# Immuno-Safety Tools Training Course: TDAR assessments

HESI IMMUNO SAFETY COMMITTEE

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# Objectives

This course will provide:

- An overview of the TDAR assessments within the preclinical drug development safety testing context, including regulatory guidelines.
- Considerations for the in-vivo experimental design for inclusion within toxicology safety studies.
- Case studies for the interpretation of the data in the context of safety or efficacity assessments.

# Definitions

- **T-Cell Dependent Antibody Response (TDAR):**
  - Used to evaluate adaptive immune function (humoral) to an antigen
  - Requires T cell implication to elicit an antibody response to the antigen

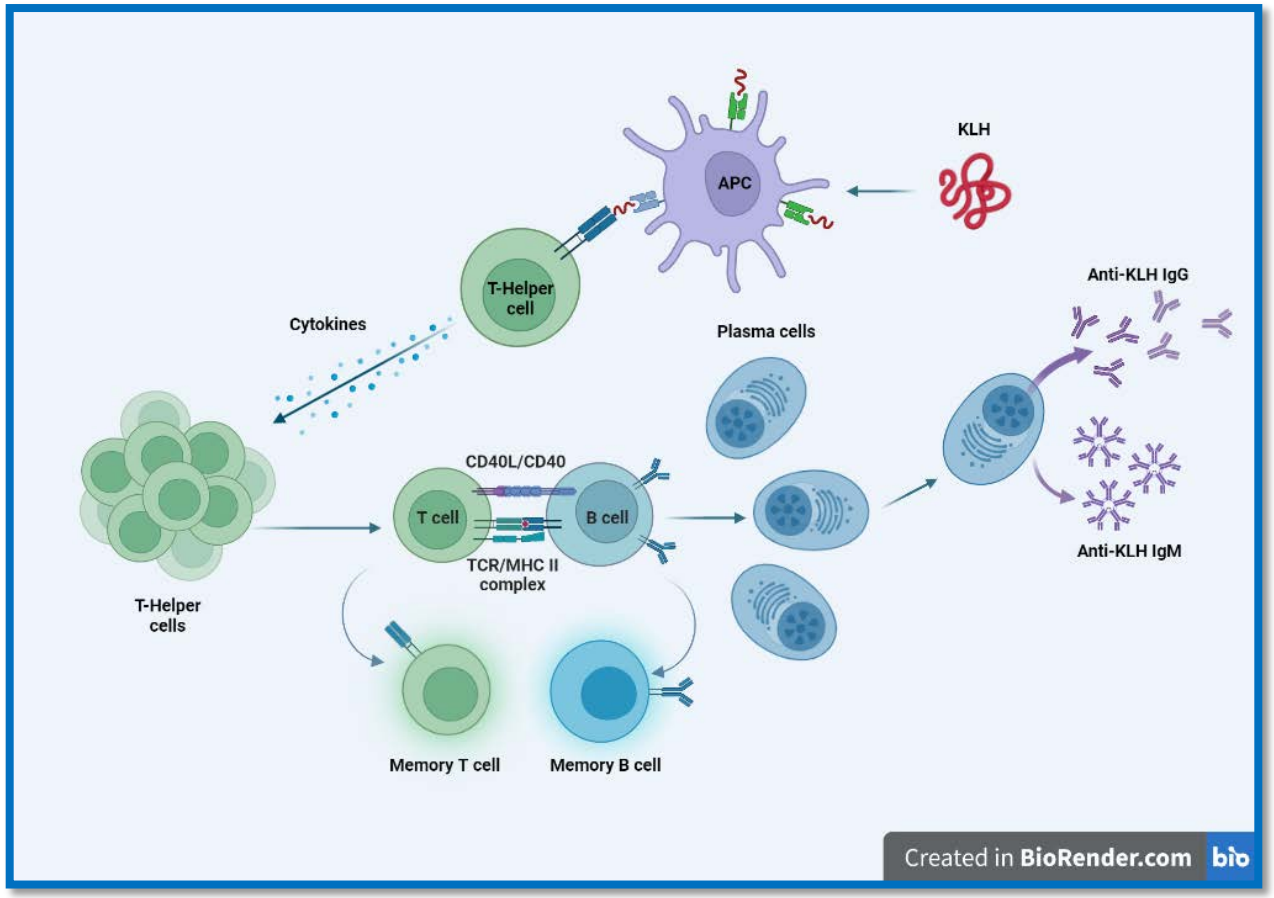
- **T-Cell Independent Antibody Response (TIDAR)** *(Not discussed further)***:**
  - Used to evaluate immune function to T-independent antigens (i.e. polysaccharide)
  - Can elicit an antibody response without the implication of T-cells

# TDAR: Principal

Antigen administration (ie. KLH) elicits TDAR, which requires multiple various immune cell types and functions:

1. Antigen presenting cells (APC):
   - Uptake of the antigen
   - Antigen processing to peptides
   - Presentation of peptide via MHCII to naïve Th cells
2. T helper cells (Th):
   - Activated Th cells proliferate
   - Activated Th cells interact and activate B cells
   - Memory T cells are generated
3. B cells:
   - Activated B cells differentiates into plasma cells producing antibodies (IgM)
   - Class switch to IgG occurs a few days later
   - Memory B cells are generated
4. Antigen specific antibodies:
   - Measured by immunoassays in serum
   - IgM: 5-7 days post immunization
   - IgG: 1 to 2 weeks post immunization

*All phases are mediated by receptor / ligand interactions and cytokines*




*A recall (secondary) response can be evaluated by repeating the antigen administration*

# TDAR: Preclinical Safety Testing

For pharmaceuticals (small molecule and biological), the inclusion of the TDAR assessment is based on a weight-of-evidence approach.

