Food and Chemical Toxicology 133 (2019) 110801

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Review

# Exploring the use of current immunological assays for the developmental immunotoxicity assessment of food contact materials

Adejoke Ogungbesan*, April Neal-Kluever, Penny Rice

*FDA/CFSAN/OFAS, 5001 Campus Drive, HFS 275, College Park, MD, 20740, USA*

ARTICLE INFO

*Keywords:*
Immune system
Immunotoxicants
Critical windows of susceptibility
Immune assays
Developmental immunotoxicity
Food contact substance

ABSTRACT

The mammalian immune system is a highly complex, interactive network of cells that facilitates innate and adaptive immune responses. The neonatal immune system may be more susceptible to chemical perturbations than that of the adult. The effects of immunotoxicants during development may not be fully detected in toxicity studies performed on adult animals. Studies characterizing the ontogeny of the immune system in developing animals have shown that there are different critical windows of susceptibility to immunotoxicants. Developmental differences are evident among species compared to humans. Functional immune assays, such as the T-cell antibody dependent response assay, in rat models have been validated for use in the assessment of immunotoxicity with other assays. Recently, published studies have explored the feasibility of using additional techniques, such as *in vitro* studies using human whole blood cells or cell lines, mostly lacking either sensitivity or proper validation for regulatory purposes. However, some techniques may be developed further to enable translation of animal toxicity findings to human risk assessment of potential immunotoxicants. This paper summarizes the information on the developing immune system in humans versus rats and how the currently available assays might be used to contribute to the safety assessment of food contact substances.

## 1. Introduction

The immune system in mammals comprises diverse components that mount protective immune responses against foreign organisms and infections, as well as transformed, potentially-cancerous cells (Holsapple et al., 2018). Immunosuppression may lead to an altered resistance to infectious diseases and neoplasia. In contrast, inappropriate stimulation of the immune system results in hypersensitivity reactions, autoimmunity, and chronic inflammation (Holsapple et al., 2018; Ruehl-Fehlert et al., 2018). The mammalian immune system is made up of both the innate (antigen-nonspecific) and adaptive (antigen-specific) immunity involving a highly complex interacting network of cells (Holsapple et al., 2018; Lawrence, 2010, 2018).

The innate immune system, unlike the adaptive immune system, functions without previous exposure to an antigen (Loberg and Archer, 2018). Cells of the innate immune system express pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs); the toll-like receptors (TLRs); and nucleotide –binding oligomerization domain-like receptors (nod-like receptors [NLRs]) (Loberg and Archer, 2018; Nakayama, 2016). Cellular and humoral responses of the adaptive immune system are mediated by T and B cells, respectively, and require prior exposure to an antigen to mature to their final effector states. Dendritic cells (DCs) stimulate proliferation and differentiation of T cells into various $CD4^+$ T helper cell subtypes and $CD8^+$ T cytotoxic cells. B cells mediate humoral immunity by the production and secretion of antibodies (Bellamri et al., 2018; Loberg and Archer, 2018).

Immune responses are modulated by genetics, age, sex (endocrine-immune interactions), mental stress (neuroimmune interactions), in addition to exposure to chemical and environmental stresses. Likewise, endocrine and nervous system functions can be modulated by the immune responses. The endocrine, immune, and nervous system interactions are designated as the immune-neuroendocrine network (INEN), which includes interactions involving the central nervous system (CNS), the sympathetic nervous system (SNS), and the immune system. The physiological mechanisms responsible for the control of INEN activity include hormones, cytokines, neurotransmitters and neuropeptides, and their reciprocal influences on each other. (Manley et al., 2018).

Developmental immunotoxicology addresses the differences in the immune responses in children relative to those of adults, particularly regarding their responses to environmental exposures. It has been suggested that adult-onset autoimmunity may result from epigenetic

* Corresponding author. Division of Food Contact Substances (DFCS), Office of Food Additive Safety (OFAS), Center for Food Safety and Applied Nutrition (CFSAN), U.S. Food and Drug Administration (FDA), 5001 Campus Drive, HFS-275, College Park, MD, 20740, USA.
*E-mail address:* adejoke.ogungbesan@fda.hhs.gov (A. Ogungbesan).

https://doi.org/10.1016/j.fct.2019.110801
Received 14 June 2019; Received in revised form 23 August 2019; Accepted 1 September 2019
Available online 06 September 2019
0278-6915/ Published by Elsevier Ltd.

changes in immune cells induced by immunotoxicants during development (Blossom and Gilbert, 2018). The developing immune system in children has been identified as a potential target for chemically mediated toxicity, and the developing immune system is generally expected to demonstrate greater susceptibility to external stressors than the developed immune system of adults (Holsapple et al., 2018; Holsapple, 2003). Developmental immunotoxicity (DIT) may lead to adverse health outcomes that present in systems other than the immune system due to the fact that the developing immune system infiltrates an entire organism (Dietert et al., 2010; DeWitt and Patisaul, 2018).

