}$ Cells	CD45, CD3, CD8
B-cells	CD45, CD20
NK Cells	CD45, CD159a
Regulatory T Cells	CD4, CD25, Foxp3

Couple with hematology

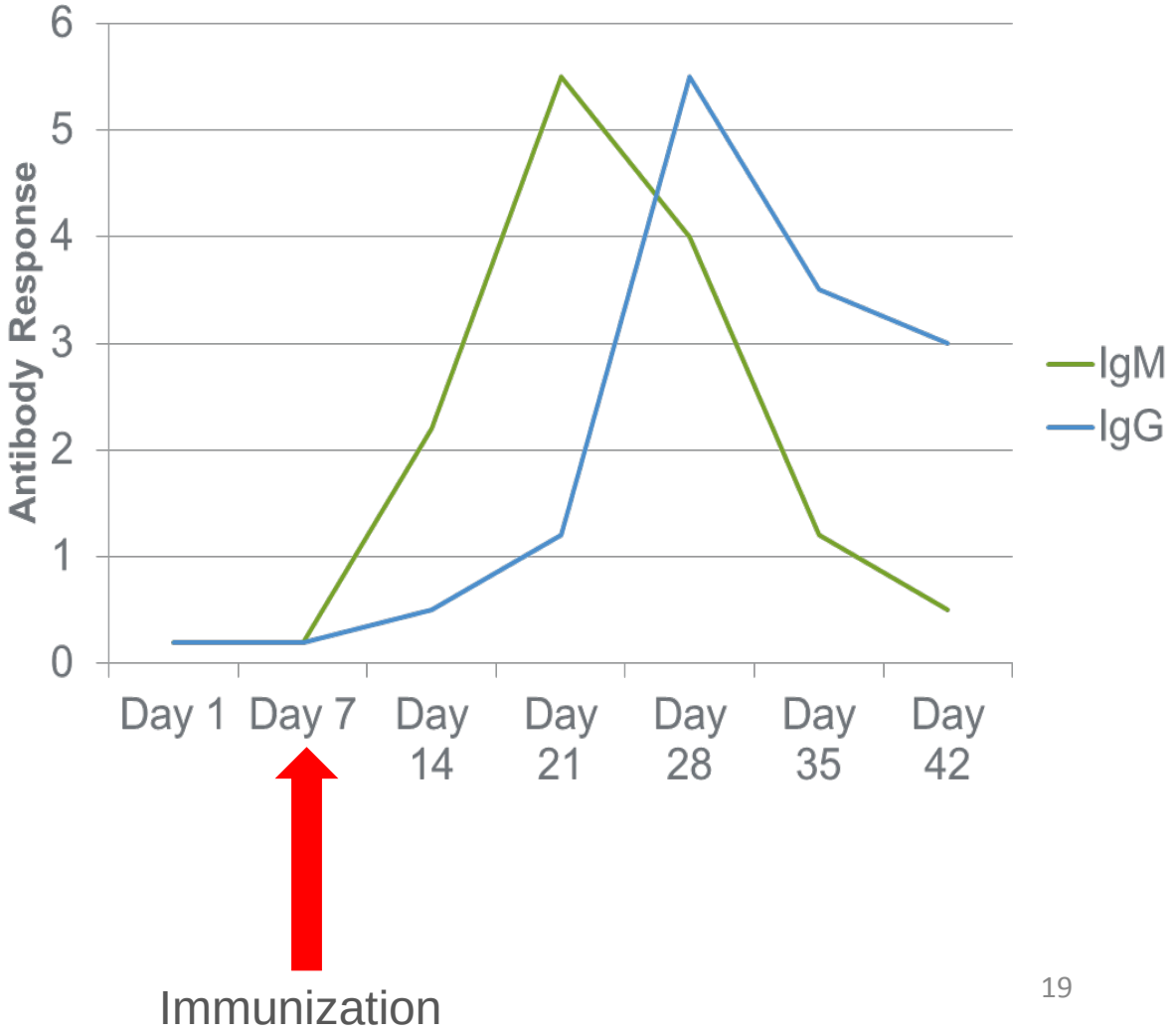
TDAR Testing

T-cell Dependent Antibody Response

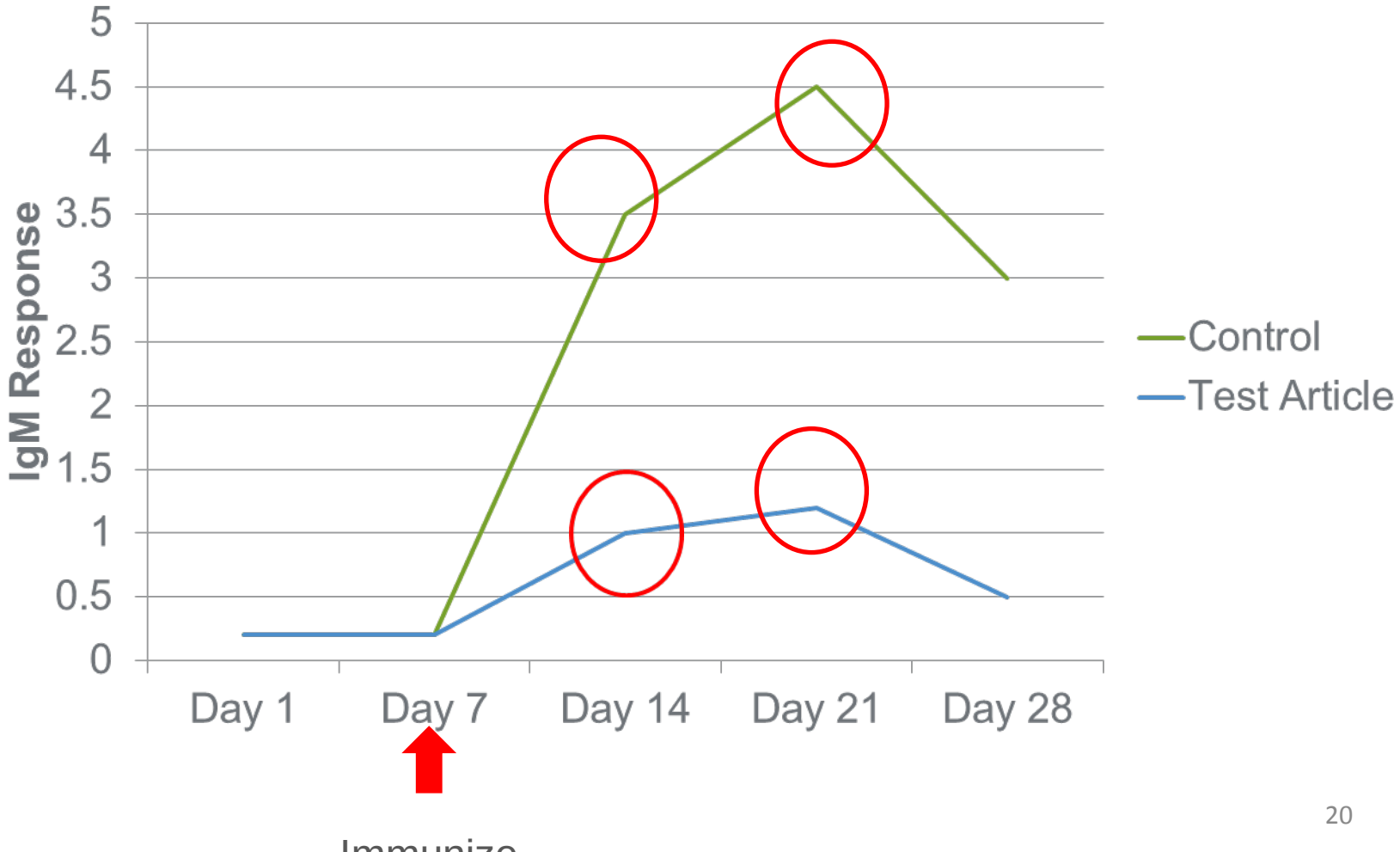
- Immune function assessment
  - Immunosuppression
- Ability to mount antibody response to standardized antigen challenge
  - Keyhole Limpet Hemocyanin (KLH)
  - Sheep Red Blood Cells (SRBC)
  - Tetanus Toxoid
- Coordinated activity of macrophages, T-helper cells, and B-cells
- Antigen-specific IgM followed 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