ICH S6(r1):
PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS

**Biologic**
(e.g: peptide, virus mAb, recombinant protein)

Standard Toxicity Studies
(2 species)
Immunogenicity assessment

**Additional**
**Immunotoxicity studies** as Appropriate based on causes for concern

**Causes for concern**

- Pharmacological properties
- Standard toxicity studies flags:
  - Haematology
  - Pathology- alteration to weights or appearance of immune organs
  - Serum globulin changes
  - Increased Infections/tumors
  - Changes to lymphocyte subsets determined by flow cytometry
  - Is the drug retained at high concentration in organs or cells of the immune system
- Intended patient population
- Known drug class effects
- Drug disposition
- Clinical information

ICH S8:
IMMUNOTOXICITY STUDIES FOR HUMAN PHARMACEUTICALS

**Small molecule**
(NCE)

Standard Toxicity Studies
(2 species)

**Additional**
Immunotoxicity studies as Appropriate based on causes for concern:
***TDAR,*** *Lymphocyte Phenotyping, NK cell cytotoxicity, Neutrophil/Macrophage function*

ICH S11: NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC PHARMACEUTICALS (refer to S8, for animals at ages of certain level of organ system maturity (e.g. TDAR after PND 45)

FDA: Nonclinical Safety Evaluation of the Immunotoxic Potential of Drugs and Biologics (Draft 2022) – Refers to the ICH S8 and S6(R1) guidelines, and addressed both immunosuppression and immunostimulation testing (e.g. TDAR).

Other regulatory agencies (i.e. EPA) also refer to the use of TDAR (KLH or SRBC) for immunotoxicology testing.

# Immunomodulation considerations

| Areas | Comments | Biologics | Small Molecules |
|---|---|---|---|
| TDAR **Immunosuppression** | **Unintended or intended consequence of drug administration** | ✓ | ✓ |
| Immunogenicity | Ability of a drug to elicit an immune response | ✓ | *N/Ap* |
| hypersensitivity/allergy | Involves IgE (Type 1)<br>IgG, IgM (Type II and III)<br>Activated T cells (Type IV) | ✓ | ✓<br>*Not applicable for Types II and III* |
| Autoimmunity | Difficult to predict | ✓ | ✓ |
| TDAR **Immunostimulation** | **Non-specific stimulatory effect of a drug or intended effect of a drug to stimulate the immune system** | ✓ | ✓ |

# TDAR:
# Preclinical Study Design considerations

## Antigens

Keyhole limpet hemocyanin (KLH)
- Most used antigen in all preclinical species
- Commercially available without adjuvant

Tetanus toxoid (TT)
- Mostly used as second antigen to KLH
- Commercial vaccine contain adjuvant
- Animals can have been previously immunized (NHP) – check health records

Hepatitis B surface antigen (HBsAg)
- Mostly used as second antigen to KLH
- Commercial vaccine contain adjuvant

Sheep red blood cells (SRBC)
- Mostly used for chemical testing in rats (EPA)

## Animals

Species
- Mice
- Rat
- Dog
- Mini-Pig
- Non-Human Primates
- Others, as required

Age

Animals should have a mature immune responses prior to immunization (e.g. Rat ≥ 49 days (post-partum)

Number of animals
- Rodent (10/sex/group)
- Dog/NHP (3-5 /sex/group)

## Intended Purpose

Immunosuppression:
- Typically, safety evaluation
- Antigen timing should be based on the WoE (Phamarcology and/or timing of previously observed effects)
- Antigen dose should be high enough to induce a robust antigen specific antibody response (IgM and IgG) and show decreases with a positive control during the validation

Immunoenhancement:
- Typically, pharmacodynamic evaluation
- Antigen timing can be concurrent to first test item dose
- Secondary response often included (> 3 weeks after the first immunization)
- Antigen dose should be low enough (suboptimal) to induced detectable, but low antigen specific antibody responses (IgM and IgG), to allow for enhanced responses to be detectable

WoE: Weight of Evidence

# TDAR:
# Preclinical Study Design considerations

## Serum collection

- Once prior to each antigen administration
- Peak IgM responses (at least 2 timepoints)
  - 5-10 days post each antigen administration
- Peak IgG responses (at least 2 timepoints)
  - 14-21 days post first antigen administration
  - 7-21 days post second antigen administration
- Serum stored at -80° until analysis
- Stable multiples months

## Analysis

- Antibodies specific to the antigen are measure by plate-based (i.e. ELISA/ECL) or flow cytometry-based assays (CBA)
- LBA assays can detect isotype specific antibodies to the antigen used (i.e. IgM or IgG)
- Antigen responses can also be measured using plate-forming assays (PFA), when using SRBC as antigen
- Assay are either developed internally, although some kits are available in some species
- Antibodies levels are reported as concentrations (ng/mL) relative to a reference material, as titers (which does not require reference material), or as plate-forming colonies (PFC)
- When validating the methods, critical parameters include:
  - Sensitivity, specificity, selectivity, parallelism (or linearity), prozone, precision, robustness and serum stability
- Proper new lot reagent qualification procedures (SOP) should be in place to maintain the performance of the assay over time