The Food and Drug Administration (FDA) regulates food additives and packaging under the Federal Food, Drug, and Cosmetic Act (FFDCA). The FDA's Office of Food Additive Safety (OFAS) in the Center for Food Safety and Nutrition (CFSAN) is responsible for ensuring the safety of all substances deliberately added to food (direct food additives) and substances that may become a part of food as a result of migration from food packaging (indirect food additives). The FDA requires premarket authorization for food additives, as stated under section 409 of the FFDCA, and any substance added to food, directly or indirectly, is a food additive unless the substance is excluded from the definition of a food additive. Food contact substances (FCSs) are considered indirect food additives and FDA's premarket approval process for FCSs evaluates the human health effects of the proposed use of a given substance in food packaging, emphasizing the evaluation of the finished food contact article. The subject of the human health risk assessment is the US population which would be consuming food that had been in contact with food packaging containing the FCS. However, the FDA recognizes that infants, especially infants under the age of 6 months (consuming a sole source of nutrition as recommended by the American Academy of Pediatricians ([https://www.aap.org/en-us/advocacy-and-policy/aap-health-initiatives/HALF-Implementation-Guide/Age-Specific-Content/Pages/Infant-Food-and-Feeding.aspx#none ]), may be particularly sensitive to any adverse effects of a given food ingredient, due to several factors that have been recently discussed (Neal-Kluever et al., 2014). Recently, the US FDA issued a new guidance to industry regarding the premarket assessment of food contact materials intended for use with infant formula or human milk (FDA, 2019); the basis for this guidance was discussed in Neal-Kluever et al. (2014). This guidance describes approaches to the infant-specific exposure and safety assessment to potential migrants from infant food contact materials such as infant bottles, nipples, and formula or milk storage materials. During a safety assessment of an ingredient for use in infant formula or food packaging products, the evaluation is focused on the potential for migrants from FCSs to elicit effects on the developing immune, nervous, reproductive, endocrine, or skeletal systems (Neal-Kluever et al., 2014). This potential may be assessed by looking at effects of the substance or suitable structural analogs in traditional teratology or postnatal toxicity studies or in repeated-dose studies carried out in adult animals. The need for specialized DIT studies may be triggered by histopathological or lymphoid organ weight findings from the above-cited studies or other factors, such as pharmacokinetic data, that would indicate a high potential for immunotoxicity during development.

This article provides an update on the state of DIT testing for FCSs, discussing the FDA's current approach to evaluating the potential for FCSs to have adverse impacts on the developing immune system.

We provide a general summary of the immune system development focusing briefly on the effects of immunotoxicants at critical windows of immune system development, consider the histopathological ontogeny of the mammalian immune system, discuss currently validated methods and approaches to assess DIT, and provide two case examples of DIT assessment of FCSs.

## 2. Critical windows of immune system development and susceptibility to developmental immunotoxicity

Common infectious or immune-related diseases occur more often and are more severe in neonates or young children in comparison to adults, due to immaturity of the developing immune system that impairs their ability to exhibit adequate immune response (Collinge et al., 2012; Sewald et al., 2015). Type and severity of adverse effects on the immune system depend on the time of exposure during the development of the immune system, the type of immunotoxicant, the applied dose, and route of exposure. According to Burns-Naas et al., 2008, the critical developmental windows or stages of the mammalian immune system that could be impacted by immunotoxicants include:

1) Formation of hematopoietic stem cell from undifferentiated mesenchymal cells (which may be impacted by toxicants causing failure of stem cell formation, abnormal hematopoiesis, and/or immune failure [partial or complete]);
2) Migration of hematopoietic stem cells to fetal liver and thymus, early hematopoiesis, and migration of macrophages to tissues (impacted by toxicants causing thymic atrophy, impaired postnatal T cell function, impaired innate immunity, inflammation in specific organs [such as brain, testes, or lung] with macrophages involvement);
3) Establishment of the bone marrow as a primary site of hematopoiesis, and the bone marrow and thymus as primary sites of lymphopoiesis for B and T cells, respectively, (impacted by toxicants causing increased risk of later-life cancer, autoimmunity, or allergy);
4) Functional development and the maturation of immunocompetence (impacted by toxicants causing shifted T helper (Th) type 1/Th2 balance [possibly responsible for loss of the conceptus during pregnancy, increased incidence of childhood viral infection, reduced response to vaccinations]); and
5) Production of mature immune responses and establishment of immunologic memory (impacted by toxicants causing increased risk of infection and cancer, allergy/atopy).

Epidemiological studies have linked adverse effects of a substance on the neonatal immune system to increased incidences of chronic immunological disorders such as autoimmunity, immune deficiency, inflammation, and allergic reactions in adults (EFSA, 2017), implying that immunotoxicity during the neonatal or juvenile period may produce latent effects that only emerge in adult animals leading to life-long impairments. This effect may not be fully detected if the immune function is only evaluated in studies where dosing only occurs in adult animals (Collinge et al., 2012; Sewald et al., 2015).