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
- ≤ 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

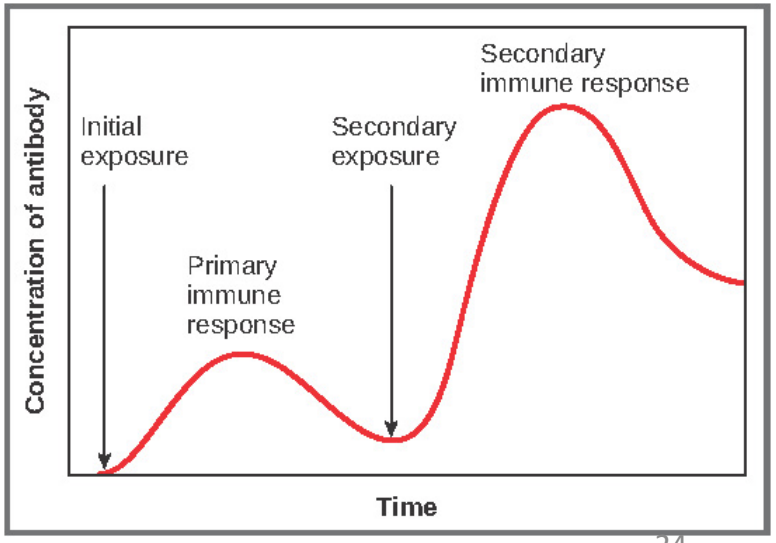
Do I need a positive control group?

- Not required

TDAR Testing

Recovery groups and secondary responses?

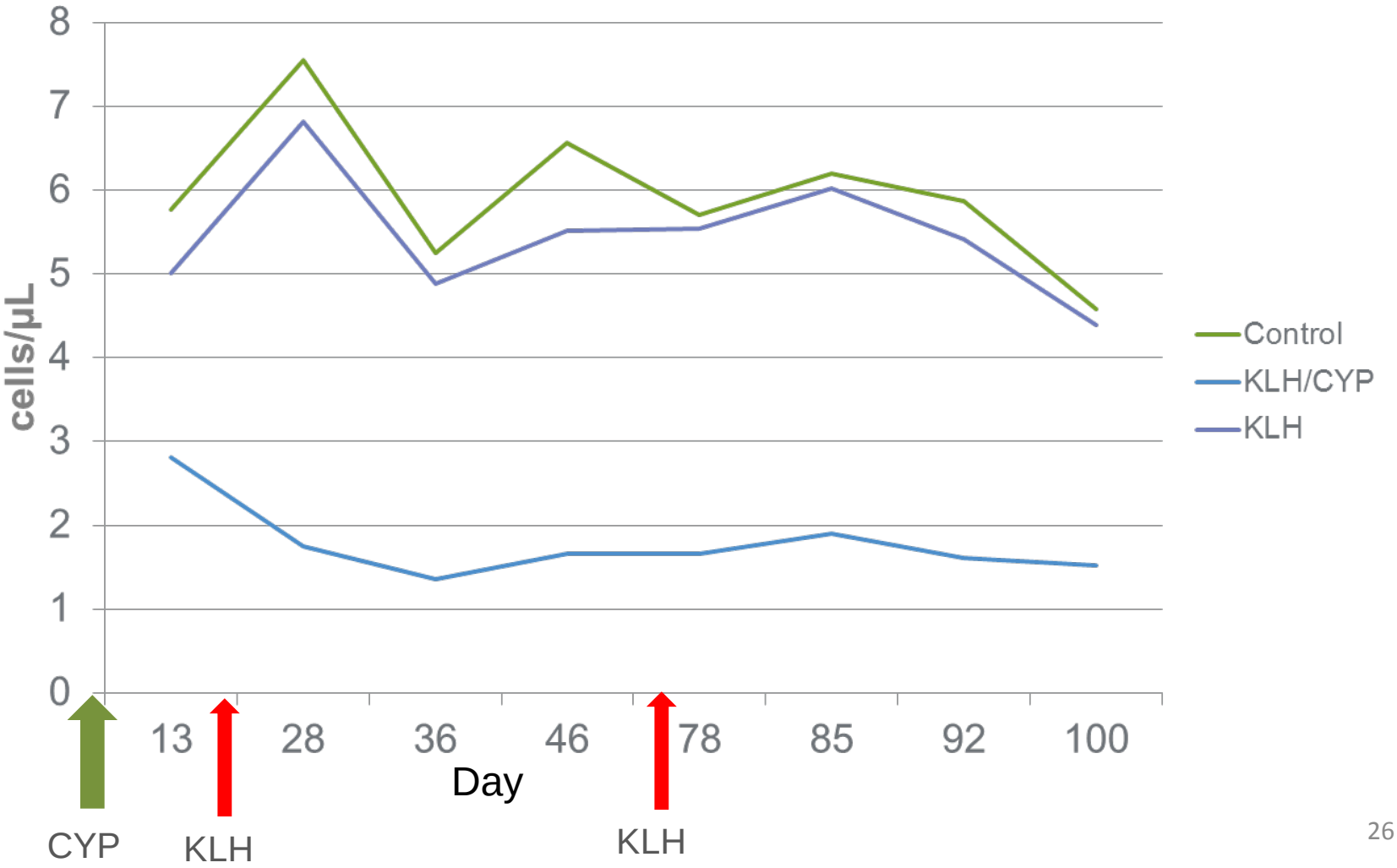
- Compound dependent
  - Must have knowledge of and account for multiple variables
    - Half life/exposure – days to months
    - 30-45+ days for antibody response to subside
    - Test system resolution
      - Lymphoid repopulation etc.
  - Then re-immunize (secondary response)
    - Faster, more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