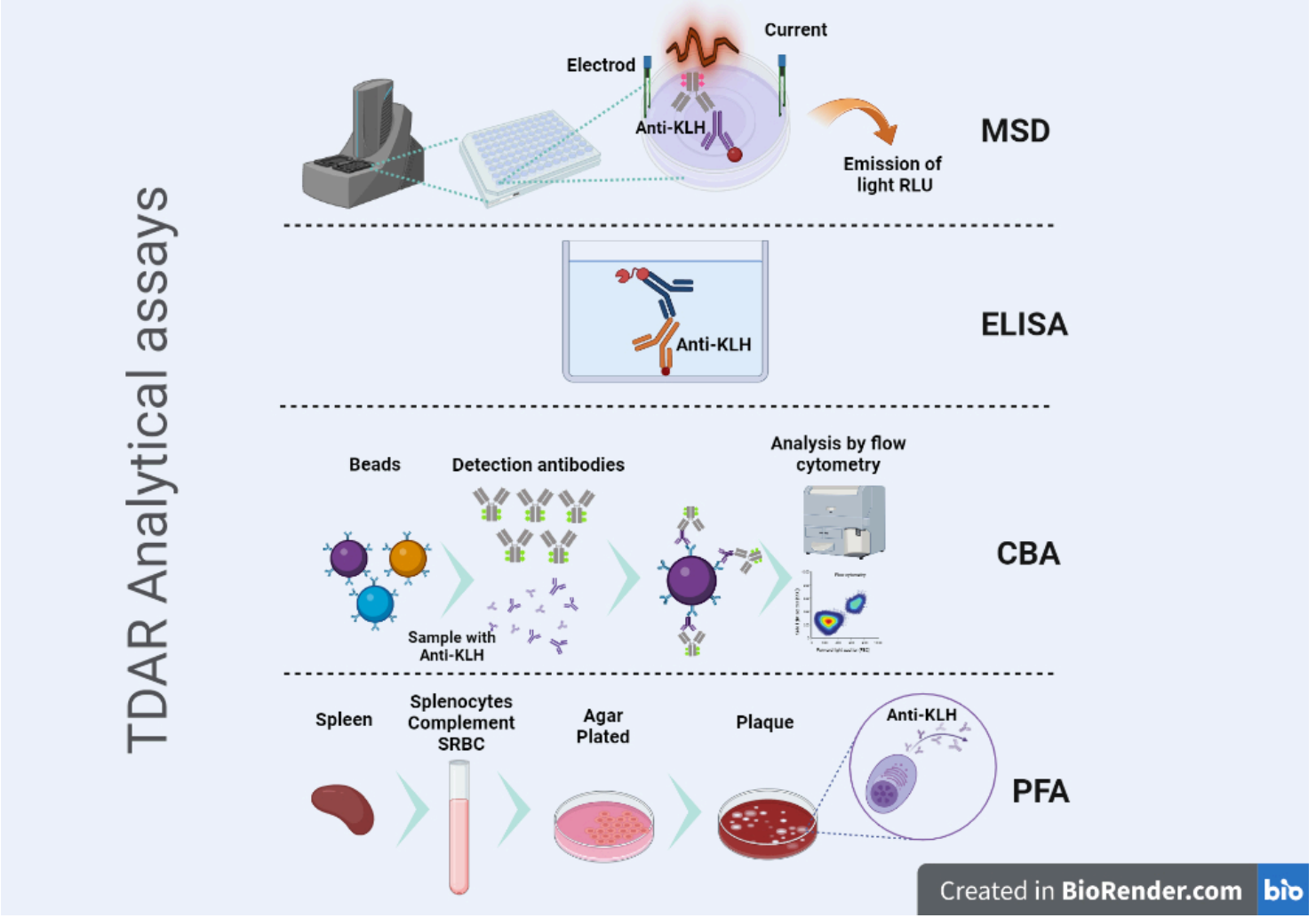
WoE: Weight of Evidence

# TDAR: Analytical methods

- **ELISA or ECL**: The antigen in coated to a plate, the test samples/controls are added, followed by the detection using species specific anti-isotype capture antibodies. The capture antibody is labeled according to the type of assay readout used. The antibody levels (µg/mL or titer) are measured using spectrophotometer (ELISA) or electrochemiluminescence (ECL).

- **CBA**: Some groups have reported using flow cytometry-based assays via antigen covered beads. instead of using an ELISA/ECL plate-based assay

- **PFA**: Immunized animal spleen cells in Agar solution containing SRBC and complement is poured in a petri dish and incubated (~3 hours). Clear areas (plaques) will be formed where B cells secreting ant-SRBC antibody capable of inducing complement- mediated lysis of SRBC. Data is reported as the counts of plaque forming colonies (PFC).



# In-life TDAR validation

- Validation is important to:
  - Select biologically relevant antigen dose levels
  - Understand the kinetics for optimal sample collection timing for each antibody isotypes
  - Understand the inter-animal variability, to determine the minimal number of animals
  - Verify the general performance of the analytical methods pre- and post-immunization
- During the validation, the animal models should be tested to with a known immunosuppressant as a positive control for verification of the model sensitivity.
- When available, the animal models can be tested to with a known immunostimulant as a positive control to select a suboptimal antigen dose level able to detect an increased response to the antigen.

