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Detailed Review Paper on In Vitro Test Addressing Immunotoxicity With a Focus on Immunosuppression

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IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

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FOREWORD

The project to develop a Detailed Review Paper (DRP) on non-animal approaches that could be used to test chemicals for their potential immunotoxic effects was initiated by the Japanese Center for the Validation of Alternative Methods (JaCVAM), who submitted a Standard Project Submission Form (SPSF) proposing the preparation of this DRP to OECD. Japan had already investigated the potential relevance and reproducibility of certain *in vitro* assays used in specific combinations to inform the assessment of immunotoxicity. Given the absence of standardized *in vitro* methods in this particular area of chemical safety testing, the Working Party of the National Coordinators of the Test Guidelines Programme recommended to start with a DRP to enable an overview of the state-of-the-science, techniques, and methods available.

The Detailed Review Paper was prepared by an international team of subject matter experts. A dedicated OECD Expert Group was formed to provide input into the draft DRP and two WNT commenting rounds were organized in 2020 and 2021 to subject the document to broad review and comments from the regulatory science community.

The Working Party of the National Coordinators of the Test Guidelines Programme approved this Detailed Review Paper at its 34th meeting in April 2022. This document is published under the responsibility of the Chemicals and Biotechnology Committee.

LIST OF ABBREVIATIONS

AOP: Adverse Outcome Pathway
CFU-GM: Colony Forming Unit-Granulocyte-Macrophage
CTL: Cytotoxic T lymphocyte
DC: Dendritic Cell
DHR: Delayed Hypersensitivity Response
DTH: Delayed Type Hypersensitivity
EAGMST: Extended Advisory Group on Molecular Screening and Toxicogenomics
ECVAM: European Centre for the Validation of Alternative Methods
FCC: Fluorescent Cell chip
HWBCRA: Human Whole-Blood Cytokine Release Assay
ICH: International Conference on Harmonisation
IL: interleukin
IL2-LA: IL-2 Luciferase activity
IL8-LA: IL-8 Luciferase activity
iPSC: induced Pluripotent Stem Cells
LOEL: Lowest Observed Effect Levels
LPS: Lipopolysaccharide
MCP-1: Monocyte chemoattractant protein-1
M-CFS: Macrophage Colony-Stimulating Factor
MIG: Macrophage-Induced Gene
MITA: Multi-ImmunoTox Assay
MLR: Mixed Leukocyte Reaction
mMITA: modified Multi-ImmunoTox Assay
NK: Natural Killer
OECD: Organisation for Economic Co-operation and Development
PGE_2: Prostaglandin E_2
SLG: Stable Luciferase Green
SLR: Stable Luciferase Red
SLO: Stable Luciferase Orange
SRB: Sulforhodamine B
TDAR: T-cell-Dependent Antibody Response
TF: Tissue Factor
TM: Thrombomodulin
TNF: Tumor Necrosis Factor
TPA: Phorbol Myristate Acetate
VCAM-1: Vascular Cell Adhesion Molecule 1

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EXECUTIVE SUMMARY

This Detailed Review Paper (DRP) aims to present and discuss the application and interpretation of *in vitro* immunotoxicity assays, mainly covering immunosuppression, and to define an *in vitro* tiered approach to testing and assessment. This project was led by Japan, as Japan has developed three Adverse Outcome Pathways (AOPs) for immunotoxicity in the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAGMST). Japan also coordinated a validation study of the Multi-ImmunoTox Assay (MITA), based on one of the proposed AOPs.

A well-functioning immune system is essential for maintaining the integrity of an organism. Immune cells are an integral part of other systems including the respiratory, dermal, gastrointestinal, neurological, cardiovascular, reproductive, hepatobiliary, musculoskeletal system, and endocrine systems. As such, exposure to immunotoxic compounds can have serious adverse health consequences affecting responses to both communicable and non-communicable diseases. It is therefore important to understand the immunotoxic potential of xenobiotics and the risk(s) they pose to humans.

In contrast to the *in vivo* testing batteries traditionally used to investigate systemic chemical toxicity, *in vitro* methods have historically been used to generate focused mechanistic information. When using *in vitro* assays for screening purposes it is likely that several assays will be required to identify immunotoxicants because of the different components of the immune system and their influences on other systems. A tiered testing strategy is proposed to assess immunotoxicity *in vitro.* In the proposed tiered approach, pre-screening for direct immunotoxicity *in vitro* begins by evaluating myelotoxicity (Tier 1). Compounds capable of damaging or destroying bone marrow cells will most likely have immunotoxic effects, as the majority of immune cells are derived from a common precursor located in the adult bone marrow. If compounds are not potentially myelotoxic, they should be tested for direct leukotoxicity, as defined as toxicity to any cell of the lymphoid or myeloid lineages (Tier 2). Compounds should then be tested for immunotoxicity at non-cytotoxic concentrations using various approaches, such as cytokine production, T cell–dependent antibody response, lymphocyte proliferation assay, mixed leukocyte reaction, and natural killer cell assay (Tier 3).

Despite the need for an *in vitro* tiered system to evaluate immunotoxicity, at present there is no consensus on which assays to use, nor how, and there are no Organisation for Economic Co-operation and Development (OECD) Test Guidelines to detect chemical immunosuppression *in vitro*. It is clear that one assay alone will not be able to cover all of the potential adverse effects of chemicals on the immune system and that a larger set of assays that will cover the spectrum of immunotoxicity is needed. The MITA is one example of such an integrated testing strategy which may be used to predict the immunotoxicity of chemicals, and may be even more powerful when combined with complementary assays. More research and investigation are needed to develop candidate assays amenable to detect immunotoxic substances without the use of animals, but available tools can already be used in an integrated fashion for that purpose.

. Introduction

1. Since the early 1980s, there has been increasing recognition that some natural and synthetic substances to which humans may be exposed are able to interfere with the function of the immune system (e.g. Vos, 1977; Dean et al 1982). As an adequately functioning immune system is essential for maintaining the integrity of an organism, immune dysregulation can have serious adverse health consequences, ranging from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. For example, it is well-known in clinical practice that treatment of patients with immunosuppressive drugs, such as those intended to prevent rejection of organ transplants, is often associated with increased numbers of infections or tumors. In addition to drugs, environmental contaminants and food additives can also target the immune system, resulting in immune dysregulation. Originally the emphasis of immunotoxicology, which is defined as the study of toxicological effects of xenobiotics on the immune system, was on immunosuppression. Later, more attention was given to chemical-induced allergies, inadvertent immunostimulation, and chemical-induced autoimmunity. The immune system comprises a complex network of different cell types located in various organs and their mediators, which operate to maintain homeostasis. Because of its complex nature, influences of chemical exposure can occur on different components of the immune system, with different mechanisms, eventually leading to adverse health outcomes. For this reason, testing has been done most often in the intact animal if not in humans themselves, and *in vitro* testing was predominantly used to unravel specific mechanisms of immunotoxicity.

2. Current practices in immunotoxicity testing are still varied and employ either or both unchallenged and challenged immune systems. Although useful information can be obtained by the histopathology of immune organs and enumeration of immune cells obtained from regular 28-day general toxicity tests, most immunotoxicity testing historically has been organized into tiers (Hinton 2000; Luster et al. 1988). Functional immune tests, which may be used in various tiers, enable the generation of data of increased quality and specificity.

3. There are several regulatory guiding principles in immunotoxicology published as pharmaceutical industry guidances (eg. ICH S8 guidance) or chemical industry guidelines for immunotoxicity. In addition to the specific OECD Test Guidelines for skin sensitizing activity (*in vivo* OECD TG 406, *in vivo* TG 429, *in vivo* TG 442A-B, *in vitro* TG 442C-E, *in silico/in vitro* GL 497), other OECD guidelines for toxicity testing include assays for assessing immunotoxic potential in the context of more general toxicity testing, such as the 28-Day Repeated Dose Toxicity Study (OECD 407), the 90-Day Repeated Dose Toxicity Study (OECD 408), and the Extended One Generation Reproduction Toxicity Study (OECD 443). The World Health Organisation/International Programme on Chemical Safety (WHO/IPCS) has published the Guidance for Immunotoxicity Risk Assessment for Chemicals (IPCS Harmonization project No. 10) oriented at immunosuppression, inadvertent immunostimulation, and autoimmunity caused by chemical exposure.

4. A workshop hosted by the International Life Sciences Institute-Health and Environmental Sciences Institute (ILSI-HESI) was held to share perspectives on immunotoxicity testing, developmental immunotoxicity, and integrated approaches to testing and assessment (IATA[1]) of immunotoxicity. The

[1] IATA Are pragmatic, science-based approaches for chemical hazard characterization that rely on an integrated analysis of existing information coupled with the generation of new information using testing strategies [See OECD

workshop summarized that standard toxicity studies, combined with trigger-based functional immune testing approaches, represent effective approaches to evaluate immunotoxic potential (Boverhof et al. 2014). To date, the chemical risk assessment practice in OECD member countries has relied to a large extent on animal data. However, these animal models and assays have many drawbacks: they are resource intensive (time, costs, and animal numbers), pose ethical problems, and have varying ability for predicting human health outcomes. For these reasons, there is a clear societal desire to minimize the use of experimental animals for toxicity testing, while ensuring adequate protection to human health and the environment. Efforts have been and are being made to replace, reduce, or refine animal-based assays as much as possible. The EU Directive (2010/63/EU) was sanctioned to achieve these goals, and some US regulatory agencies aim to phase out animal testing by 2035 (EPA, 2019).