## 3. Developmental immunotoxicology assessment in the mammalian system

The orchestrated and highly regulated developmental sequential events of the ontogeny of the vertebrate immune system are meticulously timed and well–coordinated starting in the early fetal life and continuing through the early postnatal development (Holsapple et al., 2018). Generally, the key developmental processes are the same across all mammals and define different critical developmental windows (or stages) of susceptibility to various immunotoxicants. However, there are differences in the timing of the developmental events relative to each trimester and birth, and the developmental differences *in utero* among species are evident when compared to the human (*see* Table 2). For instance, the immune system is developmentally delayed *in utero* in the rodent (the species commonly used for animal immunotoxicity and reproductive toxicity studies with FCSs) when compared to the human. Therefore, the fetal and neonatal rodents are not fully immunocompetent at birth (Johnson et al., 2018). The main human immune system development occurs in the first two trimesters, in contrast

**Table 1**
Development of immune organs in fetal rodent (mouse) vs. human[a].

| Event | Mouse | | Human | |
|---|---|---|---|---|
| | Days | % of full term (21 days) | Weeks | % of full term (40 weeks) |
| Development of fetal liver as a hematopoiesis site | 10.5 | 50 | 6 | 15 |
| Appearance of T cells in fetal liver | 14 | 67 | 6–8 | 15–20 |
| Development of Organogenesis of Thymus | 11 | 52 | 6 | 15 |
| Development of Secondary lymphoid organs | 10.5 | 50 | 7 | 18 |
| Appearance of Lymph nodes | 10.5 | 50 | 8–12 | 20–30 |
| Spleen Development | 13 | 62 | 10–14 | 25–35 |
| Development of B cell lymphopoiesis in bone marrow | 17 | 81 | 12 | 30 |
| Detection of B cells in blood | 13 | 62 | 12 | 30 |
| Detection of $CD4^+$ and $CD8^+$ T cells in spleen | 19 | 91 | 14 | 35 |
| Thymus development completed | 13 | 62 | 15–16 | 37–40 |
| Bone marrow as the major site of hematopoiesis | 17.5 | 83 | 22 | 55 |
| Expression of T cell receptor in periphery | After birth (Early post-natal) | Not applicable | 23 | 58 |

[a] Adapted from Johnson et al., (2018) (Fig.1).

to the rodent immune system in which these developmental events mostly occur during the mid-to late gestation periods and even continue to the postnatal period (Johnson et al., 2018).). Apart from species-related differences in developmental processes of the immune system, the differences between the sexes (sexual dimorphism) and the placentation of the species should all be taken into consideration in the interpretation of the data obtained from animal toxicological testing and its potential relevance to the human (Holsapple et al., 2018). The rat has been considered as a widely used (or well accepted) animal model for DIT studies (Holsapple et al., 2018). It appears that the DIT testing paradigm scheme of reproductive toxicity studies (including landmark events from gestation to weaning stages as outlined in Tables 1 and 2) for the assessment of DIT in rodents indicates that the general assessment of DIT dosing should begin prior to gestation day 7. This is to ensure that critical windows of immune system development occur during the exposure period (Johnson et al., 2018; *see* Table 1 and 2). In addition, recent interests and awareness of the relationship between toxicant exposure during development and increased risk of allergy or autoimmunity appear to suggest that the inclusion of endpoints that can detect immune system dysregulation in addition to suppression would be beneficial to the DIT assessment protocols in rodents and other animal models (Dietert and Holsapple, 2007; Johnson et al., 2018).

## 4. Histopathological ontogeny, hematopoetic development, and immunopathology/hematological and biochemical assessment of the mammalian immune system

The assessment of the mammalian immune system is known to be complex and comprises various organs and tissues such as the bone marrow and thymus (which are responsible for hematopoiesis and lymphocyte maturation, respectively), in addition to the lymph nodes and spleen (which produce responses to antigens). Other specialized tissues, such as skin-, lung-, and gut-associated lymphoid tissues located throughout the body, are responsible for producing responses to antigens or pathogens locally. These include the mucosa-associated lymphoid tissue (MALT), which is comprised of organized lymphoid tissues found in the mucosae of the gastrointestinal, respiratory, urogenital tracts, and conjunctiva (Ruehl-Fehlert et al., 2018). In man, domestic animals, and minipig, the MALT in the oronasal region is found to be significantly extensive, and the larger lymphoid nodules in the pharyngeal region representing the lympho-epithelial organs are identified as tonsils (Ruehl-Fehlert et al., 2018). The main MALT found in mouse and rat is the nasopharynx-associated lymphoid tissue (NALT). However, additional MALT found in the rat includes larynx–associated (LALT), bronchus-associated (BALT), and gut-associated (GALT) lymphoid tissues. BALT in some species such as mouse and humans is usually present when induced and therefore designated as iBALT (Ruehl-Fehlert et al., 2018). It has been reported that functional changes following toxicant exposure in these tissue-associated lymphoid tissues can produce cytokines and chemokines that can activate immune effector cells resulting in local or systemic effects (Johnson et al., 2018).