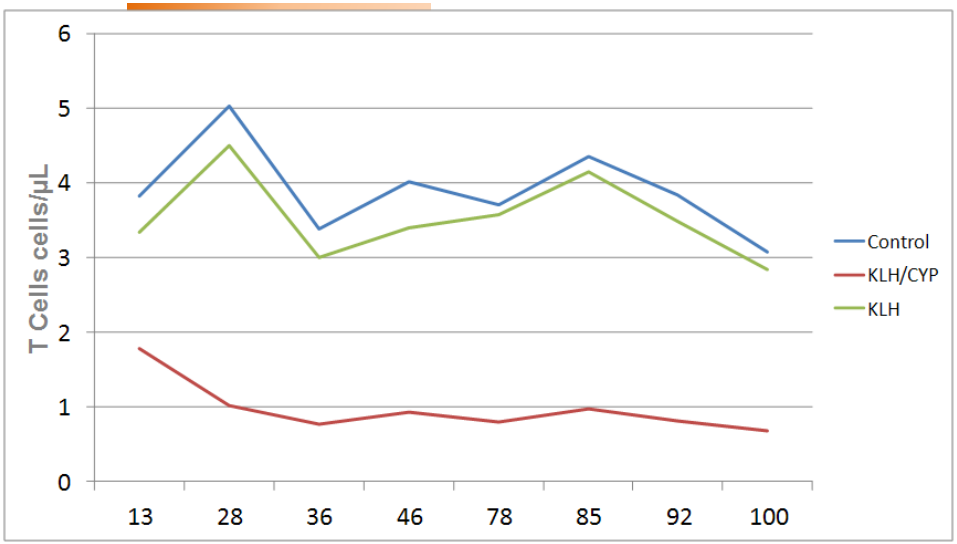
Biological Validation of ELISA Methods

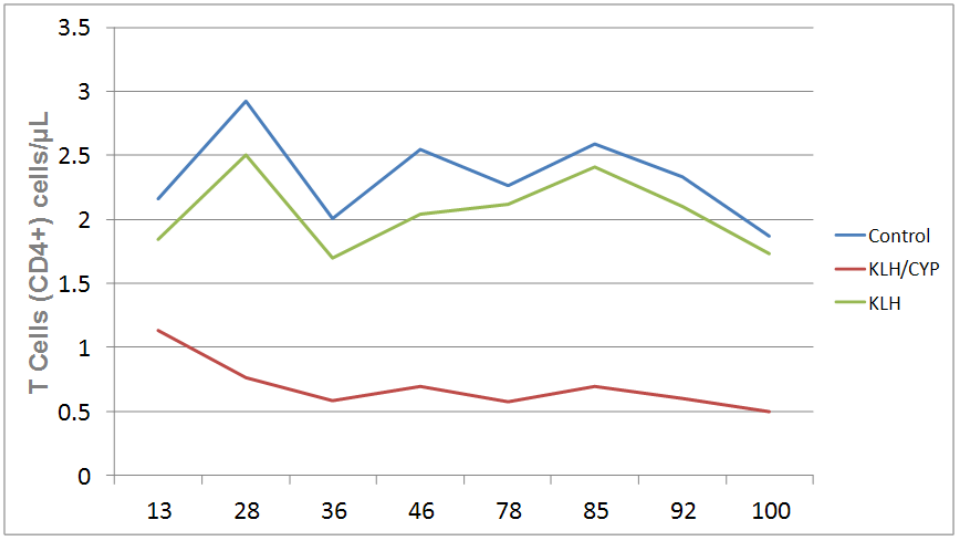
- Cynomolgus monkeys
  - Control and positive control groups
  - 6/group/sex N=36
- Challenged KLH Day 21 and 71
  - Primary and secondary responses
  - 100 days
- Positive controls group (represents test compound)
  - Cyclophosphamide beginning Day 1
- Correlated with
  - Hematology
  - Immunophenotyping - lymphocytes
  - Histopathology – lymphoid organs