5. In the regulatory context, while animal models for hypersensitivity (respiratory and skin sensitization for type 1 and type 4 hypersensitivity, respectively) and immunosuppression show an overall good correlation with human data, currently available animal models and assays are not valid to assess the potential for systemic hypersensitivity (type 2 and type 3 hypersensitivity) and autoimmunity. Because we understand the mechanism of dermal sensitization to a large degree, there has been success with the development of *in vitro* methods for hypersensitivity. Additional efforts for the development of *in vitro* assays to detect other forms of immunotoxicity (e.g. immunosuppression) are needed. One aim is that this DRP, which is focused on immunosuppression, will trigger further development of methods and approaches in those immunotoxicological aspects not covered by the current Test Guidelines. Several non-animal–based testing methods for immunotoxicity have been published, although few have reached the stage of validation and acceptance by international regulatory bodies. A workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM2) in 2003 focused on what was at that time the current status of *in vitro* systems for evaluating immunotoxicity (Gennari et al. 2005). In this workshop, a tiered approach for *in vitro* immunotoxicity testing (similar to that used for *in vivo* immunotoxicity testing) was proposed. The proposed tiered approach would begin with pre-screening for direct immunotoxicity by evaluating myelotoxicity (Tier 1). Compounds capable of damaging or destroying bone marrow will most likely have immunotoxic effects. If compounds are not potentially myelotoxic, they should be tested for leukotoxicity (Tier 2). Compounds should then be tested for immunotoxicity using various approaches, such as T cell–dependent antibody response (TDAR), lymphocyte proliferation assay, mixed leukocyte reaction (MLR), natural killer (NK) cell assay, dendritic cell (DC) maturation assay, human whole-blood cytokine release assay (HWBCRA), and fluorescent cell chip (FCC) assay (Tier 3).

6. The T lymphocyte (T cell), being a crucial cell type in function and regulation of many aspects of the immune system, has been a prime target in *in vitro* assays for immunotoxicity testing. The IL-2 Luc assay developed by Dr. S. Aiba from the Department of Dermatology at Tohoku University School of Medicine in Japan is one such assay that has reached a level of validation (Kimura, 2020). Yet, it is clear that one assay alone will not cover the entire spectrum of potential adverse effects of chemicals on the immune system. Any validated test will therefore be part of a larger set of different assays that could potentially assess all types of immunotoxicity including immunosuppression, sensitization, and autoimmunity, which together will be able to adequately predict immunotoxic action of chemicals. An integral battery of tests to achieve this goal, designated as Multi-ImmunoTox Assay (MITA), is being developed by the same institute, and is described in this document as an example of how to integrate multiple tests.

(2016) Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrated Approaches to Testing and Assessment (IATA), Series on Testing and Assessment No. 260].

7. This DRP reviews developments made in the field of non-animal-based immunotoxicity testing. Immunotoxicity by drugs or chemicals can be manifested in various ways, including dysregulation of the immune response, which could lead to immunosuppression or inappropriate immunostimulation. The latter can include unintended immune stimulation, sustained inflammation, hypersensitivity reactions and autoimmune disease. With reference to chemical-induced immunotoxicity, the effect may not be exclusively in one direction and the same substance can produce immunosuppression or immune stimulation, depending on the dose and the cellular target. Thus, it may be more appropriate to define an immunotoxic substance as any agent that can alter one or more immune functions resulting in an adverse effect for the host. In this way we focus not on the direction of the effect, but on its consequence. For this reason, we prefer to use the term immunotoxicant/immunotoxicity throughout the document, although the primary focus of this DRP will be on immunosuppression. This document is not meant to be an extensive review of immunology but does include a brief overview of the immune system, a description of commonly available assays and data that have been used to determine suppressive effects of chemicals on immune responses, and considerations for establishing assays for immunotoxicity testing. Thus, the purpose of this DRP is to provide a brief overview of the complicated nature of the immune system and assessment of immunotoxicity using cell-based methods from a regulatory standpoint. The focus of this DRP is thus on in vitro test methods that are considered ready for standardisation as OECD Test Guidelines. Although there is on-going research to further develop computational approaches, organ-on-a-chip and other technologies, these are not covered in this DRP since the level of readiness for regulatory application in the area of immunotoxicity may still be limited.

. Basic concept of immunotoxicity

8. The immune system is responsible for providing protection against foreign invaders while not reacting to self-entities. The responses required to eliminate a threat occur at the same time that responses to a non-threat must be quelled. For these reasons, immunity is dynamic, with constant surveillance needed to determine whether to initiate an immune response or to not respond. Immunity can therefore be considered a continuum along which the actions of initiating a response, resolving a response, or not responding at all are carefully balanced to achieve immune homeostasis. A tip of the balance can lead to morbidity or mortality; suppression of immune responses renders an individual susceptible to infections or cancer, while enhancement can result in hypersensitivity or autoimmune disease.

9. The immune system is comprised not only of specific immune organs, but also specialized immune cells present in most tissues. It is therefore an integral part of other systems including the respiratory, dermal, gastrointestinal, neurological, cardiovascular, reproductive, hepatobiliary, musculoskeletal, and endocrine systems. An immune response occurs through the coordination of many different cell types and can involve several tissues. The thymus and bone marrow are critical for immune cell development, while the lymph nodes and spleen are organs in which many immune responses occur.

10. Initially, the cells that are involved in detecting a threat are those belonging to the innate arm of the immune response. Innate cells, such as macrophages and neutrophils, express receptors that specifically recognize pathogen-specific patterns of proteins or lipids on foreign invaders. These innate cells can release directly cytotoxic proteins, or produce cytokines or chemokines to recruit other immune cells to the area of insult. Macrophages and dendritic cells (DCs) also serve as antigen presenting cells (APCs), which provide a bridge between the innate and adaptive arms of the immune response. The most critical APC is the DC. DCs are capable of antigen uptake, allowing for removal and destruction of pathogens. The DCs also process the antigen and present antigenic epitopes to T cells, allowing for activation of the adaptive arm of the immune response.

11. The adaptive immune response includes actions by T cells and B cells, which express receptors that recognize antigenic epitopes. Pathogen-specific T cells and B cells then undergo robust proliferation, known as clonal expansion, to ensure that a large population of cells is present to react to the current threat. Specialized subsets of T cells aid in the immune response by recruiting or activating other immune cells (TH1 or TH2 cells) or by directly killing infected cells (cytotoxic T cells, CTL). Other specialized T cells include TH17 cells, which produce high levels of pro-inflammatory cytokines that recruit innate cells, and regulatory T cells (Tregs), which help to regulate the immune response and prevent autoimmune responses. The primary role of the B cell in an adaptive response is to produce antibodies, which can neutralize foreign invaders, initiate cytolysis of infected cells, or enhance the actions of innate cells, such as phagocytosis. There are also specialized cells called innate lymphoid cells (iLCs) that play a critical role in early responses in part through robust cytokine production. Both the innate and adaptive arms of an immune response can contribute to inflammation, and even though inflammation is a normal process of pathogen destruction, it can also produce tissue damage.

12. It is clear from the above that the immune system has the functional mechanisms to eradicate threats but must also be tightly regulated to avoid inappropriate reactions. Thus, the immune system is

susceptible to toxic insults in part because of: 1) the need to maintain the delicate balance between activation, regulation, and silencing; 2) its dependence on regeneration of cells from hematopoietic stem cells in the bone marrow; 3) its requirement of clonal expansion of T cells and B cells by cellular proliferation during the adaptive response; 4) the required maintenance of appropriate levels of lymphocyte subsets, including effector, memory and regulatory subsets; and 5) its interaction with other physiological systems (i.e., gut microbiota) to maintain immune homeostasis.

13. In considering how a drug or chemical exhibits immunosuppression, the agent might alter the number of cells (innate or adaptive), the ability of the cells to produce cytokines, chemokines, antibodies or growth factors, the composition of the subpopulations of cells present at the site of the response, or the function of the cells (i.e., kill infected cells or proliferate). Signs of immunotoxic potential of agents in standard animal toxicology studies can be defined by hematological changes (i.e., leukocytopenia/leukocytosis, granulocytopenia/granulocytosis, or lymphopenia/lymphocytosis), alterations in immune system organ weights or histology, changes in serum antibodies, or changes in incidence of infections or tumors (Galbiati, et al., 2010).

14. Once it has been determined that an agent possesses immunosuppressive potential, the mechanism(s) by which an agent acts can be investigated. First, given the extensive involvement of different cell types and organs in an immune response, the cellular target(s) must be defined, which could identify that the innate or adaptive (or both) arms are sensitive to alteration by a drug or chemical. Second, it is important to define whether the parent compound or a metabolite is mediating the immunotoxic effects. Immune cells have limited capacity to metabolize chemicals, but immune cells may be targeted by metabolites generated in other organs, such as the liver. Third, it is important to determine whether the agent is directly or indirectly producing immune system toxicity. For example, there are critical interactions between the immune system and endocrine systems such that immunity is regulated by various neurotransmitters and hormones (Karmaus et al 2015*)*. Thus, the mechanisms by which an immunotoxicant acts might be different in males versus females, or the mechanism of immune suppression might involve induction of stress, as high glucocorticoid levels suppress immunity. Fourth, an immunotoxicant might alter the gut microbiome, subsequently alerting immune homeostasis. Finally, the intracellular components altered by the agent that led to immune alteration should be defined. For instance, identification of whether a drug or chemical alters specific cytokines could dictate if the agent will affect all T cells, T cells and B cells, or subpopulations of one or both cells (i.e., TH1, TH2, TH17, and/or Tregs).

15. In summary, a robust immune response requires the careful coordination of cellular interactions, subsequent recruitment and/or activation of various cells, and mechanisms for regulation at all steps. There are several cell types, immune cellular functions, and/or changes in distinct physiological systems that influence immune homeostasis and might be disrupted by an immunotoxicant. Thus, *in vitro* immune toxicity tests are critical tools for deciphering whether a drug or chemical suppresses the immune response, but it is just as important to use a battery of tests to fully characterize how an agent exhibits immunotoxicity.