### 4.1. Histological features of the fetal and postnatal rat immune system

Studies have been conducted on the histopathological ontogeny of the fetal rat immune system (including thymus, spleen, and mesenteric lymph nodes), which was found to be easily recognized at GD 20 (Holsapple et al., 2018). At PND 22, the immune system was found to be anatomically intact in the developing rat, although the histologic features indicated relative inactivity. Although the fetal thymus has distinct corticomedullary relationships at GD 20, it lacks the dense cellularity observed at PND 22 (Holsapple et al., 2018). The spleen is discernible at GD 20, but it lacks the adult-type internal structures such as periarteriolar lymphoid sheaths (PALS), lymphoid follicles, or germinal centers. By PND 22, the spleen has the typical complement of PALS, with indistinct lymphoid follicles, and few germinal centers. The mesenteric lymph nodes are discernible by GD 20, although without lymphoid follicles; the mesenteric lymph nodes have distinct primary follicles, without secondary follicles and germinal centers, by PND 22. No discernible BALT or GALT, respectively, were observed at GD 20; however, at PND 22, small distinct aggregations of BALT and GALT were evident but apparently relatively inactive compared to young adult rats. Examination of the bone marrow of the fetal rats revealed a population of hematopoietic cells at GD 20 which lacked the intense cellularity observed at PND 22. Studies of the fetal liver indicated that at GD 15, approximately equal hepatocellular and hematopoietic elements were found in the liver and subsequently, the hematopoietic activity in the liver was reduced to some extent by GD 20, and finally reduced to trace levels by PND 22. The observed reduction in the hematopoietic activity in the liver was ascribed to the migration of hematopoietic activity to the bone marrow (Burns-Naas et al., 2008).

The histological features of the immune system and organs were characterized in Sprague-Dawley rats from birth to PND 42 (Parker et al., 2015). T-cell-related compartments (primary immune system organs) typically mature prior to B-cell-related compartments (secondary immune system organs) in the following sequence: bone marrow and thymus on PND 14; mesenteric lymph node on PND 21; Peyer's patches and BALT on PND 28; mandibular lymph node, NALT, and diffuse mucosal mononuclear cell population of small intestine on PND 35; and spleen on PND 42. However, histologic maturation of the organs may not correlate with functional immunocompetence (Parker et al., 2015).

**Table 2**
Approximate timelines for the assessment of certain critical windows of the developing immune system in rodent, dog, monkey, and human[a].

| Animal | Gestation | Parturition | Lactation | Weaning | Juvenile |
|---|---|---|---|---|---|
| Rodent (rat) | Thymic primordia, Pre-B cells; T-cell receptor (TCR) expression; mixed lymphocyte response (MLR), Selective mito-responsiveness | Gestation Day (GD) 22; Small numbers of T and B cells | Splenic demarcation (red & white pulp); Delayed-type hypersensitivity response (DTH); Maximal T-independent antibody response (TIAR); Splenic germinal centers | Postnatal Day (PND) 21; Detectable T-dependent antibody response (TDAR) | Young Adult (PND 42–49); Maximal TDAR; Adult level IgG. |
| Dog | Thymic and splenic primordia; Thymic corticomedullary demarcation; Thymopoiesis begins; Splenic mito-responsiveness. | Gestation Week (GW) 9; Detectable TDAR; normal allograft rejection; thymic mitogen response | Splenic germinal centers | Postnatal Week (PNW) 21 | Young Adult (1 year); Maximal TDAR |
| Monkey | Thymic primordia; Thymic corticomedullary demarcation; TCR expression thymus; All principal cell lines (T, B, NK) present in lymphatic organs; plasma cells and IgM detected; Splenic demarcation (red & white pulp); | GW 22 | Detectable TDAR (Week 13) | PNW 16-24 | Splenic germinal centers (Week 50); Young Adult (4 years) |
| Human | Thymic primordia; Small numbers of T and B cells; Splenic mito-responsiveness; Splenic demarcation (red & white pulp); | GW 38 | Splenic germinal centers; Maximal TDAR; Maximal TIAR | PNW 52-104 | Adult level IgG; Young Adult (18 years) |

[a] Adapted from Burns-Naas et al. (2008).

### 4.2. Hematopoetic and immune development assessment in juvenile rats

Immune and hematopoetic development in the spleen, blood, bone marrow, thymus, and lungs were evaluated in Wistar rats during the first 3 weeks of life (Sewald et al., 2015). From PND 2 to 21, red cell count increased steadily, hemoglobin and hematocrit remained constant, and mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) decreased. Newborn rat erythrocytes exhibited altered size and shape, in addition to abundant polychromasia, anisocytosis, and basophilic stippling. The white blood cell count decreased from birth to PND 10, then increased greatly during the third week; lymphocytes increased while segmented neutrophils decreased during PNDs 2–21. In bronchoalveolar lavage fluid (BALF), total cell numbers increased over time, with alveolar macrophages comprising 92–98% of cells. Serum immunoglobulin M (IgM) levels increased significantly from 4 μg/mL on PND 2–31 μg/mL on PND 21; IgG levels increased slightly during this interval. IgE levels were very low. The number of splenic B-lymphocytes were stable over time, while T-lymphocytes increased from 26% on PND 2–58% on PND 17, mainly due to increasing numbers of $CD8^+$ cytotoxic T-cell and $CD4^+$ T-helper cell subsets. Peripheral blood B-lymphocytes increased toward weaning, and peripheral blood T-lymphocytes increased slowly up to PND 17 and slightly decreased toward weaning, mainly due to $CD8^+$ cytotoxic T-cells. Bone marrow lymphocytes were comprised of mainly B-lymphocytes and low numbers of T-cells without major changes over time. Pokeweed Mitogen (PWM) had stimulatory effects on isolated splenic lymphocytes and increased proliferation levels, most prominently on PND 2 and PND 4. Concanavalin A (ConA) induced only a doubling of proliferation in the newborn, with no stimulation observed thereafter (on PND 14). The size ratio between medulla and cortex in the thymus changed considerably from PND 2 to PND 21. During this time, the cortex increased in volume and cortical lymphocytes colonized the medulla, and mitosis in medullar structures decreased over time (Sewald et al., 2015).

