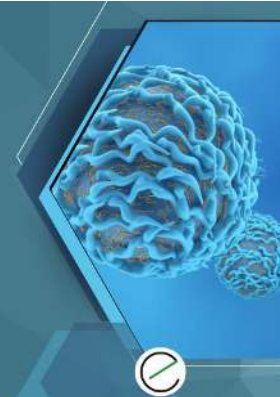


# IMMUNOTOXICITY ASSESSMENT

## ADDRESSING CHALLENGES AND ADVANCING METHODOLOGIES



## WORKSHOP FLASH REPORT

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### Background and Objectives

Immunotoxicology is gaining increasing regulatory and scientific attention due to its relevance in chemical safety assessment and its inclusion in key OECD testing guidelines. However, current approaches to evaluating immunotoxicity—especially developmental immunotoxicity (DIT)—face challenges in sensitivity, specificity, and regulatory harmonization. The ECETOC Workshop on Immunotoxicity Assessment was convened to address these challenges and explore opportunities to advance methodologies, including the integration of *in vivo* and *in vitro* endpoints, and the development of tiered testing strategies. Held in Brussels on July 9–10, 2025, the workshop brought together experts from regulatory agencies, industry, CROs, and academia to review the current regulatory landscape and scientific practices in immunotoxicity assessment, discuss practical implementation of immunotoxicity endpoints in existing studies, explore novel *in vitro* methodologies and mechanistic insights, and identify gaps and opportunities for future guidance and task force activities.

### Event Participation

Both days of the workshop were conducted in a fully hybrid format, with options for online and onsite participation. The event was attended by over 70 participants, 30 of them in person. Attendees included representatives from industry, ECHA, EFSA, NIEHS, academia, and leading CROs. The first day focused on speaker presentations while the second day focused on breakout discussions. On day two online and onsite participants were divided into three groups each addressing the same topics in three distinct sessions. Key inputs from the breakout groups were later presented in a final plenary session.

### Next Steps

ECETOC will continue the work presented at this workshop through the establishment of a new *Immunotoxicity Assessment* Task Force. The primary deliverable will be a peer-reviewed publication based on the workshop discussions and subsequent task force findings, accompanied by additional dissemination activities. The Task Force will form part of the ECETOC's immune- and neurotoxicity-related activities alongside the ECETOC *Scientific Perspective on In Vivo DNT Testing* Task Force.

## Scientific Presentations and Key Themes

Day 1 speaker presentation summaries. The summaries provided are the result of an independent expert review of the presentation and may not fully reflect the original authors' intent.

Event page: <https://www.ecetoc.org/event/immunotox/>

*Please note that some workshop slides may be unavailable or might have been modified to remove confidential information. The views and opinions expressed during the workshop are those of the participants, and do not necessarily reflect the views or policies of their employers and affiliation.*

### Immunotoxicity from a regulatory perspective – Available [here](#)

Melanie Flach – BASF, provided a comprehensive overview of OECD guidelines relevant to immunotoxicity, highlighting that while skin sensitization is well-covered, immunosuppression and DIT are only partially addressed. Autoimmunity and immunostimulation remain largely untested in regulatory frameworks. She explained that triggers for immunotoxicity testing vary across [REACH](#), [ICH S8](#), and [US EPA](#), with differences in interpretation of organ weight changes, histopathology, and hormonal effects. Divergent interpretations of adversity (e.g., [EFSA vs. EMA](#)) on TH17 cell changes from BPA exposure was discussed as an example of divergent regulatory interpretations of adversity, underscoring the need for harmonized assessment strategies.

#### Opportunities:

- Develop harmonized tiered testing strategies.
- Development and validation of New Approach Methodologies (NAMs).
- Focus on biological and human relevance of functional changes.

### REACH Implementation and DIT Cohort Triggers – Unavailable

Laura Rossi from ECHA detailed how immunotoxicity is assessed under REACH, with emphasis on the use of OECD 443 cohort 3 for DIT. She noted that ECHA has requested approximately 75 studies with DIT cohorts, primarily triggered by thymus, spleen, and hematological findings. Sex hormone activity and read-across data are used sparingly. The recent update to OECD TG 443 includes harmonized TDAR protocols and IgG measurement, aiming to improve sensitivity and reproducibility.