**The validation should demonstrate that the model is appropriate for it's intended use.**

# Example of Rodent TDAR design

Standard KLH TDAR rat study (28-day study + 28-day recovery)

- KLH (300 µg, i.v.) on Day 15 on 10 main animals/sex/group (~ 2 weeks prior to end of study)
- KLH (300 µg, i.v.) on Day 42 on 10 recovery animals/sex/group (~ 2 weeks prior to end of study)
- Serum for anti-KLH IgM collected 5 - 7 days post immunization
- Serum for anti-KLH IgG collected 7 - 14 days post immunization
- Anti-KLH antibodies detected by immunoassay (e.g. ELISA, ECL)

| Groups | Main study | Recovery | TDAR subset | |
|---|---|---|---|---|
| | | | Main study | Recovery |
| 1_Vehicle control | 10/sex | 10/sex | 10/sex | 10/sex |
| 2_Low Dose | 10/sex | 10/sex | 10/sex | 10/sex |
| 3_Mid Dose | 10/sex | 10/sex | 10/sex | 10/sex |
| 4_High dose | 10/sex | 10/sex | 10/sex | 10/sex |

- Anti-KLH IgM and anti KLH-IgG antibody detection by ELISA (Main study animals receive antigen during the dosing period, then recovery animals receive antigen during the recovery period)
- For longer study (≥ 2months, a secondary response can be included)

# Example of NHP TDAR design

Standard Cynomolgus monkey study (28-day study + 28-day recovery) – **For immunosuppression**

- KLH (**10 mg, s.c.**) on Day 15 for main study animals (~ 2 weeks prior to end of study)
- KLH (**10 mg, s.c.**) on Day 42 for recovery animals (~ 2 weeks prior to end of study)
- Serum for anti-KLH IgM collected 5 - 7 days post immunization
- Serum for anti-KLH IgG collected 7 - 14 days post immunization
- Anti-KLH antibodies detected by immunoassay (e.g. ELISA, ECL)

| Groups | Main study | Recovery study |
|---|---|---|
| 1_Vehicle control | 3/sex | 2/sex |
| 2_Low Dose | 3/sex | 2/sex |
| 3_Mid Dose | 3/sex | 2/sex |
| 4_High dose | 3/sex | 2/sex |

- Anti-KLH IgM and anti KLH-IgG antibody detection by ELISA (Main study animals receive antigen during the dosing period, then recovery animals receive antigen during the recovery period)
- For longer study (≥ 2months, a secondary response can be included)

# Reporting Considerations

- **Individual values:** Reported for each isotype and each timepoints
- **Group mean or median, and/or difference from control**: Used to describe trends
- **SD or Ranges**: Reported to describe variability
- **Area under the curves (AUC)**: Useful to calculate overall responses amongst all timepoints
- **Combining sexes**: Increases “n” value when no difference in exposure between sexes
- **Graphs**: Representation of the distribution within groups is useful to visualize trends (e.g. scatter plots)
- **Log transformation**: Used to stabilize the inter-animal’s variability (Lab specific)
- **Historical data**: Used to monitor overall immunization performance
- **Values below the detectable assay limits**: Should be given an arbitrary value for the purpose of the statistical analysis (i.e. LLOQ/2, MRD/2)

# Interpretation Considerations

The TDAR interpretation is based on:

- Comparison of the antibody levels, kinetics and class-switching from dosed group with the relevant vehicle control group
- Evaluation of changes based on trends within groups (effect can be treatment related while antibodies levels remain within range of the control group)
- Expectations for high inter-animal variability (incidence of high vs. low responders per group)
- Overall immune response (i.e. difference at an isolated timepoint may not be of concern)
- Context of the whole study data (i.e. biomarkers, immunophenotyping, clinical pathology and/or pathology data available)
- Impact of stress and/or secondary effects on the immune system can show various type of abnormal responses (i.e.  Effects on T cells, B cell, APC, cytokines, etc)
- Low incidence of pre-existing antibodies to some antigens in some species is expected (increases post immunization should still be detected  in these animals)
- *TDAR data can affect adversity levels in some cases, since it is a functional assessment*

***TDAR results are used by the regulated industry, depending on the nature of the compound, how it is regulated (environmental chemical or pharmaceutical) and its intended use (immunomodulation or not)***