. Current status of AOPs on immunotoxicity testing

~~16.~~ An Adverse Outcome Pathway (AOP) describes a logical sequence of causally linked events at different levels of biological organization, which follows exposure to a chemical and leads to an adverse health effect in humans or wildlife. AOPs are the central element of a toxicological knowledge framework, promoted by member countries through OECD, built to support chemical risk assessment based on mechanistic reasoning (OECD, 2020a). These AOPs are available in the AOP Wiki (OECD, 2020b), an interactive and virtual encyclopedia for AOP development.

17. All AOPs on immunosuppression currently available in the OECD work plan are on-going and shown in Table 1. Project 1.74: Inhibition of JAK3 leading to impairment of TDAR is under development and will not be discussed. However, two of the proposed AOPs, Project 1.38 " No. 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response" and Project 1.48 "No. 277: Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection" are undergoing peer review. No. 154 shows calcineurin (CN) activity is inhibited when CN inhibitors bind to CN with their respective immunophilins, which interferes with the nuclear localization of nuclear factor of activated T cells (NFAT), a substrate of CN. As a result, the formation of functional NFAT complexes with activator protein-1 (AP-1) that bind at the site of IL-2, IL-4 and other T cell-derived cytokine promoters is reduced, thereby suppressing production of these cytokines. Among the affected cytokines from each of the helper T cell subsets, reduced production of IL-2 and IL-4 affects the proliferation and differentiation of B cells to suppress the TDAR. AOP 277 addresses one Molecular Initiating Event (MIE), impaired IL-1 receptor signaling. The biological plausibility of the signaling cascade from the activation of IL-1 receptor to the activation of nuclear factor κB (NF-κB) is already confirmed (Verstrepen et al., 2008). In addition, the biological plausibility that suppressed NF-κB activation leads to impaired T cell activation and antibody production leading to increased susceptibility to infection is supported by several published works (OECD, 2020b). To recapitulate some aspects of the *in vivo* immunotoxic responses by using *in vitro* methods, it will be very important to more closely mimic respective *in vivo* situations based on individual AOPs, although this may be complicated and laborious.

Table 1. Ongoing AOPs for Immunosuppression in the OECD work plan

Project 1.38: The Adverse Outcome Pathway on Binding of FK506-binding protein (FKBP12) by calcineurin inhibitors leading to immunosuppression	
Lead: Inclusion in work plan: Current situation:	Japan 2015 No. 154: Inhibition of Calcineurin Activity Leading to Impaired T-Cell Dependent Antibody Response , External review completed as presented in EAGMST meeting 2020.
Project 1.48: The Adverse Outcome Pathway on Dysregulation of IL-1 transcription leading to immunotoxicity	
Lead: Inclusion in work plan: Current situation:	Japan 2016 No. 277: Inhibition of IL-1 binding to IL-1 receptor leading to increased susceptibility to infection, External review completed as presented in EAGMST meeting 2020.
Project 1.74: Inhibition of JAK3 leading to impairment of TDAR	
Lead: Inclusion in work plan: Current situation:	Japan 2018 No. 315: Inhibition of JAK3 leading to impairment of T-Cell Dependent Antibody Response, Under Development

. State-of-the-art knowledge in the field of *in vitro* or non-animal assays

18. As mentioned in the previous sections, an immunotoxic compound is a compound that can alter one or more immune functions resulting in an adverse effect for the host (Luster et al., 1992). Any alteration in immune functions (e.g., antigen presentation, cytokine production, cell proliferation) that significantly deviates from control values and that can be linked to a downstream immunotoxic effect, should be considered as adverse if the immunomodulation is unintended. Considering this definition and that many functional immune tests following *in vivo* exposure are de facto *ex vivo* tests, attempts are being made to recapitulate the immune response following chemical exposure *in vitro* considering as much as possible the complexity of immune function and integration with multiple cells and soluble mediators. When interpreting the *in vitro* results, as discussed in section V, any limitations of the assay(s) (e.g. metabolic capacity) should be taken into consideration.

19. Although outside the scope of this DRP, the success in replacing animal testing for assessment of skin sensitization is a noteworthy accomplishment in the field of immunotoxicology. In the past two decades, thanks to the mechanistic understanding of the skin sensitization process that made it possible to define the first AOP (OECD (2014), *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins*, OECD Series on Testing and Assessment, No. 168, OECD Publishing, Paris, https://doi.org/10.1787/9789264221444-en) incredible progress has been made into the development and validation of non-animal models to detect skin sensitizers, and several OECD test guidelines have been published (TG 442C-E and GL 497). In addition, several reviews have been recently published on this topic (de Avila, 2019; Casati et al., 2018; Corsini et al., 2018).

20. With the successful development of new approach methodologies for the assessment of skin sensitization, the next step must be the development of a strategy to address immunosuppression without the use of animals. We recognize that the level of complexity and our understanding of the mechanistic pathways that lead to immunosuppression are less clear than those for hypersensitivity, and additional studies are needed to prove the possibility and feasibility to address immunotoxicity using *in vitro* approaches (Corsini and Roggen, 2009; Lankveld et al., 2010; Galbiati et al., 2010; Luster and Gerberick, 2010; Hartung and Corsini, 2013). Nevertheless, we must acknowledge that important progress has been made in the development of *in vitro* assays for the assessment of immunotoxicity, with the HWBCRA and the MITA representing significant achievements (Langezaal et al., 2001; Kimura et al., 2018).

In vitro opportunities to identify immunosuppressive agents

21. As noted above, factors such as age at onset, gender, dose, duration, and route of exposure may result in differing effects on the immune system and skew the adverse response in the direction of immunosuppression or immunostimulation. Thus, while this DRP focuses on immunosuppression, all

the assays described in this section could lead to results demonstrating either no effect, immunosuppression and/or immune enhancement when compared to a control group, with the last two indicating an immunotoxic effect. Before beginning any evaluation, it would be useful to collect all available information in the literature, including information from sources such as the CompTox Dashboard, and the Integrated Chemical Environment data integrator, which include data from multiple endpoints, several of which may be relevant for immunotoxicity (Bell et al., 2017; Naidenko et al., 2021).

22. Due to the complexity and diversity of the immune responses, it was generally assumed that it would be very difficult to reproduce all the key events and processes *in vitro*. To a large extent, *in vitro* systems do not consider the interactions of the different cellular and soluble components involved in the immune response, nor the potential for neuro-immuno-endocrine interactions. Therefore, the assessment of *in vitro* immunotoxicity has often been valuable only in the cases of a direct immunotoxicant (Gennari et al., 2005). Several isolated processes can be studied *in vitro* including antigen presentation, lymphocyte proliferation, cytokine production, phagocytosis, lytic functions, and even primary antibody production, offering the possibility to assess immunotoxicity *in vitro.* Recently there has been incredible progress in 3D models with engineered immune tissues and organs, such as bone marrow, thymus, lymph nodes and spleen being described (see review by Gosselin et al., 2018), and microfluidic body-on-a-chip, and in the future it may be possible to identify both direct and indirect immunotoxicants using an integrated model of the whole human immune system (Shanti et al., 2018).

23. Primary human immune cells such as monocyte-derived DCs, T cells, and B cells obtained from human peripheral blood may be useful materials for *in vitro* testing and are highly clinically relevant. However, the use of human primary cells for developing a testing assay may have several issues regarding ethics, donor-to-donor variability, versatility, and reproducibility. Variability reflects diversity in individual immune capability that requires consideration, and it is important to understand and ensure that it is reflected in *in vitro* systems developed using non-primary cells. Variability and predictive capacity are important considerations for establishing scientific confidence for individual or combinations of *in vitro* methods. Use of induced pluripotent stem cells (iPSC) technology may further improve the versatility of *in vitro* assays. Several human immune cells including T cells, B cells, DCs, and NK cells have been generated from iPSC (Vizcardo et al., 2013; French et al., 2015; Senju et al., 2011; Kitayama et al., 2016). In the future, iPSC technology might be used to provide different populations of bone marrow cells such as iPSC-derived hematopoietic stem cells and mesenchymal stem/stromal cells to obtain a more complete picture of myelotoxicity *in vitro*.

24. When assessing the potential immunotoxicity of xenobiotics, bioavailability should also be considered as part of *in vitro* testing. If a compound is not systemically available, a direct adverse effect on the immune system should not be expected as the compound would not reach immune cells or tissues. However, local effects at the site of exposure would still be possible. For example, it is important to consider that the immune system is closely linked and influenced by the microbiota. A substance taken orally could influence the microflora and mucosal DCs, and even if it is not absorbed into the systemic circulation, it may in turn influence the immune response (Belkaid and Hand, 2014). *In vivo* toxicokinetic studies, if available, or physiologically based pharmacokinetic (PBPK) models should be used to assess or predict absorption. For consistency, any alternative means to obtain information on systemic bioavailability without *in vivo* animal data should be preferred. At this regard, the ECHA Guidance in information requirements and chemical safety assessment Chapter R.7c (available at: https://echa.europa.eu/documents/10162/13632/information_requirements_r7c_en.pdf/e2e23a98-adb2-4573-b450-cc0dfa7988e5) contains a section on how information on systemic bioavailability can be gathered.