#### Regulatory Gaps:

- Adult animals' data may not be sensitive enough to trigger DIT.
- Need for validated NAMs and readiness criteria.
- International collaboration (e.g., CAAT, PARC) is key.

For further information see:

- EOGRTS review with satellite project: [EOGRTS review project](#)

- Update of [Test No. 443: Extended One-Generation Reproductive Toxicity Study | OECD](#)
- KARC – update [da33bf25-2b75-1fe9-c308-53043f9b9a28](#)
- CAAT: [DIT Alternatives Group - The Johns Hopkins Center for Alternatives to Animal Testing](#)
- [PARC Projects | Parc](#)

### Practical considerations for the inclusion of additional immunotoxicity endpoints in existing regulatory studies – Available [here](#)

Kirsten Hartman-Van Dycke from Charles River discussed the feasibility of adding immunotoxicity endpoints to regulatory studies next to standard endpoints: organ weights, histopathology, haematology and TDAR assay. She explained that while many assays are available, there are various challenges in including these endpoints in classical regulatory studies. These challenges include the logistics for cell culture, blood volume and housing duration. There are only a few examples of new approaches which were validated under GLP or supported by historical control data, both of which are essential to interpreting results. She emphasized that dose selection and stress-related confounding effects are critical considerations when interpreting immune endpoints.

Suggested immunotoxicity endpoints were highlighted, which included those which are currently standard (e.g. histopathology of lymphatic organs, including bone marrow cellularity and weighing lymphoid organs), and enhancement of read outs in blood and spleen.

### Overview of *in vitro* methodologies for immunotoxicity assessment – Available [here](#)

Emanuela Corsini – University of Milan, emphasized that the current landscape of *in vitro* methods provides a feasible foundation for exploring the immunosuppressive potential of chemicals. She advocated for a focus on endpoints that reflect the functional integrity of the immune system, rather than relying solely on structural or cytotoxic markers.

Given the complexity of immunotoxicity and the breadth of *in vivo* data typically required to classify a compound as immunotoxic, Corsini suggested that multiple *in vitro* assays will be necessary. These should be incorporated into Integrated Approaches to Testing and Assessment (IATA) to enhance predictive accuracy and regulatory relevance: several *in vitro* assays targeting different immune cell types and functions is likely to yield better predictive performance than any single assay alone. These could include whole blood assays, PBMC-based tests, and the validated IL-2 luciferase assay ([OECD TG 444A](#)).

Adverse Outcome Pathways (AOPs), key characteristics of immunotoxicants, and computational tools as complementary approaches for hazard identification were also highlighted. The OECD Detailed Review Paper ([DRP, OECD 2022](#)) “In Vitro Test Addressing Immunotoxicity with a Focus on Immunosuppression” discusses promising models for developmental immunotoxicity and was highlighted as a key document for reference.

She emphasized the need for more chemicals to be tested, standardized protocols, and multi-omics for biomarker discovery.

**Gaps identified for *in vitro* testing:**

- Larger datasets are needed to distinguish general immunosuppression biomarkers from those specific to certain chemical classes.
- Assays should target a broader range of immune cell types.
- Indirect immunotoxicity must be considered, potentially through advanced models such as 2D/3D cultures, that better mimic physiological conditions.
- A deeper mechanistic understanding is required to define clear toxicological endpoints.
- Protocol standardization and the development of high-throughput instruments are essential for reproducibility and scalability.
- The use of serum-free media should be prioritized to reduce variability and reliance on animal-derived components.
- Finally, international acceptance and harmonization of these methods will be critical for regulatory uptake.

**Transition from *in vivo* to *in vitro* immunotoxicity prediction: methodology, practical insights, and *in vivo* implementation opportunities – Available [here](#)**

Raymond Pieters from IRAS, and the University of Applied Sciences in Utrecht, presented a mechanistic perspective on immunotoxicity, emphasizing the importance of innate immunity and immune cell migration in understanding immune-mediated effects. He introduced Drug-Induced Liver Injury (DILI) as a relevant model for studying immune-related toxicity, particularly in the context of idiosyncratic responses. For example, trovafloxacin a fluoroquinolone antibiotic, was shown to impair immune cell migration by suppressing ATP release through the pannexin-1 channel, interfering with immune cell recruitment and contributing to cholestatic liver toxicity.