### 4.3. Histopathology/immunopathology assessment of the immune system

Both gross and microscopic examination of the primary (thymus) and secondary lymphoid tissues (spleen and lymph nodes), particularly lymphoid organs that undergo extensive proliferation and self-renewal, were found to play an important role in the assessment of potential immunotoxicants. It was observed that histological lesions of the thymus are sensitive indicators of immunotoxicity, with a reported good correlation of the thymic cortex lesions with altered antibody production (Johnson et al., 2018). Although histopathology evaluations have been the major endpoint of standard nonclinical toxicological testing and could be applied to the evaluation of DIT studies, some modifications are most probably needed due to the size and age of the animals, in addition to the special histological features of the immune system. The typical immunologically important organ weights (such as those of spleen, thymus, adrenal glands, prostate gland, liver, and brain) considered during a standard nonclinical toxicology study play a very essential part in the detail assessment of the immune system in DIT studies (Burn-Naas et al., 2008). Overall, it was concluded that immunopathology, including hematology, lymphoid organ weight and histology, and immunophenotyping, are important to characterize effects, but should not substitute for functional assessments (Dietert and Holsapple, 2007; Collinge et al., 2012).

### 4.4. Hematological and biochemical assessment of the immune system

The evaluation of hematological and biochemical is another endpoint of a standard non-clinical toxicity study which comprises total white cell count and differential leukocyte count in addition to serum albumin, globulin, total protein, and albumin:globulin (A:G) ratio determination (Burn-Naas et al., 2008). The evaluation of the various

changes in the levels of these entities following a toxicant exposure has been found to be useful in the assessment of immunotoxicity in toxicity studies. For instance, the administration of immunosuppressant compounds has been found to result in significant white blood cell counts (such as lymphocytes) reductions. Additionally, inflammatory disease processes may manifest as increased white cell counts (particularly a significant increase in granulocytes). Considering the fact that sample size limitations in DIT studies may hinder a complete pathology evaluation, the evaluation of hematology parameters can be more useful and ascribed a higher priority than clinical chemistry parameters evaluation (Collinge et al., 2012).

## 5. Immune assays and developmental immunotoxicity (DIT) assays

Various immune assays are often currently used to assess humoral or cell-mediated immunity in adult laboratory animals in addition to histopathology usually conducted in immunologically important organs (Johnson et al., 2018; Dietert and Holsapple, 2007; FDA Redbook, 2000). These assays include T-cell-dependent antibody response (TDAR), delayed type hypersensitivity (DTH) or contact sensitivity, T-cell cytotoxicity (CTL), Natural Killer (NK) cytotoxicity, cytokine production flow cytometry/cell counting immunophenotyping, and lymphoproliferation in response to mitogens (*see* Table 3). Some of these assays can be used to assess innate immunity as well. As noted below based on toxicity studies in rats, TDAR has been found to be more useful for PND 45 and older pups when compared with younger pups. Therefore, the other assays such as DTH, CTL, NK cytotoxicity, immunophenotyping, and lymphoproliferation assays can be combined with TDAR and developed further to assess developmental immunotoxicity in younger pups (under PND 45).

### 5.1. T-cell-dependent antibody response (TDAR)

The TDAR is particularly useful in assessing humoral immunity or antibody formation (Dietert and Holsapple, 2007; Hessel et al., 2015; Holsapple et al., 2018; Wang et al., 2018). The assay is a marker for immunosuppression hazard identification which usually involves antibody synthesis or response to various infectious agents and toxins through a complex series of events involving multiple cell types (Johnson et al., 2018). The evaluation of TDAR involves the use of various methods (such as antibody- or plaque-forming cell assay [AFC or PFC], enzyme-linked immunospot assay [ELISPOT], enzyme-linked immunosorbent assay [ELISA], or hemagglutination assays) to assess antibody responses induced by immunization with whole cells. However, considering the predictivity of the TDAR across the immune system developmental windows, a few studies indicated that younger pups (at PND 4 or 8) did not produce a detectable antibody response when compared with the older pups (PND 45) (Dietert and Holsapple, 2007; Ladics et al., 2007).