25. As a general strategy, *in vitro* testing for direct immunotoxicity should be done in a tiered approach (adapted from Gennari et al., 2005; Corsini and Roggen, 2009), with the first tier measuring myelotoxicity (Tier 1). Myelotoxicity or bone marrow toxicity represents the decrease in production of cells responsible for providing immunity (leukocytes), carrying oxygen (erythrocytes), and/or those

responsible for normal blood clotting (thrombocytes). In the context of immunotoxicity, myelotoxicity would refer to toxicity to precursors of immune cells. Compounds that are capable of damaging or destroying the bone marrow will have a profound immunotoxic effect, since the effectors of the immune system itself will no longer be available. Therefore, if a compound is myelotoxic, according to the specific assay performed, the chemical will *de facto* be an immunotoxicant. *Ex vivo* colony forming assays are used to assess bone marrow toxicity in animal models. The methodologies for evaluating myelotoxicity *in vitro* using bone marrow culture systems are well-characterized and scientifically validated for reproducibility and predictive capacity (Pessina et al., 2003; 2005; Rich and Hall, 2005; Haglund et al., 2010), but they are not required for regulatory testing or widely accepted as a standard screening tool due to technical challenges. Results of a pre-validation study showed that the *in vitro* colony forming unit-granulocyte-macrophage assay (CFU-GM) is linear and highly reproducible within and between laboratories (Pessina et al., 2001; Pessina et al., 2010). In an international blind trial (Pessina et al., 2002), the model correctly predicted the human maximum tolerated dose (MTD) for 20 drugs out of the 23 tested (87%).

26. Compounds that are not directly myelotoxic may still selectively damage leukocytes (defined as cells of lymphoid or myeloid lineage), which are the primary effectors and regulators of immunity, so the next step in evaluating potential immune toxicity *in vitro* is testing for leukotoxicity (Tier 2). If the agent is cytotoxic to immune cells at concentrations relevant for human exposure, the agent should be considered an immunotoxicant. If not, data on leukotoxicity will be used for the selection of the concentration range to be used in Tier 3, in which only non-cytotoxic concentrations should be used (cell viability > 80%). The choice of the cellular model to be used in Tier 3 will depend on the target identified in Tier 2 and the functional test to be performed (e.g., B or T cells, DCs, NK cells). There are several methods that can be used to assess cytotoxicity, among which the fluorometric microculture cytotoxicity assay to screen for leukotoxicity can be mentioned. This assay is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes. 20This method has been used in primary peripheral blood mononuclear cells (from different species) by Hassan et al. (2007) to measure cell survival following exposure to cytotoxic drugs and shown to correlate well with CFU-GM data. Cytotoxicity may result from the destruction of rapidly dividing cells by necrosis or apoptosis. Alternatively, chemicals may cause cell death by interfering with cell activation by affecting signal transduction pathways. A variety of well-established and accepted methods are available for assessing cell viability (e.g., colorimetric, flow cytometric assays), and several of these assays are integral parts of currently accepted OECD TGs. If leukotoxicity occurs at concentrations relevant to the expected *in vivo* human exposure, then cytotoxicity remains a relevant effect associated with immunosuppressive potential. If expected *in vivo* concentrations are unknown, the use of PBPK models should be considered to predict pharmacokinetic parameters. After consideration of myelotoxicity and leukotoxicity, which if positive are sufficient to classify the compound as immunotoxic, basic immune cell functionality may then be assessed by performing specific functional assays that identify targeted cells and processes (Tier 3). These assays (i.e., proliferative responses, lytic activity, cytokine production), should be conducted using concentrations of the test chemical that are not cytotoxic and provide acceptable viability for the specific assay.

27. Alternative *in vitro* methods have the potential to reduce animal use and testing cost, to facilitate immunotoxicity screening, and prioritization efforts (Luebke, 2012). Several *in vitro* assays that evaluate specific functions or functional correlates of the immune system (e.g., CTL activity, NK cell activity, antibody production, cytokine production, cell proliferation) have been used to assess immunotoxicity. A significant response in any of these assays should be interpreted as the chemical possessing the potential for immunotoxicity and should warrant further investigation. In Table 2, relevant immune components, and opportunities for *in vitro* assessment of immunotoxicity (immunosuppression) are reported, and readers are referred to the cited works for further details. The tests shown in Table 2 refer to what has been published with the specific purpose of identifying immunotoxic substances *in vitro*. Other aspects of immune function may be evaluated (e.g,. phagocytosis, production of lysozyme,

microbicidal activity) to identify the immunotoxic potential of a substance, but have been used less frequently for screening purposes. In addition, while there are numerous immune cell subtypes, (e.g., Th subpopulations or DC subsets), involved in the different immune responses, there is a need to establish validated methods to study how alterations in their functional capabilities contribute to immunotoxicity. Considering the complexity of the immune response, more than one *in vitro* test will likely be needed to define the immunotoxic potential of a xenobiotic. Table 2 also includes the source of cell used (i.e. human vs animal, or primary vs cell line). While some of the methods reported involve the use of primary cultures of animal origin, the partial replacement still allows for a reduction and refinement in the use of animals. Currently, the main issue for most of the *in vitro* models mentioned in Table 2 is the limited number of chemicals tested. Some of the most promising tests will be described in more detail in the following paragraphs, either because they have been validated or are in the process of validation or because they measure the production of antibodies, which in animal models is considered the most predictive parameter.

Table 2. Key targets in chemical-induced immunosuppression and *in vitro* test opportunities

KEY IMMUNOLOGICAL TARGETS (TIER)	*IN VITRO* OPPORTUNITIES	CELL MODEL	REFERENCES
Bone marrow (Tier 1)	Human lympho-hematopoietic colony-forming assay for myelotoxicity (e.g. CFU-GM)	Human bone marrow and umbilical cord blood; rodent bone marrow	Pessina et al., 2003; 2005; 2010; Rich and Hall, 2005; Haglund et al., 2010
Leukotoxicity (Tier 2)	Cell viability (e.g., MTT, LDH release assay, flow cytometry)	Rodent splenocytes; human peripheral blood mononuclear cells	Hassan et al 2007; GIVIMP, 2018
Innate immunity (Tier 3)	NK cell activity	Rodent splenocytes; human peripheral blood mononuclear cells	Lebrec at al., 1995
	Monocytes/macrophages cytokines	Human peripheral blood mononuclear cells (e.g. whole blood assay); rodent splenocytes; cell lines (e.g. THP-1)	Langezaal et al., 2001; Langezaal et al., 2002; Carfì et al., 2007; Vessillier et al., 2015; Kimura et al., 2018
	Mast Cells/Basophils	Human basophils	McGowan et al., 2013
Cell mediated immunity (Tier 3)	T cell proliferation	Rodent splenocytes; human peripheral blood mononuclear cells	Lebrec at al., 1995; Carfì et al., 2007
	Mixed leukocyte response (MLR)	Rodent splenocytes; human peripheral blood mononuclear cells	Lebrec at al., 1995
	Cytotoxic T lymphocyte (CTL)	Rodent splenocytes; human peripheral blood mononuclear cells	Lebrec at al., 1995
	Cytokine production	Rodent splenocytes; human peripheral blood mononuclear cells (e.g. HWBCRA); human cell lines (e.g. Jurkat T cells)	Langezaal et al., 2001; Langezaal et al., 2002; Ullerås et al., 2005; Carfì et al., 2007; Ringerike et al., 2005; Stølevik et al., 2010; Kimura et al., 2018
	Transcriptomic profiles	Human peripheral blood mononuclear cells; human cell lines (e.g. Jurkat T cells)	Hochstenbach et al., 2010; Shao et al., 2014; Schmeits et al., 2015
	In vitro antigen presentation to T cells	Mouse cell lines (e.g. 3A9; Ch27B)	Lehmann and Williams, 2018

Humoral immunity (Tier 3)	B cell proliferation	Rodent splenocytes; human peripheral blood mononuclear cells	Carfì et al., 2007
	In vitro antibody production	Rodent splenocytes; human peripheral blood mononuclear cells	Keoper and Vohr, 2009; Lu et al., 2009; Collinge et al., 2010; Fischer et al., 2011

The table reports methods that have been proposed as alternatives to animals for the identification of immunotoxicants.

28. Among these assays, the HWBCRA has the advantage of comprising multiple cell types in their natural proportion and environment, allowing the evaluation of both monocyte and lymphocyte functions by using selective stimuli (Langezaal et al., 2001 and 2002), while “omics” techniques can provide additional mechanistic understanding and hold promise for the characterization of classes of compounds and prediction of specific toxic effects (Hochstenbach et al., 2010; Shao et al., 2014; Schmeits et al., 2015). The IL-2 Luc assay also allows high-throughput analysis (Kimura et al., 2018), which will greatly expand the opportunities for *in vitro* testing. The CFU-GM assay, the HWBCRA as a pyrogen test, and the IL-2 Luc assay have undergone validation for reproducibility and predictive capacity.

29. The whole blood assay provides a more physiological environment, as compared to isolated peripheral mononuclear cells, which may allow for a broader assessment of immune functions. In addition to cytokine production as in the HWBCRA, the whole blood assay can be used to address many other relevant immunological endpoints, including NK cell activity, lymphocyte proliferation, and antibody production. The cost of performing these assays depends on the endpoints, but it is overall relatively inexpensive compared to *in vivo* studies, and feasibility is high due to extensive use of this methodology (Hartung and Corsini, 2013). In the *in vitro* pyrogen test (i.e., HWBCRA), which is used in the same way as the Limulus test to analyze the possible presence of contamination of Gram negative bacteria or their remnants in drugs, and in the analysis of water and industrial raw materials, samples are incubated with fresh or cryopreserved human whole blood for the detection of the production of the proinflammatory cytokine IL-1β by enzyme-linked immunosorbent assay (Hartung and Wendel, 1995). In addition, the HWBCRA has also been adapted for immunotoxicity testing, to permit the potency testing of immunostimulants and immunosuppressants (Langezaal et al., 2001 and 2002). In this case, tested compounds are incubated in the presence of lipopolysaccharide (LPS) to activate monocytes or staphylococcal enterotoxin B to activate lymphocytes (mainly CD4+), and the release of IL-1β and IL-4, respectively, are subsequently measured by ELISA. Results are then expressed as IC50 values for immunosuppression, or SC(4) (stimulatory concentration resulting in a four-fold increase) values for immunostimulation, depending on the results observed. Thirty-one pharmaceutical compounds were used to 2222optimize and standardize the method. The *in vitro* results correlated well with *in vivo* data, and the test appears to reflect immunomodulation, meaning that both immunosuppression and immunostimulation can be detected. Results were reproducible (CV = 20 +/- 5%), and the method could be successfully transferred to another laboratory. A sensitivity of 67% and a specificity of 100% for the combined endpoints were calculated, where “sensitivity” refers to correctly identifying positive immunotoxicants, and “specificity” refers to correctly identifying negative immunotoxicants (reviewed in Hartung and Corsini, 2013).