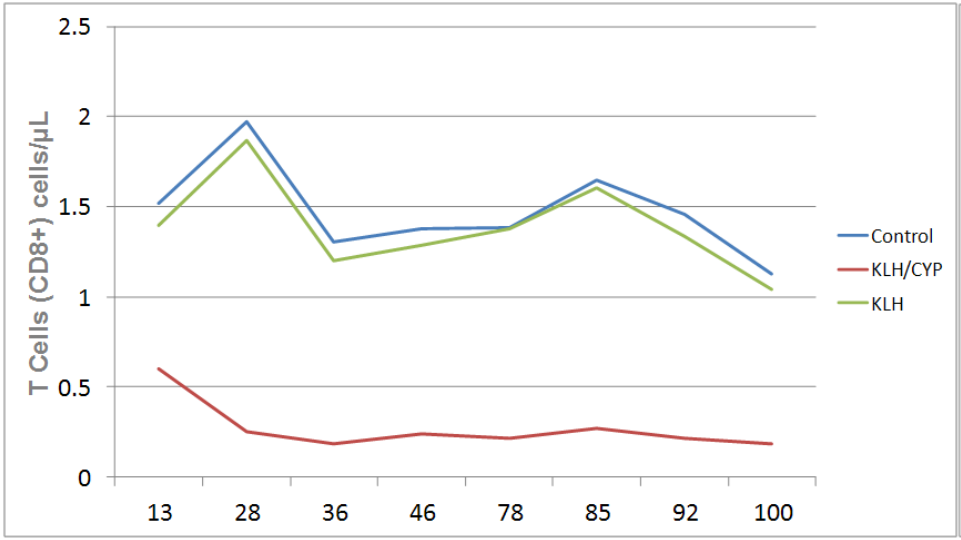
Lymphocyte Counts – Pooled Sexes

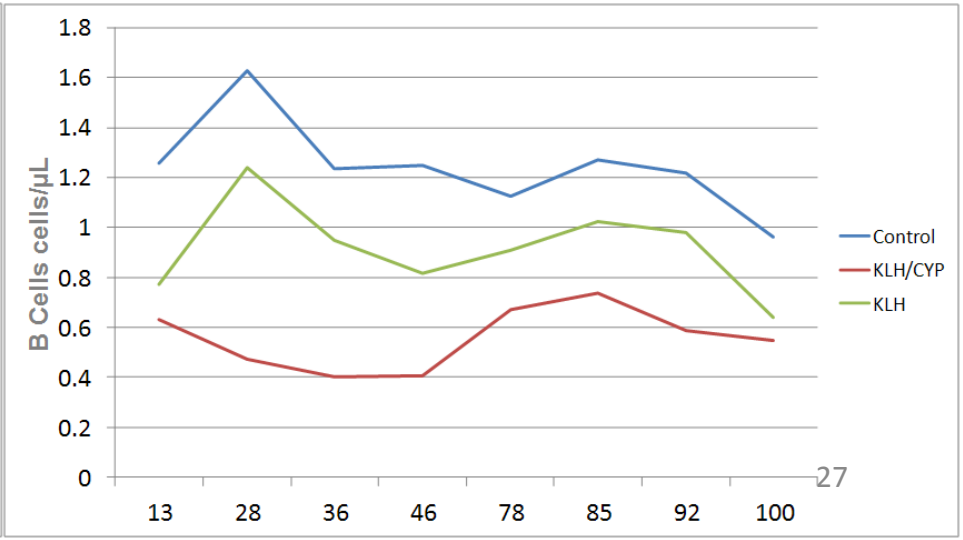


Immunophenotyping Results









Pathology – Organ Weights

Test Article-related Organ Weight Changes - Terminal

Male and Female (Percent change relative to control)