He further discussed how kinase inhibition, particularly of ROCK and MLCK, affects immune cell migration and contributes to immune dysregulation. These pathways are critical for cytoskeletal dynamics and cellular movement, and their disruption can lead to impaired immune surveillance and response.

Pieters emphasized that personalized factors, such as genetic predisposition and concurrent infections, significantly influence immune responses and susceptibility to immunotoxic effects. This context-dependency underscores the complexity of immunotoxicity and the limitations of one-size-fits-all models.

**Recommendations:**

- Use of *in vitro* assays to de-risk compounds before proceeding to animal testing.
- Migration and signalling pathways as critical endpoints for immunotoxicity assessment because immune effects are multifactorial and context-dependent, requiring integrated approaches for accurate hazard identification.

**Experimental and mechanistic *in vitro/in vivo* evaluation of immune effects – Available [here](#)**

Marc Pallardy – University Paris-Saclay. The presentation focused on the challenges and approaches for evaluating immune effects in experimental and mechanistic studies, both *in vitro* and *in vivo*.

The speaker began by emphasizing how certain pharmaceuticals have provided clear mechanistic insights into immunosuppression due to their molecular targets being well understood. These examples illustrate how knowing the target simplifies testing strategies. However, for many chemicals, such as PFAS or Bisphenol A, the targets remain unknown, making evaluation more challenging and results difficult to interpret.

The review continued with established *in vivo*-approaches, such as OECD tiered testing and the T-cell Dependent Antibody Response (TDAR), which are highly predictive for immunosuppressive effects. Also discussed was the evolution of immune monitoring technologies, including multiplex cytokine assays, advanced flow cytometry, and single-cell transcriptomics, which offer precise characterization of immune cell populations and functions. These tools, widely used in human studies, could be adapted to animal models to improve mechanistic understanding and maximize the read outs from these studies.

A key point was the identification of “key characteristics” of immunotoxicants, which can guide assay development. While it is impractical to create a test for every key characteristic, focusing on central functions, such as T-cell dependent responses, can provide broad predictive value. Generic assays are thus desirable. NAMs were also explored, particularly those using human cells, induced pluripotent stem cells, and 3D tissue models.

In conclusion, the immune system’s complexity does not make testing impossible, but it requires a combination of mechanistic insight, validated assays, and innovative technologies. Leveraging human-based models and decision trees built on diverse cell systems represents a promising direction for future immunotoxicology research and to maximize the information obtained from current OECD rodent assays.

**Application of alternative methods for immunotoxicity assessment – Available [here](#)**

Victor Johnson from NIEHS and Burleson Research Technologies, with support from Dori Germolec NIEHS, introduced a human whole blood-based closed culture system (TrueCulture) designed to assess all three major immune functions: innate, humoral, and cell-mediated immunity. This system offers a human relevant risk assessment and simplifies operation by requiring a single heat block, eliminating the need for traditional cell culture incubators.

The platform includes a comprehensive toolbox of assays, such as immunophenotyping, NK cell function, cytokine profiling, and antigen-specific T-cell activation. These endpoints can be collected from single donors, which reduces variability in data interpretation. Immunophenotyping further supports the contextual analysis of functional assay outcomes.

Johnson emphasized the value of using virus peptide pools to stimulate whole blood cultures, noting that combining this with serology data from the same donors provides meaningful insight into immune responsiveness. Case studies with dexamethasone and benzo(a)pyrene (BaP) demonstrated dose-dependent suppression of immune function. In particular, the BaP study highlighted the importance of

incorporating metabolic activation (e.g., S9 fraction) into in vitro systems to detect immunotoxicants that require bioactivation ([Johnson et al., 2025](#)).

#### Scientific Takeaways:

- Evaluate all three major immune functions: innate, humoral, and cell-mediated; as in in vivo hazard identification.
- Whole blood NAMs are highly translatable and portable.
- Individual donors serve as internal controls to reduce variability.
- Integration of multiple endpoints from a single sample enhances mechanistic insight.
- *In vitro* metabolism is essential for assessing certain compounds.

#### Development of a human in vitro TDAR assay – Available [here](#)

Lenie van den Broek (Mimetas) & Sofie Pattyn (IQVIA Laboratories, formerly known as ImmunXperts) described the development of a 3D lymph node-on-a-chip using cryopreserved Peripheral Blood Mononuclear Cells (PBMCs) in a microfluidic platform.