30. Progress in *in vitro* testing for direct immunotoxicity includes validation of existing assays and selection of the assay (or combination of assays) that performs best, as described in the last section of this document (section VIII). In particular, the two luciferase assays that comprise the MITA are undergoing official validation studies; the IL-2 Luc assay that evaluates the effects of chemicals on the IL-2 promoter activity in response to stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io), and the IL-1 Luc assay that evaluates the effects of chemicals on the IL-1β promoter activity in response to stimulation with LPS. During the validation studies, the lead laboratory evaluated

the predictivity of the IL-2 Luc assay by examining 25 chemicals in the validation studies and 60 chemicals in the test data set. The predictivity of the IL-2 Luc assay was 75% in 25 chemicals and 82.5% in 60 chemicals, respectively. This predictivity is not optimal to predict immunotoxicity of chemicals as a stand-alone test method. Combination with other immune function tests, in particular myelotoxicity tests or leukotoxicity tests, will increase thepredictivity of the IL-2 Luc assay, as it is unable to detect myelotoxicity or antiproliferative effects (Kimura et al., 2014; Kimura et al., 2018).

31. In experimental animals, the TDAR is considered the “gold standard” to identify immunotoxic compounds (Luster et al., 1992; Lebrec et al., 2014). The TDAR has been the consensus choice for a functional endpoint to identify immunotoxicity hazard in most, if not all, regulatory guidelines, because the TDAR requires many of the cellular components of an immune response and thus, is a sensitive indicator of the overall immunotoxic potential of chemicals. Koeper and Vohr (2009), Lu et al. (2009), Collinge et al. (2010, 2020), and Fischer et al. (2011) reported the possibility to assess *in vitro* antibody production in the context of immunotoxicity. Antibody production provides a holistic summation of antigen processing, presentation and recognition, gene transcription and rearrangement, cell proliferation and differentiation, and ultimately, the production of antibodies, the effector molecules (Luebke, 2012). Koeper and Vohr (2009) and Fischer et al. (2011) used the *in vitro* antibody response (Mishell-Dutton culture) as an alternative to the existing animal tests to predict different immunosuppressants. Using this model, they were able to show that cell sources from both rats and mice were able to correctly predict all of 11 tested compounds and to clearly distinguish immunosuppressants from negative control substances. In another model proposed by Lu et al. (2009) a polyclonal immunoglobulin M (IgM) antibody-forming cell (AFC) response model to directly characterize immunotoxicity in primary mouse or human B cells was developed. CD40 ligand (CD40L) is used to activate B cells and to mimic T cell-dependent antibody responses *in vivo*. Antibody production, proliferation, and phenotypic changes characteristic of B cell activation as well as the plasma cell phenotype are measured. Two well-characterized immunotoxicants, arsenic and benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide, were tested. The novel model proposed by Lehmann and Williams (2018) to evaluate effects on antigen presentation, which is a key step in successful immunization is also of interest. Even if it is based on the use of two mouse T and B cell lines (3A9 hen egg lysozyme-specific I-A^k restricted T cell hybridoma cell line and the mouse Ch27 B lymphoma cell line), the method allows the evaluation of the effect of chemical exposure on several integrated events critical for immunization, including uptake, processing and presentation of antigen by antigen presenting cells, and antigen recognition and IL-2 production and secretion by T cells (Lehmann and Williams, 2018). However, these assays do not address all aspects of the humoral immune response, and it will be necessary to develop methods which assess the ability of B cells to undergo somatic hypermutation, affinity maturation, and class-switch. In the future, the gold standard TDAR might be replaced with *in vitro* coculture systems using iPSC-derived DCs, T cells, and B cells, or ultimately a microphysiological system of human immune system-on-a-chip consisting of these cell types to capture all aspects of humoral immunity (Miller at al., 2020). Further explorations of these models are recommended.

32. Finally, the BioMapTM Diversity Plus platform can be mentioned (Singer et al., 2019). The BioMap® Diversity Plus platform consists of several human primary cell-based assays modeling complex tissue and disease biology of organs (vasculature, immune system, skin, lung) and general tissue biology. Among the 12 systems, monocyte activation (readouts: MCP-1, VCAM-1, TM, TF, CD40, E-selectin, CD69, IL-8, IL-1α, M-CSF, sPGE2, SRB, sTNFα), T cell activation (readouts: MCP-1, CD38, CD40, E-selectin, CD69, IL-8, MIG, PBMC Cytotoxicity, Proliferation, SRB), and B and T cell autoimmunity (readouts: B cell Proliferation, PBMC Cytotoxicity, Secreted IgG, sIL-17A, sIL-17F, sIL-2, sIL-6, sTNFα) are likely to be relevant to identify chemical-induced immunotoxicity. However, its predictivity and reproducibility for chemicals inducing immunotoxicity, has not been demonstrated with a diverse set of environmental contaminants. Further explorations are recommended.

. Performance factors of *in vitro* assay(s)

33. To be appropriate for regulatory use, alternative *in vitro* assay(s) examining immunotoxicity should be characterized using the Reference Compound List (Table 3). The list is not exhaustive but is a compilation of environmental contaminants and drugs that have been shown to induce immunotoxicity in non-clinical studies and/or humans. When evaluating assay performance, to be in line with other validation studies, typically at least 20 compounds in total should be considered, but the required number of chemicals will depend on the specific test being performed and the regulatory guidance for the assay type. Compounds other than those in the reference list may be included, but their use should be justified according to the selection factors listed in section VII. The compounds in Table 3 have been selected from multiple classes, covering a wide range of biological and chemical modalities that have multiple immune targets. While it is beneficial to use compounds from multiple classes with different immune-related targets, some consideration of the cell type or response evaluated in the assay is necessary to interpret the sensitivity and specificity of each test. An approximate 2:1 ratio of positive to negative compounds, selected from different chemical classes, should be tested to ensure selectivity with the limited number of reference materials available.

34. A range of concentrations should be tested in each assay, and clearly described, as the same compound may be immuno-stimulatory or immunosuppressive depending on the concentration level. A certain degree of toxicity is expected at the highest concentration tested, which indicates that the chemical is doing something to the cells (similarly to the maximum tolerated dose in animal studies). However, significant toxicity should be avoided as it is difficult to ascertain whether the compound is toxic to a particular cell type, or if it decreases the proliferative capacity of those cells in the assay. When available, information on internal dose from *in vivo* exposure should be used to guide dose selection. Ideally, sensitivity and specificity would be 100%. However, these levels cannot be standardized and are dependent on the assay and chemicals being used. In general, the sensitivity to detect a positive compound in an assay(s), when applied on chemicals from the Reference Compound List (Table 3), should be at least 75%, with evidence of sufficient specificity (i.e., differentiating between true positives and true negatives, OECD GD286, 2018).

35. Inter-laboratory reproducibility and transferability between laboratories is required for the purpose of validation to establish an OECD test guideline if a particular assay is to be used in more than one laboratory. The minimum and maximum number of laboratories needed for a comprehensive assessment of the validity of the test method will depend on the type of test, the questions being addressed, and/or the overall amount of testing required of each laboratory. In many cases, three or four laboratories per test method may be an adequate number for an assessment of the inter-laboratory reproducibility (OECD GD34, 2005). Evaluation should also include assessments of accuracy and reproducibility over time. The performance characteristics of each assay, as well as the performance of the combined battery (if used) should be specified.

36. When interpreting results, the applicability domain and any limitations of the assay(s) should be taken into consideration (i.e., solubility, stability in culture media, metabolism). If the compound requires metabolism to exert its effects, but the system lacks metabolic enzymes, the results need to be considered in this context. Immune cells are generally considered to have low metabolic capacity, therefore, the use of S9 or other alternative metabolic activation systems should be considered by test

developers (Ooka et al, 2020). If not, it must be clearly stated that compounds that require metabolic activation fall outside the applicability domain of the test, and in case of negative results, one must be sure before classifying the compound as non-immunotoxic that the compound does not undergo bioactivation.

. Performance information of *in vitro* assay(s) to be provided to health authorities

37. To enable evaluation of an alternative assay(s) for use in immunotoxicity risk assessment for regulatory purposes, the following information should be provided: a detailed description of the predictive model including the *in vivo* endpoints for which it is trying to predict an outcome, and its use in the context of a tiered approach and integrated testing strategy. The *in vitro* model can consist of a single assay or a battery of assays together (a battery of tests measuring different immune endpoints is more likely to be predictive). If a battery of tests is used, each individual endpoint should be fully described with how the assessment of validity is made, including how the endpoints were selected.

38. The details of the prediction model used for determining positive and negative outcomes from the assay, including the borderline results and their interpretation should be presented for each assay. The model should correlate concentrations tested in the *in vitro* assays to the *in vivo* internal dose required to result in immunotoxicity in the species being predicted. For example, concentrations associated with immunotoxic effects should be interpreted in the context of expected *in vivo* exposure parameters such as C_{max} or AUC. If available, PBPK models can also inform concentration ranges.

39. The compound list used to qualify the assay performance should be presented. For purposes of establishing the predictive capacity (i.e., sensitivity, specificity and accuracy) of proposed test methods to be used by several laboratories, typically at least 20 compounds encompassing multiple chemical classes (examples listed in Table 3) in total should be tested by multiple laboratories, recognizing that the number of chemicals and testing laboratories will depend on the variability of the specific test being performed. In each laboratory, the chemicals should be tested in three independent runs performed with different cell batches on multiple days. Each run should consist of at least three concurrent replicates for each test chemical, negative, and positive control.