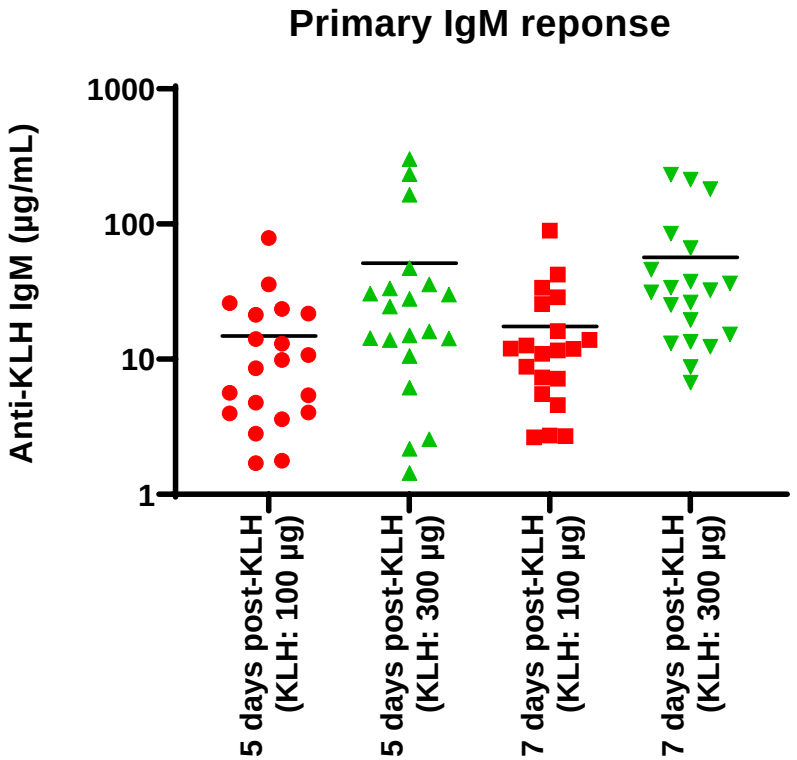
# Case study 1: Rat TDAR validation

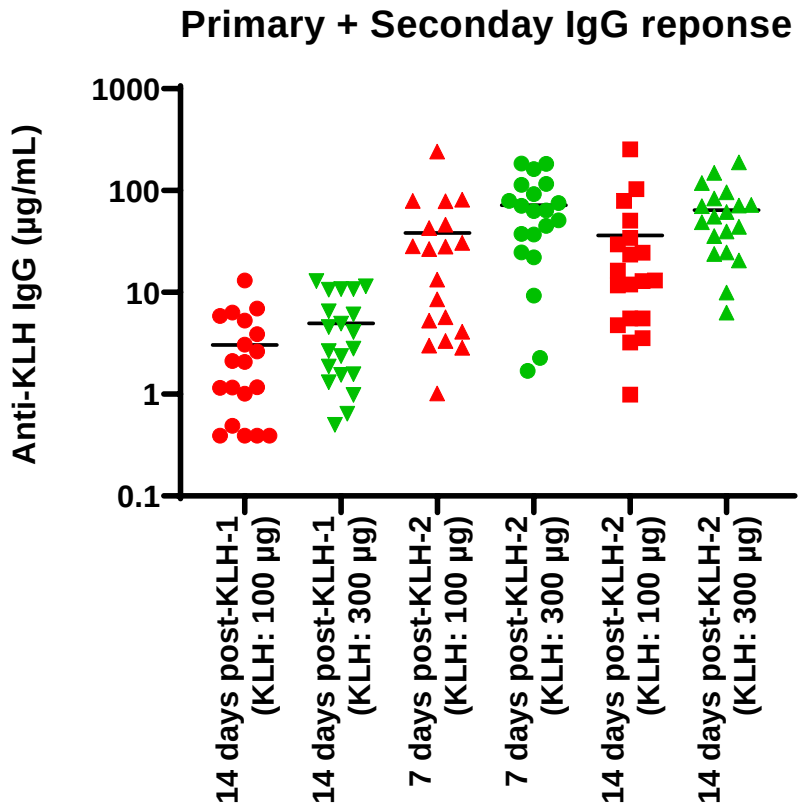
- **KLH**: Administered *i.v.* on Day 1 and Day 15
- **Cyclophosphamide**: Administered *i.p.* daily, starting 4 days prior to KLH administration
- Blood was collected (Serum): 5, 7, 10 and 14 days after each KLH administrations
- Anti-KLH IgM: Measured pre-KLH, 5 and 7 days post KLH
- Anti-KLH IgG: Measured pre-KLH, 10, 14 post KLH-1, and 5, 7, 10 and 14 days post KLH-2

| Groups | KLH dose (µg/dose) | PC dose (mg/kg/day) | Sprague Dawley rats | |
|---|---|---|---|---|
| | | | Males | Females |
| 1_Low KLH | 100 | 0 | 10 | 10 |
| 2_High KLH | 300 | 0 | 10 | 10 |
| 3_Low KLH + Cyclophosphamide | 100 | 10 | 10 | 10 |
| 4_High KLH + Cyclophosphamide | 300 | 10 | 10 | 10 |

# Case study 1: Rat TDAR validation

## KLH dose levels comparison




***All animals dosed with cyclophosphamide had undetectable anti-KLH IgM and IgG antibody levels after both KLH injections (completed inhibition of TDAR)***