The model includes B cells, T cells, APCs, and endothelial cells with direct cell-cell interaction. The main challenges are donor variability and antigen penetration through the extracellular matrix, which can affect consistency and sensitivity. Despite these limitations, the model shows strong potential for applications in immunogenicity prediction, B-cell response modeling, and future integration into safety assessments for biologics and vaccines.

#### Scientific Takeaways:

- The model shows promise for mimicking recall responses.
- Donor variability and antigen penetration are key challenges.
- Potential future applications in immunogenicity prediction and B-cell assays.

#### DIT: A Perspective from Agrochemical Safety – Available [here](#)

Joseph Enriquez (Corteva) and Christian Strupp (Gowan) discussed the integration of Developmental Immunotoxicity (DIT) into agrochemical safety assessment. They explained that immunotoxicity in agrochemicals was previously deprioritized but is now regaining attention due to regulatory shifts.

Agrochemical safety assessments typically involve high animal use of up to 9,000 animals per data package. They advocated for embedding immunotoxicity endpoints, such as TDAR, within standard 90-day studies to reduce the need for standalone animal tests. They also recommended developing tiered testing strategies that are triggered by specific findings in early studies. Importantly, they suggested prioritizing immune suppression as a key consideration in carcinogenicity assessments, given its mechanistic relevance.

Scientifically, they concluded that immunotoxicity assessment in agrochemicals must strike a balance between regulatory requirements and scientific feasibility. While *in vitro* tools developed in the pharmaceutical sector may be adapted for agrochemical use, these methods require validation and regulatory acceptance. The speakers stressed that early and ongoing dialogue with regulators is essential to avoid unnecessary testing and to ensure that immunotoxicity endpoints are appropriately integrated into safety evaluations

**Scientific Takeaways:**

- Agrochemical immunotoxicity assessment must balance regulatory needs with scientific feasibility.
- *In vitro* tools from pharma may be adapted to agrochemical use but require validation first.
- Early dialogue with regulators is essential to reduce animal use and unnecessary testing.

**The Evolution of Chemical Safety Assessment: A Transformational Program – Available [here](#)**

Bennard van Ravenzwaay from ECETOC with support from Phil Botham (Syngenta) presented the tiered approach framework to hazard identification first published in [Ball et al. 2022](#) and later expanded in [Doe et al. 2025](#).

This framework is divided in several tiers, starting with a Threshold of Toxicological Concern (TTC), followed by *in silico* assessments (QSAR, AOPs, AI), *in vitro* assays, and enhanced “smart” *in vivo* studies. Ben advocated for the integration of omics technologies to bridge *in vivo* and *in vitro* data and emphasized the need for practical, incremental improvements rather than revolutionary changes. He highlighted that more refined *in vivo* studies could reduce animal use while enhancing data quality. Immunotoxicity endpoints should be embedded in these broader safety strategies and benefit from a tiered approach to hazard identification.

## Main Takeaways from Day 1 – Scientific Opportunities

**Key Learnings:** The TDAR assay remains a cornerstone of functional immunotoxicity testing, with recent updates improving its robustness. Functional immune assays using human whole blood offer high translational value and can reduce donor variability. 3D models and organ-on-chip systems show promise for mimicking immune responses but require further validation. Integration of immunotoxicity endpoints into agrochemical safety assessment is feasible and necessary. Tiered testing strategies and smarter study designs can enhance regulatory relevance while reducing animal use. Harmonization of protocols and early stakeholder engagement are critical for successful implementation.

### Maximizing Data from Existing Animal Studies

One of the clearest opportunities identified during the workshop is the enhancement of current regulatory animal studies, such as OECD 422, 408, and 443, by adding immunotoxicity-relevant endpoints without requiring new standalone studies:

- Including histopathology of immune-relevant organs (e.g., thymus, spleen, bone marrow cellularity).
- Expanding hematological parameters to include differential leukocyte counts and immune cell subtypes.
- Incorporating splenic lymphocyte subpopulation analysis (already part of OECD 443 cohort 1A).
- Embedding TDAR assays within 90-day studies to assess functional immune responses.