### 5.2. Delayed type hypersensitivity (DTH) or contact sensitivity/cytokines release

This assay is very useful in cell-mediated immunity assessment. Cell-mediated immunity involves reactions mediated by T cells. Cytokines released by antigen-specific T cells, such as $CD4^+$ T cells, provide the activation signals necessary for the bactericidal or cytolytic effects of macrophages for the elimination of the infection (Johnson et al., 2018; Dietert and Holsapple, 2007). The DTH response is useful in providing a comprehensive assessment of the response of T cells to intracellular infections, and the assay depends on at least two distinct functional components: 1) antigen specific T cells produced by Th 1 driven acquired immune responses; and 2) the recruitment of primed T cells to the challenge site or periphery by the tissue macrophage/dendritic cells (Dietert and Holsapple, 2007). Contact basophil hypersensitivity (CBH)

**Table 3**
Immune assays.

| Assay name | Endpoint/Immune Response assessed | Age of animals | Cell types involved | Readout method |
|---|---|---|---|---|
| TDAR | Adaptive (Humoral) immunity; T-cell dependent antibody production to KLH, SRBC, BSA, TT, bacteriophage φ X174, HsBSA | PND 45 and older | Antigen-presenting cells (APC; dendritic cells, macrophages); T helper cells, B cells | Plaque-formation (PFC assay) ELISPOT, ELISA, hemagluttinin assay |
| DTH | Adaptive immunity; Cell-mediated antigen-specific immune response to KLH, SRBC, egg protein, BSA | All ages | $CD4^+$ T helper cells, $CD8^+$ T cells, tissue APC | Measurement of swelling at injection site following challenge |
| Cytotoxic T-lymphocyte (CTL) response | Adaptive immune system cell-mediated cytotoxicity; against T-dependent antigens against SRBC, BSA, KLH; evaluating effective antigen presentation via the major histocompatibility complex (MHC) classes I and II. | All ages | APC, $CD4^+$ T helper cells, and $CD8^+$ T effector cells. | Measurement of CTL responses/proliferation by flow cytometry or other colorimetric, fluorometric, radioisotopic, or luminescence assays |
| NK cell assay | Adaptive (Cell-mediated) immunity and Innate immune system cell-mediated cytotoxicity (lack antigen-specific surface receptors); cytokines (e.g.tumor necrosis factor-α) release; viral infections control; Incubation of target cells with NK cells | All ages | NK cells; MHC class I alleles; macrophages | Measurement of NK cells cytotoxicity activity by flow cytometry or other assays |
| Flow cytometry/Cell counting immunophenotyping | Adaptive (Cell-mediated) immunity | All ages | Peripheral blood lymphocytes (e.g. B cells, T helper cells, T suppressor cells) and other leukocytes (such as neutrophils, eosinophils, basophils, monocytes) | Measurement by fluorescence detectors of fluorescence emitted from positively stained cells or particles |
| Mitogen-induced lymphoproliferation | Adaptive immunity; cell-mediated antigen non-specific immune response; mitogens: Con A, PWM, PHA, LPS | All ages | Peripheral blood mononuclear cells,T and B lymphocytes | Measurement of lymphocyte proliferation by flow cytometry or other colorimetric or radioisotopic assays. |
| Complement activation tests | Innate immunity; role in development of inflammation and tissue damage in autoimmune and inflammatory conditions. | All ages | Peripheral lymphocytes, antibodies (IgM, IgG), complement activation products or anaphylatoxins (C3, C3a, C4, C5a, etc.); detection antibodies | Quantification of target cells lysed or living cells by flow cytometry, or other colorimetric or radioisotopic assays (radioimmunoassays) |

has also been used to assess DIT cell-mediated immunity. The DTH assay can be very useful in complementing the TDAR in a functional test battery for DIT, particularly since DTH has been assessed in both juvenile and young adult rodents (Dietert and Holsapple, 2007). Monitoring changes in these antigen-specific T cells and cytokine release by immunotoxicants has a potential for the development of immunological biomarkers. However, there are certain limitations (including variability due to multiple genetic, physiological, and environmental factors) in the direct application of cytokine release as a robust biomarker in developmental immunotoxicology (Luster et al., 2005).

### 5.3. Cytotoxic T lymphocyte or T cell cytotoxicity (CTL) and Natural Killer (NK) cell cytotoxicity assays

Tumor cell lysis by NK cells has been found to be important in the assessment of innate immunity with regard to the impact of immunotoxicants (Johnson et al., 2018). The tumor cell lysis test involves the assessment of the lytic function by the quantification of the proportion of tumor cells (target cells) lysed following co-incubation with NK cells (effector cells) obtained from the spleen or peripheral blood (Johnson et al., 2018). The CTL and NK cell cytotoxicity assays are useful in assessing cell-mediated immune (CMI) response and are considered to be useful in complementing TDAR as primary screening assays as potential alternatives to the DTH response (Dietert and Holsapple, 2007). The NK cell cytotoxicity assay is also useful in assessing innate immunity in rodents and has been used to complement TDAR in evaluating DIT in rodents (Holsapple et al., 2018). Cytotoxic T lymphocytes (CTLs) are responsible for destroying host cells that are chemically or virally modified as well as neoplastic cells displaying tumor antigens. The CTL assay has been used in the assessment of influenza-specific CTL responses in laboratory animals as well as in the peripheral blood of humans, and targeting CTL responses is expected to play a very important role in the improvement of vaccine strategies for viral infections (Johnson et al., 2018; Quinones-Parra et al., 2014).