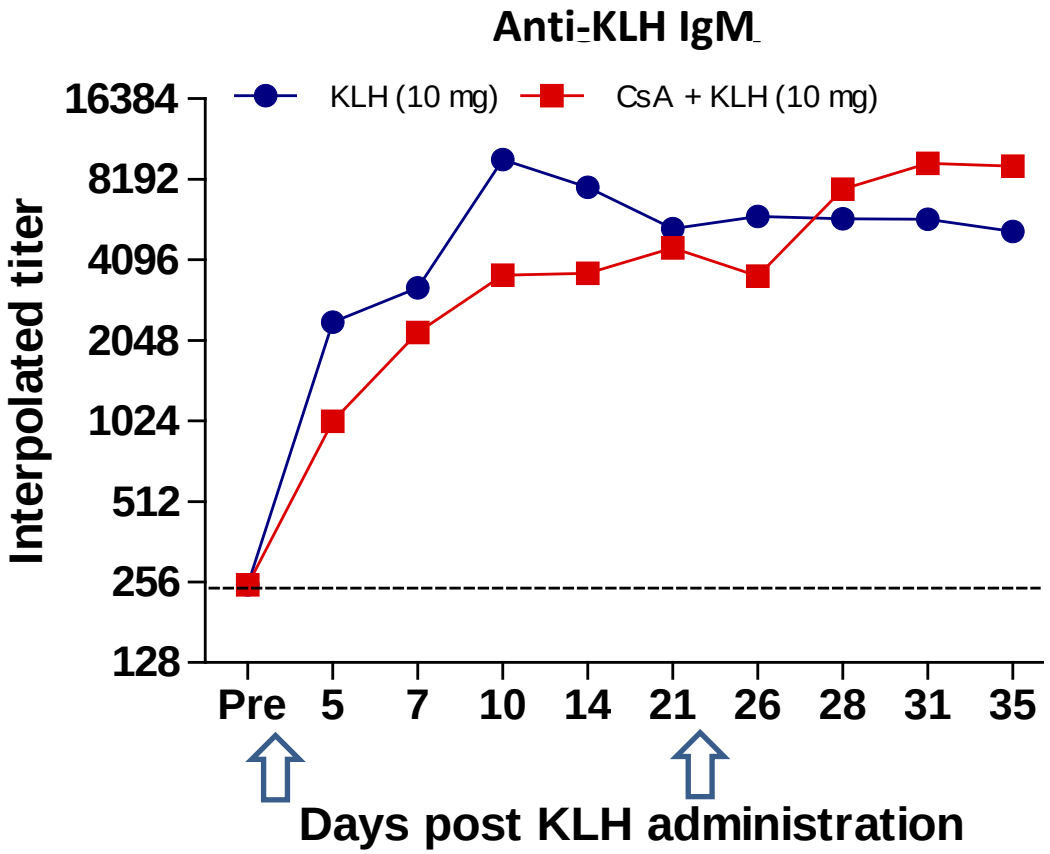
# Case study 2: NHP TDAR validation

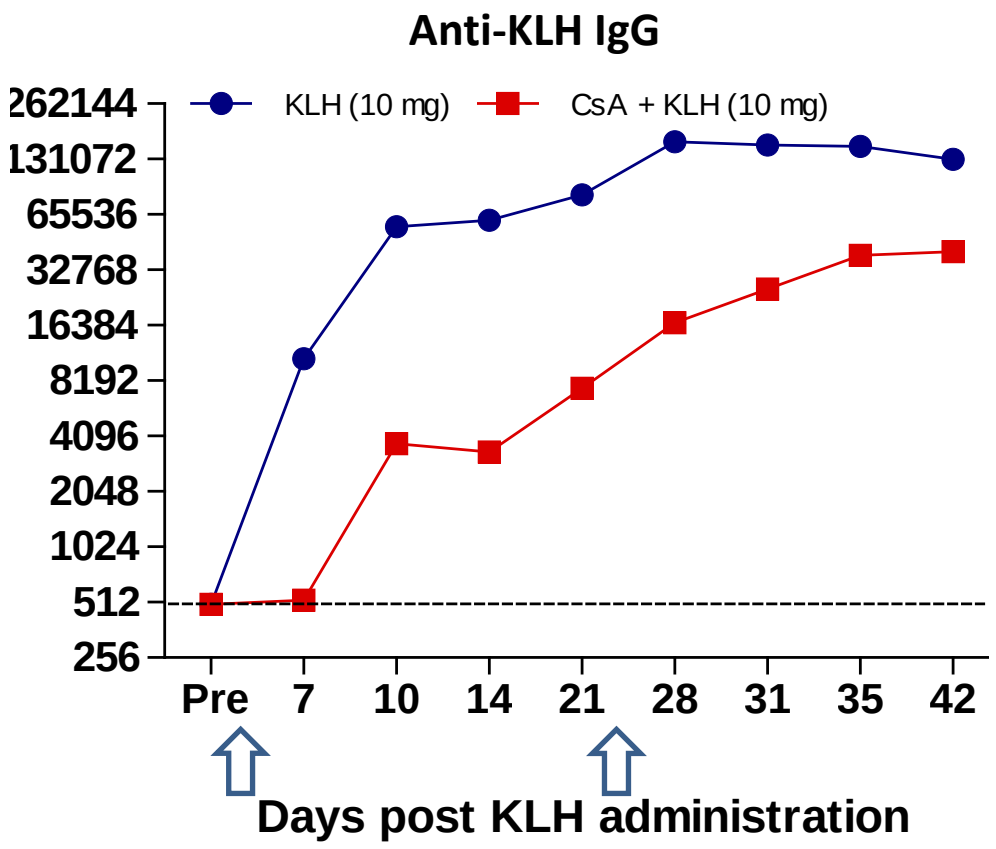
- **KLH**: Administered *s.c.* on Day 1 and Day 21
- **Cyclosporine**: Administered *oral gavage* daily, starting 7 days prior to KLH administration
- **mAbx**: Administered *i.v.*, starting 7 days prior to KLH administration
- Blood was collected (Serum): 5, 7, 10, 14 and 21 days post each KLH administrations
- Anti-KLH IgM: Measured pre-KLH, 5, 7, and 10 days post each KLH
- Anti-KLH IgG: Measured pre-KLH, 7, 10, 14 and 21 days post each KLH

| Study Phase | Groups | KLH dose (mg/dose) | PC dose (mg/kg/dose) | Number of animals (Sexes combined) |
|---|---|---|---|---|
| Immunosuppression Phase | 1_KLH | 10 | 0 | 4 |
| | 2_KLH + Cyclosporine | 10 | 37.5 *(BID)* | 4 |
| Immunostimulation Phase | 3_KLH | 1 | 10 | 4 |
| | 4_KLH + mAb X | 1 | 10 *(every 2 weeks)* | 4 |