40. The calculation of the accuracy, sensitivity, and specificity values of the proposed *in vitro* test method for a single assay or battery of assays, should be equal to or better than the target values derived from the validated *in vivo* reference method(s). If a battery of assays is being proposed, the above information for each individual test method should be included. The combined accuracy, sensitivity, and specificity of the battery of assays should be greater than that of each of the individual assays. Typically, the sensitivity should be ≥75% (OECD 286). Any participating laboratory may misclassify an *in vivo* non-immunotoxic chemical as long as the final specificity of the test method is within the acceptable range.

41. The source of all reagents, biologic materials, and test compounds should be included. Test compound purity, stability and CAS number should be documented if available. The source/reference of all *in vivo* exposure data used for comparison should also be provided. Assays should be developed with the understanding that regulatory studies should generally be conducted in compliance with current

Good Laboratory Practice (GLP). Consultation with the relevant health authority(ies) is highly recommended to determine the level of validation needed.

. Selection factors for the reference compounds to be used in development of the in vitro assay(s)

42. The Reference Compound List (Table 3) contains environmental contaminants and drugs that have been shown to induce immunotoxicity in nonclinical studies and/or humans. The list includes representative chemicals from a number of classes of compounds that have been demonstrated to be immunotoxic (i.e. perfluoroalkyl substances, polycyclic aromatic hydrocarbons, organotins), known immunosuppressive therapeutics and other substances, but is not a comprehensive listing of all immunotoxicants. These compounds, as well as others, can be used to support qualification of an alternative assay or battery of assays. Many of the suggested reference compounds have been evaluated using tiered testing panels of *in vivo* assays in rodents (Luster et al., 1988; Vos and Van Loveren, 1989) and have been reported to suppress functional immune responses or modulate disease resistance. Modulation of observational measures, such as organ weights and cell subpopulations have also been described. It should be noted that while these compounds generally have immunosuppressive effects, depending on the exposure concentration and experimental design, some of these compounds may exhibit immunostimulatory effects, particularly when they act upon regulatory cells. In addition, positive control compounds may have different potency or target different cell types *in vitro* and an understanding of their mode of action and *in vivo* exposure parameters such as internal dose and metabolism may inform effects on specific cell populations. For a limited number, there is evidence from human epidemiology (i.e. ethanol, lead, and 2929 perfluorooctanoic acid) or clinical studies (cyclosporine A, dexamethasone, and diethylstilbestrol). In addition, there is a growing body of *in vitro* evidence supporting the immunotoxicity of these reference materials, and for many there are data with human cells or cell lines. The selected compounds target a variety of cell types and processes and will thus be useful to identify defined approaches for the *in vitro* assessment of immunotoxicity. The proposed negative compounds have been tested in a full battery of immune function assays in rodents as described in Luster et al (1988) and were negative under the conditions of those studies.

Table 3. Reference Compound List

Positive Controls	CAS Number	Reported Immune Targets
Aflatoxin B1*	1162-65-8	DTH, Cell Proliferation, Innate Immunity
2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine*	105650-23-5	Cell Proliferation, Antibody Response, Cytokine Production
Atrazine	1912-24-9	Cell Proliferation, Lymphoid Organ Weights, Clinical Pathology
Azathioprine*	446-86-6	Antibody Response, CTL
Benzidine*	92-87-5	Antibody Response, NK Cell Activity, Cell Proliferation, CTL
Benzo(a)pyrene*	50-32-8	Antibody Response, Cell Proliferation, Host Resistance, NK Cell Activity, Lymphoid Organ Weights
Cadmium Chloride	10108-64-2	Antibody Response, Cell Proliferation, Cytokine Production
Chloroquine*	54-05-7	Innate Immunity
Chrysene*	218-01-9	Antibody Response
Cyclophosphamide*	50-18-0	Antibody Response, Cell Proliferation, DTH, Lymphoid Organ Weights
Cyclosporine A	59865-13-3	Antibody Response
Deoxynivalenol	51481-10-8	Antibody Response, Cytokines
Dexamethasone	50-02-2	Macrophage Function, NK Cell Activity, Clinical Pathology, Lymphoid Organ Weights, Cytokines
2,4 Diaminotoluene*	95-80-7	Antibody Response, DTH
Dibromoacetic acid	631-64-1	Antibody Response, NK Cell Activity, CTL
Dichlorodiphenyltrichloroethane (DDT)	50-29-3	Antibody Response, Cell Proliferation, Cytokine Production, Lymphoid Organ Weights
Dideoxyadenosine	4097-22-7	Antibody Response, Cell Proliferation
Diethanolamine	111-42-2	Antibody Response, CTL, Clinical Pathology
Di(2-Ethylhexyl) Phthalate	117-81-7	Antibody Response, Innate Immunity, Cytokines
Diethylstilbestrol*	56-53-1	Antibody Response, Cell Proliferation, Cytokine Production
Dimethylbenz(a)-anthracene*	57-97-6	Antibody Response, Cell Proliferation, Lymphoid Organ Weights
Diphenylhydantoin*	630-93-3	NK Cell Activity, Lymphoid Organ Weights
Ethanol*	64-17-5	Antibody Response, NK Cell Activity, Cytokine Production
Ethyl carbamate*	51-79-6	Antibody Response, Cell Proliferation
Ginseng	50647-08-0	NK Cell Activity, Lymphoid Organ Weights

Glycidol	556-52-5	Antibody Response, NK Cell Activity, Clinical Pathology
Hexachlorobenzene*	118-74-1	Antibody Response
Hexachlorobiphenyl 153	35065-27-1	Antibody Response, CTL, DTH
γ-Hexachlorocyclohexane (Lindane)*	58-89-9	Cell Proliferation, CTL, NK Cell Activity
1,2,3,6,7,8-Hexachlorodibenzo-p-dioxin	57653-85-7	Antibody Response, Cell Proliferation, DTH, Lymphoid Organ Weights
Indomethacin	95-83-0	Cell Proliferation
Lead acetate	6080-56-4	Antibody Response, DTH
Methadone Hydrochloride	1095-90-5	NK Cell Activity, Lymphoid Organ Weights
2-Methyoxyacetic Acid	625-45-6	Antibody Response, Cell Proliferation, Lymphoid Organ Weights
Morphine Sulfate	64-31-3	Antibody Response, NK Cell Activity, Cell Proliferation, Lymphoid Organ Weights
Mycophenolic Acid	24280-93-1	Antibody Response, Cell Proliferation
Nitrobenzene*	98-95-3	Antibody Response, NK Cell Activity, Cell Proliferation
n-Nitrosodimethylamine*	62-75-9	Antibody Response, NK Cell Activity, Cell Proliferation, DTH
m-Nitrotoluene*	99-08-1	Antibody Response, Cell Proliferation, DTH
Ochratoxin A	303-47-9	NK Cell Activity, Lymphoid Organ Weights
Parathion	56-38-2	Host Resistance, Antibody Response
Pefluorooctanoic Acid	335-67-1	Antibody Response, NK Cell Activity, Cytokine Production
Perfluorooctanesulfonic acid	1763-23-1	Antibody Response, NK Cell Activity, Cell Proliferation, Cytokine Production
3,3',4,4',5-Pentachlorobiphenyl*	57465-28-8	Antibody Response, NK Cell Activity, Cell Proliferation, Lymphoid Organ Weights
Pentachlorophenol*	87-86-5	Antibody Response, NK Cell Activity, Cell Proliferation
Prednisolone	50-24-8	Macrophage Function, NK Cell Activity, Clinical Pathology, Lymphoid Organ Weights, Cytokines
Propanil*	709-98-8	Antibody Response, NK Cell Activity, CTL, Cell Proliferation, Cytokine Production
Tetrabromobisphenol A	79-94-7	Cytokines, Innate Immunity
2,3,7,8-Tetrachlorodibenzo-p-dioxin	1746-01-6	Antibody Response, CTL, Thymus Weight,
δ 9- Tetrahydrocannabinol	1972/8/3	Antibody Response, Cell Proliferation, Cytokine Production
Thalidomide	50-35-1	Antibody Response, CTL, Clinical Pathology
Tributyltin	56-24-6	Antibody Response, Lymphoid Organ Weights, Cytokine Production
Tributyltin Chloride	1461-22-9	Antibody Response, NK Cell Activity, CTL, Cell Proliferation, Lymphoid Organ Weights
Tributyltin Oxide	56-35-9	Antibody Response, NK Cell Activity, CTL, Cell Proliferation, DTH, Lymphoid Organ Weights

		Negative Controls
Chloramine	10599-90-3	
4-Chloro-o-phenylenediamine	95-83-0	
Dichloroacetic acid	79-43-6	
Methyl carbamate	598-55-0	
Nitrofurazone	59-87-0	
Oxymethalone	434-07-1	
Patulin	149-29-1	
Sodium Bromate	7789-38-0	
Sodium Chlorite	7758-19-2	

*Denotes compounds which require metabolic activation for immunotoxicity to manifest. Because there is a large body of literature for each of these individual compounds the reader is referred to compilations of information or data on immuntoxicity that review these effects including: Cohen et al 2000; Corsini and van Loveren, 2015; Descotes 2004; Dewhurst et al 2015; House et al 2007; Kaplan et al 2019; Kimura et al 2020; Luster et al 1988; 1992; Tryphonas et al 2005; Vohr 2005, WHO 1996

. *In vitro* immunotoxicological assessments using combinations of cell types or cell lines

8.1. Lessons from immunotoxicological assessments using rodents

43. The use of tiered testing panels of *in vivo* assays in rodents has been the most common methodology for assessment of immunotoxicity since the inception of the discipline (Luster et al., 1988; Van Loveren and Vos, 1989). The *in vivo* tiered approach proposed by the National Toxicology Program at the NIH contains both screening assays to detect immunologic effects (Tier I) and a comprehensive suite of assays to provide an in-depth assessment of immune function and host resistance endpoints (Tier II), as listed in Table 4 (modified from (Luster 1998)). Chemicals are judged as immunotoxicants based on whether they produced a significant dose-response effect ($p<0.05$) in a measure of a functional immune response (rather than an observational measure such as a change in body or organ weights), or if they significantly altered two or more test results at the highest dose of chemical tested ($p<0.05$). Based on the ability of various immune tests to predict increased susceptibility in disease resistance assays, Luster et al. (1992), demonstrated that: 1) a number of the immune tests provided a relatively high association with changes in host resistance (i.e., > 70%), such as the TDAR, delayed hypersensitivity response (DHR), cell surface immunophenotyping markers, and CTL assay. In contrast, several of the tests, such as leukocyte counts and lymphoproliferative response to LPS were poor predictors, with concordance values of approximately 50%; and 2) the combination of two immune tests significantly increased the predictive value from that obtained using individual tests. Pair-wise combinations which included either the plaque forming cell (PFC) response (a TDAR endpoint), cell surface immunophenotyping markers, or DHR gave consistently higher concordances and combinations of two or three immune tests involving these measures could give more than 90% concordance with effects on disease resistance.