These add-ons may be logistically feasible and could be implemented with minimal disruption to study design, especially when supported by harmonized protocols and extending of historical control data.

### Improving Functional Readouts in Routine Studies

The use of flow cytometry and cytokine profiling in blood and spleen samples collected during routine toxicity studies can provide early indicators of immune modulation and are increasingly compatible with GLP environments. Opportunities include:

- Immunophenotyping to detect shifts in immune cell populations.
- Cytokine multiplex assays to assess immune activation or suppression.
- NK cell activity assays as early markers of innate immune function.

### Leveraging In Vitro Systems to Complement In Vivo Data

*In vitro* assays, particularly those using human whole blood or PBMCs, can be used to interpret or validate findings from animal studies.

- Use donor-matched controls to reduce variability.
- Apply virus peptide pools and serology to contextualize immune responses.
- Incorporate metabolic activation (e.g., S9 fraction) to detect immunotoxicants requiring bioactivation.

These systems are portable, scalable, and increasingly standardized, making them ideal for bridging mechanistic gaps in animal data.

### Targeting Mechanistic Endpoints in Study Design

- Mechanistic insights highlighted the importance of cell migration and signalling pathways (e.g., ATP release via pannexin-1, kinase inhibition). Including markers of immune cell trafficking and activation in study endpoints.
- Using omics technologies (transcriptomics, metabolomics) to identify early immune perturbations.
- Designing studies that can detect context-dependent immune effects, such as those influenced by infection or genetic background.

### Advancing Developmental Immunotoxicity (DIT) Assessment

While DIT remains challenging, opportunities include:

- Supporting refinement of cohort 3 protocols in OECD 443, including IgG measurement and booster immunization.
- Exploring stem cell-derived models and organoids for developmental immune function.
- Investigating critical windows of immune development using zebrafish or microphysiological systems.

These efforts should be aligned with regulatory needs and readiness criteria to ensure future uptake.

### Driving Harmonization and Regulatory Confidence

Through building trust in immunotoxicity data by:

- Contributing to the development of AOPs for immune endpoints.
- Participating in inter-laboratory validation efforts.
- Engaging with regulators early to align study designs with evolving expectations.

## Day 2 Break-out-groups outcomes

### Adult Immunotoxicity Assessment

The discussion centred on how to improve the detection of immunotoxic effects within existing repeat-dose toxicity studies, such as OECD Test Guidelines (TG) 407, 408, and 422, without increasing animal use or compromising study integrity.

One of the most practical and scientifically supported proposals was the inclusion of immunophenotyping, particularly using splenocyte analysis. Parameters such as CD4, CD8, and NK cell populations could be assessed via flow cytometry, offering valuable insights into immune modulation. This approach was considered especially feasible in 90-day studies, where sample sizes and study design allow for more robust data collection.

However, limitations were noted for shorter studies like the 28-day OECD 407 and combined OECD 422, particularly due to small group sizes and confounding factors such as lactation in females, which may affect immune parameters and complicate interpretation.

The group also discussed TDAR. While it is a cornerstone of immunotoxicity testing, it was deemed impractical to integrate directly into core studies due to regulatory constraints, especially under ICH S8 which requires satellite animals and prohibits immunization within main study groups.

A promising development highlighted during the session was the recent update to OECD 407 and 408, which now allows for cryopreservation of samples. This opens the door to omics-based follow-up analyses (e.g., transcriptomics, metabolomics) without disrupting the original study design or requiring additional animals. Such approaches could provide mechanistic insights and support weight-of-evidence evaluations for immunotoxicity.

#### Opportunities:

- **Add-on immunophenotyping** in 90-day studies using spleen samples is feasible and informative.
- **Corticosterone measurements** could help distinguish stress-related thymus changes from direct immunotoxicity.
- **Cryopreserved tissues** offer potential for transcriptomic/metabolomic analysis to support mechanistic insights.
- **Functional assays (e.g., TDAR)** should be triggered but non mandatory; those could be applicable when supported by NAMs or ex vivo data.

### Developmental Immunotoxicity (DIT) Assessment

The group engaged in a critical evaluation of the current approach to developmental immunotoxicity within the OECD TG 443 extended one-generation reproductive toxicity study, with particular focus on cohort 3 and the TDAR assay.