### 5.4. Flow cytometry/cell counting immunophenotyping

Flow cytometry is a laser- or impedance-based, biophysical technology used in cell counting, cell sorting, biomarker detection, and protein engineering. Flow cytometric analysis is important in assessing immunophenotyping of peripheral blood lymphocytes (such as B cells, T helper cells, T suppressor cells, NK cells and total T cells) in rats and can be combined with the evaluation of lymphocyte subpopulations in tissues to assess immunotoxicity (Luster et al., 1992). Flow cytometry plays an important role in measuring NK cell number, and although immunophenotyping has been found to be the best single predictor of immunotoxicity, the overall predictivity is improved immensely with its combination with other assays (Johnson et al., 2018).

### 5.5. Mitogen-induced lymphoproliferation

Polyclonal mitogens are non-specific agents that stimulate lymphocytes from the Go phase (resting phase) to subsequently enter the S phase of the cell cycle. Another indication of the overall immune system health is the potential proliferation of lymphocytes in response to mitogens. The polyclonal mitogens can be used to stimulate the proliferation of T cells, B cells, or both types of lymphocytes such that T and B cells are stimulated by different polyclonal mitogens (Johnson et al., 2018; Murphy et al., 2008). For instance, T lymphocytes are usually stimulated by mitogens such as phytohemagglutinin (PHA) and Con A, while B cells are stimulated by lipopolysaccharide (LPS). Both T and B lymphocytes are stimulated by PWM (Murphy et al., 2008). However, this assay is considered as a non-specific measure of the adaptive immunity and of limited predictive values since antigen receptors are not activated (Johnson et al., 2018).

### 5.6. Complement activation tests

The evaluation of complement activation is another test that has been found to play an important role in the assessment of innate immunity. The impact of immunotoxicants on the complement system can be conducted *in vitro* using human or animal serum and *ex-vivo* in serum and/or plasma following *in vivo* exposures (Johnson et al., 2018). The common endpoints for evaluation include the analysis of complement activation split products (such as C3a and C5a) by ELISA in addition to complement activity using the hemolytic assay (Johnson et al., 2018).

## 6. Functional/validated tests in juvenile animals and regulatory applications

There are several immune assays that have been validated for use in standard toxicity test paradigms for assessing adverse effects of a test article on the immune systems of adult animals. However, these assays have not been specifically validated for use in juvenile animals, and traditional developmental toxicity protocols assess few parameters related to immunotoxicity and no parameters directly measuring functional immunocompetence. With the increased awareness of the high sensitivity of the developing immune system to toxic insults, several groups have proposed strategies to assess DIT via modification of existing toxicity test protocols or by addition of specialized assays to the developmental toxicity test battery.

A workshop on juvenile testing evaluated the feasibility of adding a DIT component to the extended one-generation reproductive toxicity study (EOGRTS), Organization for Economic Cooperation and Development Testing Guidance document 443 (OECD TG 443) (Piersma et al., 2012). The workshop participants stated that since the ultimate goal is to predict the exposure risks for children, it is desirable that the protocol is relevant to the immunological experiences of children. Therefore, in addition to direct gavage dosing of the pups, the DIT assessment should include an immune challenge (e.g., immunization, vaccination, infectious challenge). The protocol should include evaluation of humoral (e.g., TDAR), cell-mediated (e.g., DTH), and innate immune function (e.g., macrophage production of metabolic and cytokine mediators) of a challenged immune system and enable supplementation of these data with histological, cell-counting and cytokine information (Piersma et al., 2012).

Most agreement is found with the recommendation of TDAR to measure humoral immunity (Dietert and Holsapple, 2007; Hessel et al., 2015; Wang et al., 2018). TDAR can be assessed in rat weanlings but is optimal after PND 42; in non-human primates, TDAR can be assessed as early as 2–4 weeks of age (Collinge et al., 2012). Rat weanlings show a sufficient PFC antibody response to SRBC, however, a high background was observed with ELISA for this age of animal (Ladics, 2007).

Choice of antigen to use for immunization should be carefully considered when conducting TDAR (Wang et al., 2018). The SBRC is usually the preferred antigen for the assessment of immunotoxicants in rodents; however, the use of SRBCs is not recommended in dogs due to hypersensitivity reactions (Wang et al., 2018). In rats, immunization with SRBC does not appear to alter hematology or clinical pathology parameters, but less is known about potential effects of using KLH; some evidence shows that KLH does not alter these parameters in non-human primates (Collinge et al., 2012).

For the assessment of cellular immunity, the DTH response is usually recommended. Potential alternatives to DTH are T cell cytotoxicity (CTL) and NK cell cytotoxicity assays, but studies comparing different ages using these assays have not been done (Dietert and Holsapple, 2007). Dietert and Holsapple (2007) reviewed the results from approximately 20 DIT studies and suggested options for effective DIT screening. As DIT may manifest as changes in immune balance or immunosuppression, functional information is needed. The tests considered to be effective and with good predictability were TDAR complemented with DTH, NK, or CTL. Dietert and Holsapple (2007)

suggested that supporting information could be obtained from cytokine production and flow cytometry/cell counting and considered histopathology of immune organs to be helpful but not a substitute for functional tests. The authors recommended the rat as the model for DIT assessment because of its history in toxicity testing, indicated that DIT should be incorporated into DART protocols where possible, and noted that the inclusion of both sexes is critical. Finally, they recommended dosing during all non-adult windows from gestation through lactation followed by direct dosing of pups after weaning (Dietert and Holsapple, 2007).