# Case study 2: NHP TDAR validation

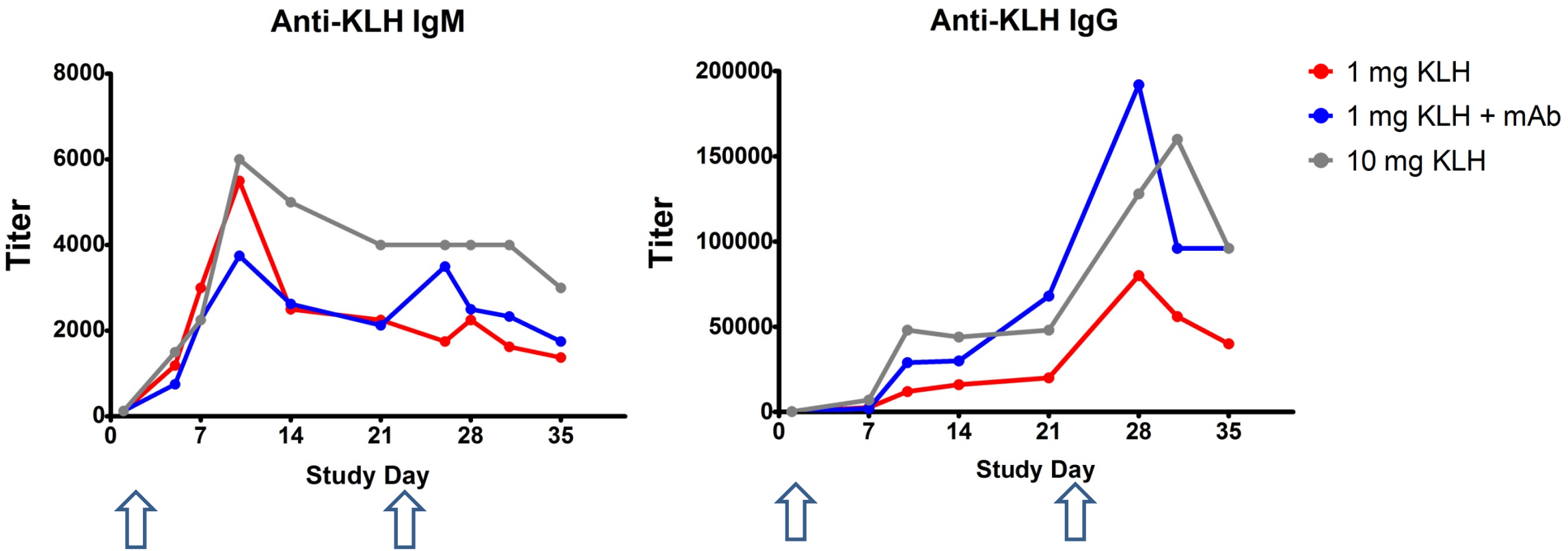
## Immunosuppression positive control



# Case study 2: NHP TDAR validation

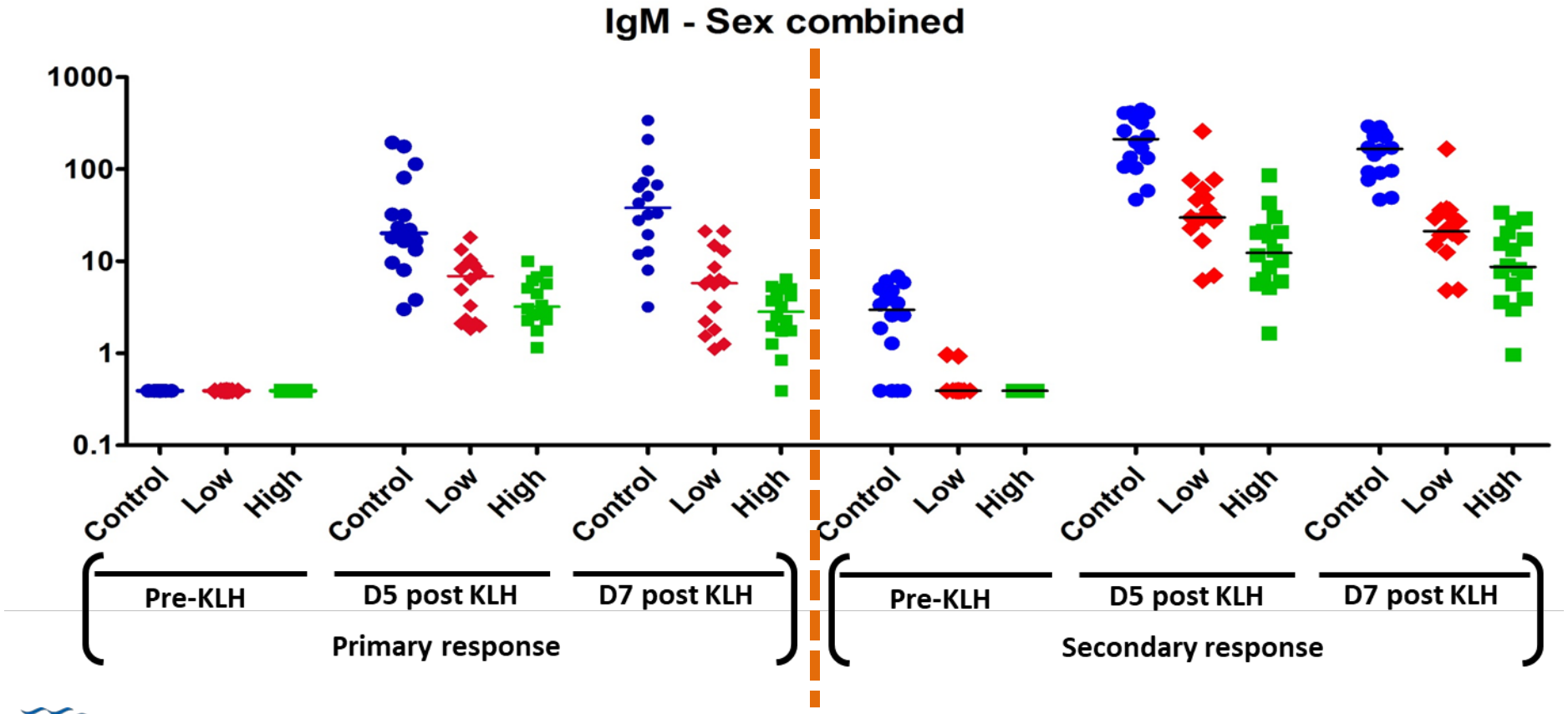
## Immunostimulation positive control



# Case study 3: Rat TDAR interpretation

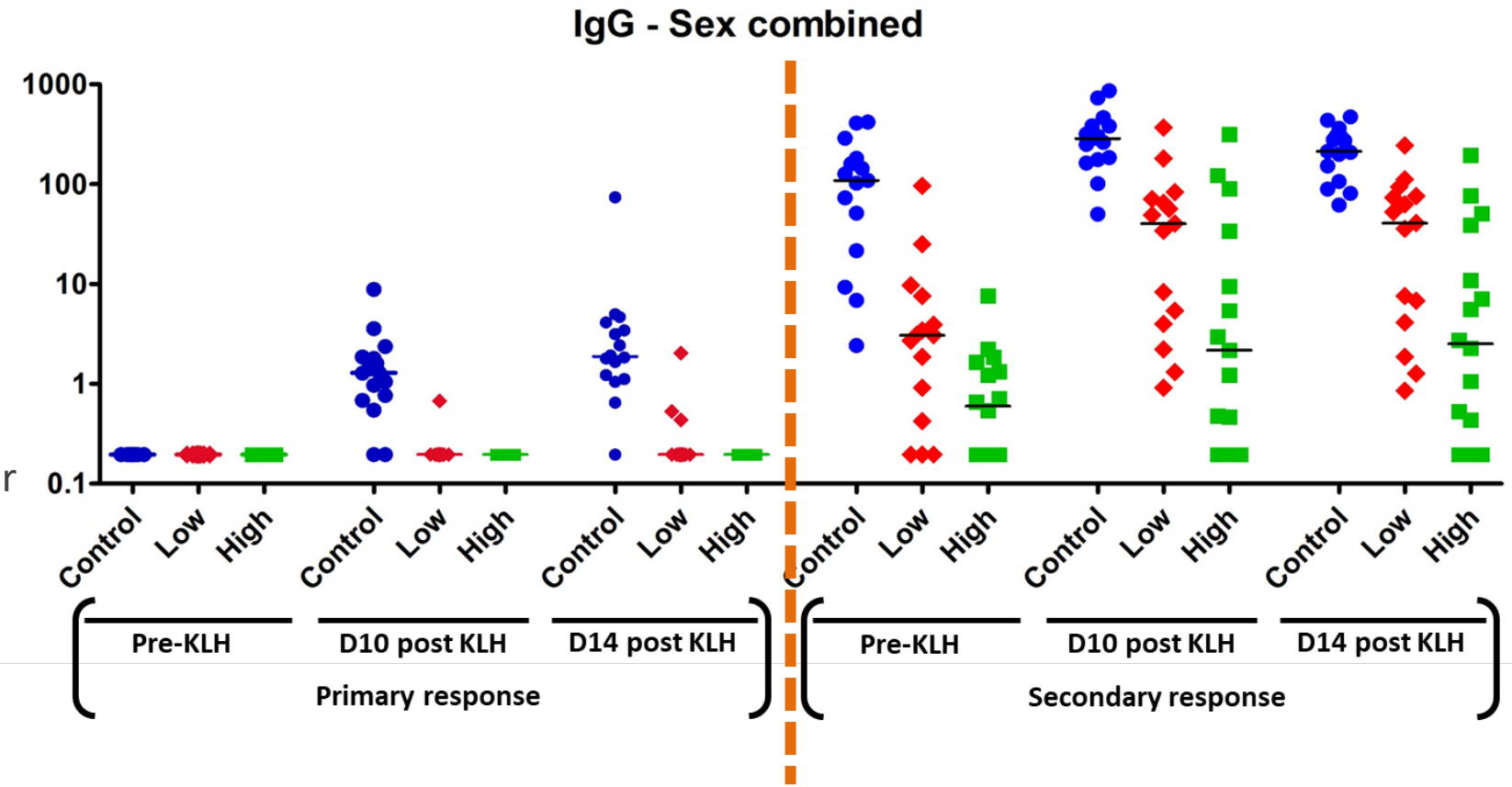
**In this study, KLH was administered (i.v.) at on Day 28 and Day 70 (42 apart)**

- All dosed animals had a detectable anti-KLH IgM antibody response
- However, a dose-dependent decrease in the antibody levels was observed (Primary and secondary)



# Case study 3: Rat TDAR interpretation

- **After the first KLH administration:** Although all dosed animals had detectable anti-KLH IgM levels, the anti-KLH IgG antibodies were undetectable for most dose animals (Class switching affected)
- **Prior to the second KLH administration:** Anti-KLH IgG antibodies were detected, indicating class switching occurred between 14 to 42 days post KLH.
- **After the second KLH administration**: The anti-KLH IgG levels increased for most dose animals, indicating some recall response, although there was a dose-dependent decreases in the response.



# Conclusion

- TDAR assessments only needs to be conducted if there is a cause for concern
- TDAR can be used for immunosuppression or for immunostimulation assessment
- TDAR can be included in standard toxicology studies (Same animals for large animals, satellite animals for rodents)
- TDAR is available for most preclinical species
- Analytical methods allow detecting IgM and IgG isotype specific to the antigen
- Different antigen can be used
- TDAR data needs to be interpreted with the complete toxicology data set and taking into account biological variability

# Reference

Regulatory Toxicology and Pharmacology 69 (2014) 7–21

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## The T-cell-dependent antibody response assay in nonclinical studies of pharmaceuticals and chemicals: Study design, data analysis, interpretation

Hervé Lebrec [a,*], Brigitte Molinier [b], Darrell Boverhof [c], Mark Collinge [d], Wendy Freebern [e], Kristin Henson [f], Daniel T. Mytych [g], Hans D. Ochs [h], Ronald Wange [i], Yung Yang [j], Lei Zhou [g], Joshua Arrington [k], Marie Soleil Christin-Piché [l], Jacintha Shenton [m]