44. Several regulatory guidelines or guidance documents have since been developed for the assessment of immunotoxicity of pharmaceuticals or industrial products (e.g. ICH S8). The majority of these suggest that standard toxicity studies, combined with trigger-based functional immune testing, represent an effective approach to evaluate immunotoxic potential (Boverhof et al., 2014). Among the various functional immune tests, the TDAR has been the consensus choice for a functional endpoint to identify immunotoxicity hazard in most, if not all, regulatory guidelines (Fischer et al., 2011; Koeper and Vohr, 2009). These approaches affirm the need to evaluate multiple aspects of the immune response to accurately predict immunotoxicity.

Table 4. *In vivo* tiered approach for detecting immune alterations in rodents

	Procedures
Tier I	
	Hematology
	Organ Weights – Spleen, thymus
	Cellularity – Spleen and bone marrow
	Histology of lymphoid organs
	IgM antibody plaque-forming cells (PFCs)
	Lymphocyte blastogenesis
	Natural killer cell activity Surface markers (peripheral or tissue immunophenotyping)
Tier II	
	IgG antibody PFC response
	Cytotoxic T lymphocyte cytolysis
	Delayed hypersensitivity response (DHR) Macrophage/neutrophil functional assays
	Host resistance (syngeneic tumors, bacterial, viral, and parasite models)

2. *In vitro* immunotoxicological assessments using primary cells

45. To maximize relevance to human immunotoxicology and to avoid inter-species extrapolation, it is recommended to use human cells for all *in vitro* tests. Although the use of primary human cells, which are available from peripheral blood or from buffy coats, are of highest clinical relevance, consideration can be given to the use of sufficiently well-characterized and largely used cell lines for certain aspects of the test systems (Gennari et al., 2005). Although continuous cell lines are not physiologically equivalent, many have proven to be valid surrogates but require appropriate characterization to ensure they are accurately recapitulating the normal immunological responses and functions (Boverhof et al., 2014). However, as cell lines are subject to genetic drift over excessive passages, it is important to control the stability of the cell line used. Another aspect that should not be disregarded, is that some compounds' immunotoxicity, especially drug-induced hypersensitivity, has been associated with specific HLA-types (Fan et al. 2017). Thus, the haplotype of the cell lines used should also be considered. If human cells are not available or human cell lines cannot be used, the use of non-human cells may be considered if the method proves to have acceptable predictive capacity or if the same response is expected both qualitatively and/or quantitatively, similar to what is expected using animals in toxicology (Corsini and Roggen, 2017; Lankveld et al., 2010).

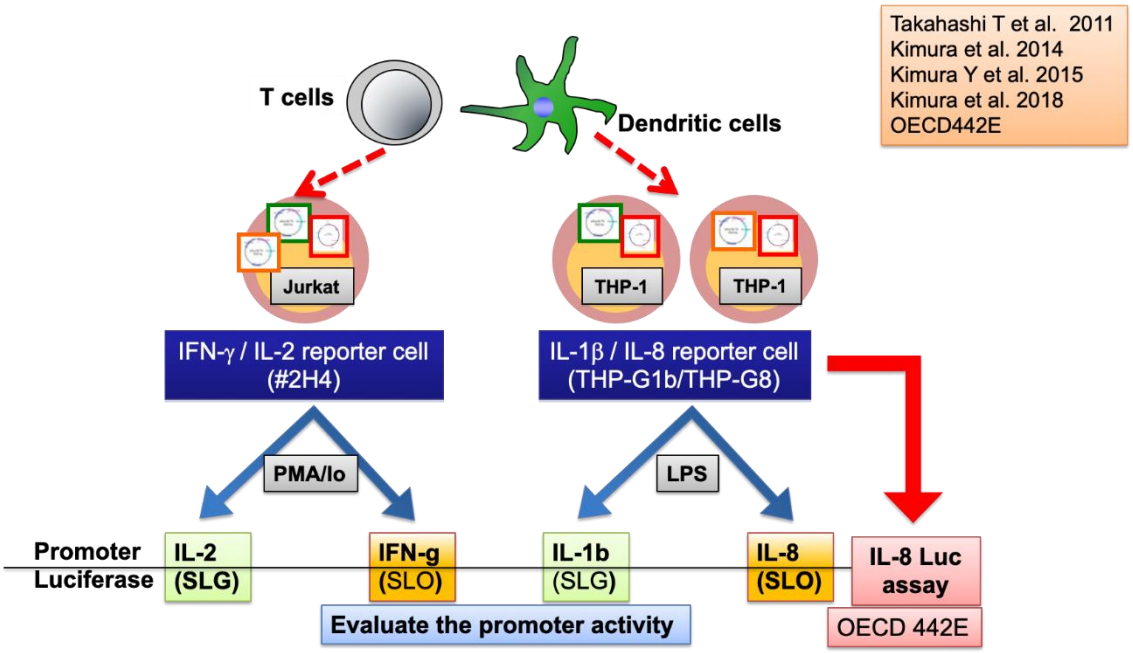
3. *In vitro* immunotoxicological assessments using cell lines

46. Most of the *in vitro* immune tests described in section IV use animals or human samples to obtain immune cells. The use of primary human cells for *in vitro* tests can entail challenges and

inconsistencies due to constraints around securing human samples, large variability among donors, poor reproducibility, inability to control donors' disease state and environmental factors, or the need to differentiate progenitor cells. Although continuous cell lines are not the physiological equivalent of primary cells or animals, many have proven to be valid surrogates if appropriately characterized to ensure they are accurately recapitulating the normal immunological responses and functions (Boverhof et al., 2014).

47. Almost a decade ago, a luciferase reporter assay system, MITA, was established to evaluate the effects of chemicals on the human immune system using stable reporter cell lines instead of primary human cells (Figure 1). The MITA is composed of 3 stable reporter cell lines: 1) 2H4 derived from Jurkat cells containing luciferase genes regulated by the IL-2, IFNγ, and G3PDH promoters (Saito et al. 2011), 2) THP-G8 cells derived from THP-1 cells containing luciferase genes regulated by the IL-8 and G3PDH promoters (Takahashi et al., 2011), and 3) THP-G1b cells derived from THP-1 cells containing luciferase genes regulated by the IL-1β and G3PDH promoters (Kimura et al. 2018). Using these cell lines, it has been demonstrated that MITA can reflect the effects of immunosuppressive drugs on cytokine expression by T cells or macrophages, and that the evaluation of drugs using the MITA was consistent with those obtained using the mother cell lines (Jurkat and THP-1 cells) or by using stimulated human whole blood cells ((Kimura et al. 2014) and Table 5).

Figure 1. Multi-ImmunoTox Assay (MITA)



MITA is an approach to detect immunotoxic chemicals using a combination of 3 luciferase reporter cell lines. 2H4 cells are used to evaluate the effects of chemicals on IL-2 and IFNγ promoter activity under stimulation with PMA/ionomycin. THP-G1b cells evaluate IL-1β promoter activity under stimulation with LPS, while THP-G8 cells evaluate IL-8 promoter activity under stimulation with LPS. The IL-8 Luc assay (OECD 442E) is an *in vitro* skin s35ensitization test that determines the induction of IL-8 promoter activity by chemical-treated THP-G8 cells. A modified version of the MITA (mMITA) is composed of the MITA and the IL-8 Luc assay (OECD 442E). In support of the initiative to increase the number of available *in vitro* immunotoxicity assays, two of the four reporter assays comprising MITA (IL-2 Luc and IL-1 Luc) are currently undergoing validation studies (Kimura et al. 2020).

48. When the performance of the IL-2 reporter assay was evaluated by examining immunosuppressive drugs whose effects in humans have been well-established (Table 5, reviewed by Allison 2000), the results demonstrated that the majority of the known agents caused reductions in IL-2 transcription including tacrolimus, cyclosporine A, dexamethasone, chloroquine, minocycline, sulfasalazine, ruxolitinib, tofacitinib, and baricitinib. However, decreased IL-2 transcription was not observed with several immunosuppressants whose mechanism of action is dependent on the inhibition of DNA synthesis or anti-proliferative effects on T cells, such as rapamycin, cyclophosphamide, azathioprine, mycophenolic acid, mizoribine, and methotrexate. Thus, it is critical to define the applicability domain for any proposed *in vitro* testing strategy to understand if the assay can appropriately assess the effects of certain compounds (e.g., those that require metabolism or target particular pathways).