Group:	KLH/CYP		KLH	
Sex	M	F	M	F
Number Examined	6	6	6	6
Spleen (g)	↓21.67[a]	↓10.52	↓2.58	↓3.13
Thymus (g)	↓62.51[a]	↓61.25[a]	↑7.30[a]	↓12.21

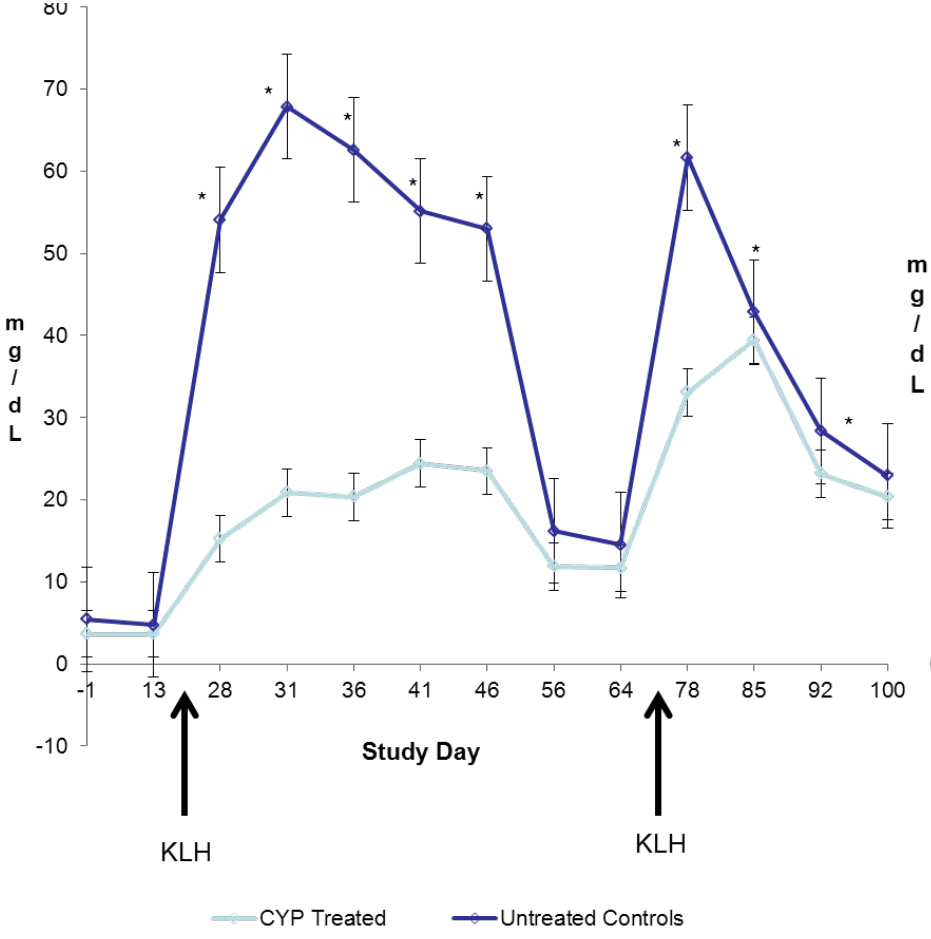
[a] Significantly different from Antigen 1 Vehicle; ($p<0.05$)