Participants expressed concern over the sensitivity and reliability of TDAR, noting that the assay suffers from high variability and lacks standardized protocols across laboratories. Although cyclophosphamide is used as a positive control, the wide spread of individual responses often requires logarithmic scaling to visualize data, and even then, results are frequently inconclusive. A recent update to the guideline now includes a second immunization step, but few labs have implemented it due to its novelty and the absence of historical control data.

Beyond technical limitations, the group raised important questions about the relevance of rodent models for assessing human immune development. Differences in immune system maturation between species such as the timing of thymus populations, make extrapolation challenging. Continuous exposure throughout gestation and lactation in OECD 443 further complicates interpretation, as it becomes difficult to distinguish developmental effects from acute or adult immunotoxicity.

To address these issues, the group proposed several scientific and regulatory improvements. These included conducting retrospective analyses of TDAR triggers and outcomes under REACH, developing a standalone DIT guideline, and considering segmented exposure protocols to better isolate developmental windows. There was also strong support for integrating human-relevant *in vitro* assays, such as those based on human blood or stem cell-derived models, to improve translational value.

The discussion emphasized the need for human-relevant AOPs to guide future testing strategies and reduce reliance on rodent-specific data. Participants also called for clearer definitions of adversity versus adaptive immune responses, noting that current regulatory frameworks often label any change as adverse without sufficient mechanistic context.

### Opportunities:

- **Retrospective analysis** of TDAR data and re-evaluation of DIT triggers could refine study design and reduce unnecessary animal use.
- **Standalone DIT studies** with segmented exposure (gestation vs. lactation) may improve specificity.
- **Species comparison reviews** and pharmaceutical data mining could enhance human relevance.
- **Development of human-relevant AOPs** for DIT is essential to guide future NAMs and regulatory decisions.

### Tiered Testing Strategy and NAMs

These discussions explored how to structure a scientifically sound tiered testing strategy for immunotoxicity, integrating NAMs while balancing regulatory feasibility and biological relevance. The discussion emphasized that scientific questions must guide technology selection, not the other way around.

The group reviewed the IL-2 luciferase assay (OECD TG 444A) as a Tier 1 tool for assessing T-cell activation. While validated and reproducible, it was considered insufficient as a standalone assay, as it

only captures a narrow slice of immune function. To build a more comprehensive Tier 1 screening strategy, participants proposed expanding the toolbox to include:

- **NK cell activity assays**
- **Neutrophil and macrophage function**
- **Antigen presentation and cytokine profiling**
- **B/T cell differentiation and immunoglobulin release**

These endpoints could be assessed using human PBMCs or whole blood, which offer high translational value and can be normalized across donors to reduce variability. However, the group acknowledged practical limitations, including:

- **Solubility and chemical stability** issues *in vitro*
- **Limited metabolic competence** of immune cells
- The need for **longer culture durations** (e.g., TDAR assays require ~2 weeks)
- Challenges in **applicability domains**, especially for complex or poorly soluble chemicals

The group also discussed organ-on-chip and organoid models, such as thymus organoids and lymph node-on-a-chip systems. These models show promise for DIT assessment but are not yet optimized or widely validated. Zebrafish and *C. elegans* were considered for innate immunity screening, though their use is constrained by dosing consistency and chemical compatibility.

As part of the upcoming immunotoxicity assessment task force a proposal was made to develop a coherent NAM strategy anchored in:

- **AOPs** tailored to immune system components
- **Key characteristics of immunotoxicants**
- **Applicability domain mapping** for NAMs
- **Decision trees** to guide when and how NAMs should be used

The task force would also explore how NAMs could support Next Generation Risk Assessment (NGRA), particularly in confirming or refuting findings from animal studies and helping to demonstrate negative results with confidence.

#### **Opportunities:**

- **Expand tier 1 NAMs** to include assays for innate immunity (e.g., NK, neutrophil, macrophage function).
- **Use PBMCs and whole blood assays** for multi-endpoint screening (e.g., cytokines, immunoglobulin, proliferation).

- **Develop thymus organoids or MPS** for DIT modelling.
- **Integrate metabolic activation** (e.g., S9 fraction) into *in vitro* systems to detect bioactivated immunotoxicants.
- **Map NAMs to AOPs** and define applicability domains for immunotoxicity testing.