Table 5. Evaluation of immunomodulatory drugs by MITA (modified from the original report by Kimura et al 2014 and Kimura et al 2018)

Principal Mechanism of Action	Drug	Effects of Transcriptional Activity			
		IL-2	IFNγ	IL-1β	IL-8
					IMMUNOSUPPRESSIVE DRUGS
Regulation of gene expression	Dexamethasone	↓	—	↓	↓
Kinase and phosphatase inhibitors	Cyclosporin A	↓	↓	—	—
	Tacrolimus	↓	↓	—	—
	Rapamycin	↑	—	—	—
JAK inhibitors	Ruxolitinib	↓	N	N	N
	Tofacitinib	↓	N	N	N
	Baricitinib	↓	N	N	N
Nrf-2 inhibitor	Dimethyl fumarate	↓	N	N	N
PDE4 inhibitor	Apremilast	↑	N	N	N
Alkylation	Cyclophosphamide	—	—	—	—
Inhibition of de novo purine synthesis	Azathioprine	—	—	—	—
	Mycophenolic acid	↑	↑	—	—
	Mizoribine	—	—	↑	↑
Inhibition of pyrimidine and purine synthesis	Methotrexate	↑	↑	—	—
					OFF-LABEL IMMUNOSUPPRESSIVE DRUGS
	Sulfasalazine	↓	↓	↓	↓
	Colchicine	↑	—	↑	↑
	Chloroquine	↓	—	—	↓
	Minocycline	↓	↓	—	—
	Nicotinamide	↑	—	↓	↓
					NON-IMMUNOMODULATORY DRUGS
	Acetaminophen	↑	—	—	↑
	Digoxin	↓	↓	—	—
	Warfarin	↑	—	↓	↓

JAK=Janus kinase, Nrf-2=nuclear factor erythroid 2-related factor 2, PDE4=phosphodiesterase 4

↑=stimulation, ↓=suppression, —=no effect, N=not tested

4. Combination of *in vitro* assays and clustering analysis has the potential to increase predictivity

49. As stated throughout this document it is likely that several assays will need to be used in combination to increase the ability to predict immunotoxicity using in vitro tests. For example, while the predictivity of the IL-2 Luc assay is high when testing T cell-targeting chemicals, combination with other immune function tests, in particular myelotoxicity tests or leukotoxicity tests, will be essential to cover global immunotoxicity. Methods for evaluating myelotoxicity *in vitro* using bone marrow culture systems (Haglund et al., 2010; Pessina et al., 2003) could be combined with the MITA to provide a more complete assessment. In addition, a novel luciferase assay designated as the IL-2 Luc LTT has recently been developed, that can detect the antimitotic effects of chemicals and can correctly identify immunosuppressive chemicals that were negative in the IL-2 assay (Kimura et al., 2021). Thus, the combination of the IL-2 Luc assay and the IL-2 Luc LTT can overcome some of the limitations of the IL-2 Luc assay alone (Kimura et al., 2021) and may be even more predictive when combined with a myelotoxicity assay.

50. Another example of this combinatorial approach is the addition of the MITA (IL-2 reporter assay in response to PMA/ionomycin [IL-2LA] plus the IL-8 reporter assay in response to LPS [IL-8+LPS]) with the IL-8 reporter assay for skin s37ensitization test (IL-8-LPS, OECD 442E), denoted as the modified MITA (mMITA). Sixty chemicals with well-known immunotoxic profiles were examined by the mMITA and were classified based on multiple approaches into a final group of six clusters with distinct characteristics. **Cluster 1:** chemicals that preferentially suppressed IL-8+LPS and showed a negative IL-8-LPS (preferential IL-8+LPS suppression); **Cluster 2:** those that suppressed IL-2LA and showed a positive IL-8-LPS, but did not affect IL-8+LPS (IL-2LA suppression and IL-8-LPS(+)); **Cluster 3:** those that suppressed both IL-2LA and IL-8+LPS and showed a positive IL-8-LPS (IL-2LA and IL-8+LPS suppression and IL-8-LPS(+)); **Cluster 4:** those that did not suppress either IL-2LA or IL-8+LPS and showed a negative IL-8-LPS (all negative); **Cluster 5:** those that suppressed both IL-2LA and IL-8+LPS but showed a negative IL-8-LPS (IL-2LA and IL-8+LPS suppression); and **Cluster 6:** those that preferentially suppressed IL-2LA and showed a negative IL-8-LPS (preferential IL-2LA suppression). The power of this approach is highlighted by the fact that although there were less well characterized chemicals tested, their potential *in vivo* effects could be inferred by comparing to well understood medicinal drugs that landed in the same cluster, such as sulfasalazine for Cluster 1, chloroquine for Cluster 2, colchicine for Cluster 3, acetaminophen for Cluster 4, dexamethasone for Cluster 5, and cyclosporine A and FK506 for Cluster 6 (Kimura et al., 2014; Kimura et al., 2018).

51. Overall, it is crucial to examine the correlation between *in vitro* assays and their *in vivo* effects. As demonstrated using the example of the mMITA, the clustering of chemicals using only three parameters may be inadequate to detect every aspect of their immunotoxic effects. However, such clustering in the context of a battery of complementary assays can be a first step to profile the immunotoxicity of chemicals.

. Discussion and Conclusion

52. Besides the presence of specific immune organs, immune cells are an integral part of other systems including the respiratory, dermal, gastrointestinal, neurological, cardiovascular, reproductive, hepatic, and endocrine systems. Consequently, exposure to immunotoxic compounds can have detrimental effects on the response to both communicable and non-communicable diseases. It is therefore important to understand the immunotoxic potential of xenobiotics and the risk they pose to humans.

53. Current practices in overt immunotoxicity testing are still varied and employ either experimental animals or humans themselves, while *in vitro* testing has predominantly been used to study specific mechanisms of immunotoxicity. There are several regulatory guiding principles in immunotoxicology, including IPCS/WHO Guidance for Immunotoxicity Risk Assessment for Chemicals (Harmonization project No. 10). All of these guidelines involve animal testing, and the current practice of risk assessment of chemical exposure is based to a large extent on animal testing.

54. While a number of non-animal-based testing methods for immunotoxicity have been published, not many have reached the stage of validation and acceptance. In accordance with the promotion of alternative testing methods and the global desire to reduce the use of laboratory animals, the purpose of this document is to provide the state-of-the-art knowledge for non-animal testing in immunotoxicology, and a way forward. Currently, a tiered approach is the most appropriate means to assess immunotoxicity, as described above. In the proposed tiered approach pre-screening for direct immunotoxicity *in vitro* begins by evaluating myelotoxicity (Tier 1). Compounds capable of damaging or destroying bone marrow cells will most likely have immunotoxic effects, as all immune cells derive from a common precursor located in the adult bone marrow. If compounds are not myelotoxic, they should be tested for direct leukotoxicity (Tier 2). Compounds should then be tested for immunotoxicity using various approaches, such as TDAR, lymphocyte proliferation assay, MLR, and NK cell assay (Tier 3).

55. It is likely that multiple assays will be required to define immunotoxicants because of the complexity and varied components of the immune system (e.g,. innate or adaptive immune responses). The combined use of several in vitro assays in IATAs or defined approaches, such as those used for skin sensitization, should increase the ability to predict immunotoxicity over an individual assay. It is critical to define whether the parent compound or a metabolite is mediating the immunotoxic effects, as immune cells have limited capacity to metabolize drugs. Even upon determining that a compound is immunotoxic, it is important to remember that there are interactions between the immune, nervous, and endocrine systems such that immunity is regulated by various neurotransmitters and hormones, which could also result in sex differences in the sensitivity to immunotoxicity.

56. At present there is no consensus on which assays to use, or how, and there are no OECD test guidelines to detect chemical immunotoxicity *in vitro*. Any validated test should therefore be part of a larger set of different assays that preferably covers all types of immunotoxicity including immunosuppression, sensitization, and autoimmunity. The MITA described in Section VIII, is one example of a combination of in vitro assays which may be used to predict the immunotoxicity of chemicals (and could be considered a potential future Tier 3 approach), with the potential for including additional endpoints which address gaps in the assessment of innate and humoral immunity. Comparing unknown chemicals to known immunotoxicants through clustered analyses such as those used in the

mMITA, information can be obtained on potential cellular targets and mode of action. Considering the incredible progress in 3D models, with engineered immune tissues and organs, such as bone marrow, thymus, lymph nodes and spleen being described, and microfluidic body-on-a-chip, it is reasonable to think that in the future it will be possible to assess immunotoxicity in an integrated model of the whole human immune system. While additional studies are certainly needed to define the possibility of identifying immunotoxic substances without the use of animals, the road is being paved for the use of integrated testing strategies that together hold the promise of being able to adequately predict the immunotoxic action of chemicals *in vitro*.

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. Appendix

Antibody Response: Alterations in antigen-specific T cell-dependent antibody responses. Antigens most commonly used include sheep erythrocytes and keyhole limpet hemocyanin.
Cell Proliferation: Alterations in the proliferative response following stimulation with mitogenic compounds such as concanavalin A, phytohemagluttinin, lipopolysaccharide or with allogenic leukocytes in a mixed lymphocyte reaction.
Clinical pathology: Indicates alterations in the leukogram or hematology parameters, such as leukocytopenia/leukocytosis, granulocytopenia/granulocytosis or lymphopenia/lymphocytosis.
CTL: The cytotoxic T lymphocyte assay measures cytotoxity against tumor or virally infected cells. For the reference compounds above, the majority of studies used either *ex vivo* or *in vitro* assays.
DTH: Alterations in delayed type hypersensitivity were measured by assessing the cell-mediated immune response to a soluble antigen such as keyhole limpet hemocyanin following sensitization and secondary challenge.
Lymphoid Organ weights: Decreases in the weight of spleen, thymus or relevant lymph nodes.
NK cytotoxicity: Natural killer cell cytotoxicity was measured using either *ex vivo* or *in vitro* assays which quantitate cytotoxicity against tumor cell lines or virally infected cells.