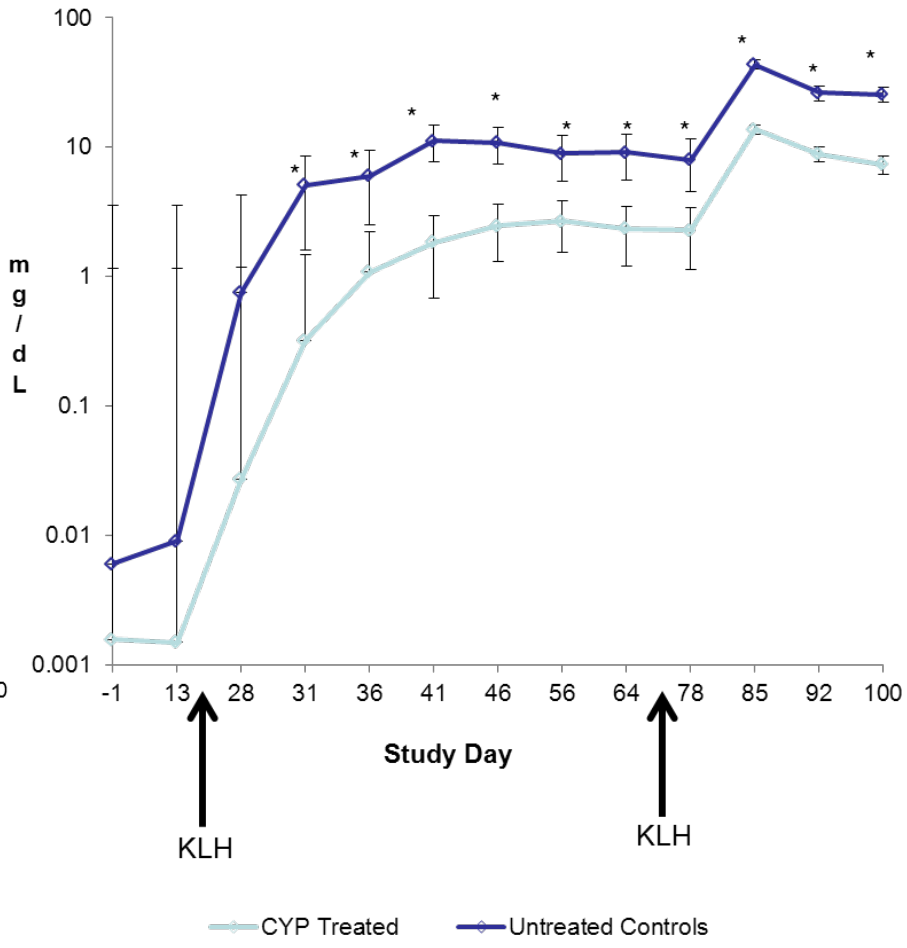
↑ - Increased
↓ - Decreased
M – Male
F – Female

Microscopic Pathology

Test Article-related Microscopic Observations – Terminal Pooled Lymph Nodes (iliac, popliteal, inguinal, and mandibular)						
Group:	Control		KLH/CYP		KLH	
Sex	M	F	M	F	M	F
Number Examined	47	47	46	43	47	48
Lymph nodes (pooled)						
Depletion, lymphoid, generalized (minimal to mild)	0	0	7	6	0	3
Depletion, lymphoid, germinal center						
-minimal	0	0	0	0	1	0
Hyperplasia, lymphoid, germinal center						
-minimal	0	0	0	0	5	2
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

	Day 28	Day 78
Lymphocytes	-74%[a]	-70%[a]
T Cells	-77%[a]	-78%[a]
CD4+	-70%[a]	-73%[a]
CD8+	-87%[a]	-84%[a]
B Cells	-62%	-27%
NK Cells	-92%[a]	-87%[a]
KLH IgM	-72%[a]	-46%[a]
KLH IgG	-84%[b]	-60%[a]

[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^{neg}) and plasmacytoid (CD11c-/CD123^{+})
  - 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 *in vitro* human studies in cases with mechanistic relations

Messaoudi I et. al. (2011) *Antioxid Redox Signal*
Stebbings R et. al. (2007) *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

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