A 2012 International Life Sciences Institute Health and Environmental Sciences Institute (ILSI HESI) workshop on DIT testing of pharmaceuticals recognized the importance of functional DIT evaluations on a case-by-case basis (Collinge et al., 2012). For the most robust assessment of DIT, the workshop participants recommended a combination of immunopathology with an examination of humoral immunity (e.g., TDAR) and an evaluation of cellular immunity such as DTH, CTL, or innate immunity such as NK cell assay. However, they noted that while the TDAR assay has been fully validated, the NK cell, DTH, and CTL assays have not, although they are well characterized in animals (Collinge et al., 2012).

Hessel et al. (2015), reviewed nine compounds, (methyl mercury [MeHg], di octyl tin chloride [DOTC], ethanol [EtOH], di(2-ethylhexyl) phthalate [DEHP], heptachlor, tributyl tin oxide [TBTO], 2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD], methoxychlor [MXC], chlorpyrifos [CPF]), that have been extensively evaluated in multiple assays to compare the lowest observed effect level (LOEL) values for effects on DIT parameters with those of general developmental parameters. In conclusion, Hessel et al. (2015) noted that immune parameters were more sensitive endpoints than general developmental toxicity endpoints; the functional immune responses (such as TDAR and DTH) to challenge were more sensitive than histopathology; and that the data reviewed support the inclusion of multiple parameters covering both the antibody-mediated (or humoral) and cell-mediated immune responses.

## 7. Other studies- *In vitro* and "-omics" assays

Several alternatives to *in vivo* rodent immunotoxicity assays have been proposed in the literature, many based on human cells or transfected immune cell-derived cell lines. Methods for the assessment of the potential for inducing dermal sensitization using cell lines, *in vitro* reconstituted human epidermis, and/or in silico predictive software are the most developed, with many validated methods listed in the OECD test method database (Corsini and Roggen, 2017). These batteries of alternative methods, when used to assess single chemicals, reportedly have an accuracy level of 70–90% when compared to *in vivo* data (Settivari et al., 2017). However, this battery is not appropriate for use in testing complex mixtures, and individual tests are generally considered to be insufficient as standalone assays to capture the full *in vivo* response (Settivari et al., 2017). A corresponding battery of *in vitro* test methods have been proposed to assess the potential to induce respiratory sensitization, such as the Genomic Allergen Rapid Detection (GERD) assay and the Direct Peptide Reactivity Assay (DPRA), but none of these assays have been validated for use in predicting respiratory sensitization potential.

Two *in vitro* methods for assessment of cytotoxicity to immune cells—colony forming units-granulocyte/macrophage assays in human or mouse primary bone marrow cells and the human whole blood cytokine release assay have been validated for use in a tiered approach for predicting the ability of a given chemical to induce myelotoxicity or lymphotoxicity, respectively (Germolec et al., 2017). However, it should be noted that these assays are not considered valid for use in quantitative risk assessment of immunotoxicity and are only considered useful as hazard identification information and/or information on potential mechanisms of action of the immunotoxicant in question (Settivari et al., 2017). Additional human cell-based methods under development include the Multi-Immunotox Assay (MITA), which uses human immune cell lines stably-transfected with cytokine-responsive luciferase genes, and the Fluorescent cell chip (FCP) assay, which similarly uses transfected cell lines expressing cytokine-inducible green fluorescent protein. Others are *in vitro* models of B and T cell activation, differentiation, and function using purified human blood-derived B or T cells, such as the mixed lymphocyte reaction assay, the anti-CD3 T cell proliferation assay, and the cytotoxic T lymphocyte assay; and *in vitro* assays using human umbilical cord blood-derived hematopoetic stem cells to examine the effects of chemicals on human immune cell development (Phadnis-Moghe and Kaminski, 2017; Aiba and Kimura, 2017; Germolec et al., 2017; Holsapple et al., 2017). The Mishell-Dutton assay, the *in vitro* equivalent to the rodent TDAR assay, is conducted with primary rodent spleen cells and considered highly sensitive and predictive of immunotoxic potential, although this assay has not currently been validated for regulatory use (Settivari et al., 2017). Genomics data and enhanced flow cytometric data are also being explored as potential alternative methods to assess immunotoxicity; however, these data are not currently validated for regulatory use (Germolec et al., 2017). Zebrafish and other fish species have also been explored as potential non-mammalian animal models for use in human health risk assessment due to their fast generation times, ease of culture, and homology to human physiology in many aspects, including the immune system. However, as with the cell-based models above, zebrafish models have not been validated for use in the prediction of immune hazard in humans (Planchart et al., 2016; Johnston et al., 2017).

Systems biology approaches (involving simultaneous integration of different biological components and interactions of multiple organ systems mapped out for a given biological process) are being adapted to immunotoxicology and are potentially useful in identifying various immune biomarkers, in addition to gaining insights into the effects of agents on immune function such as immunostimulation or immunosuppression (Yucesoy and Gallucci, 2018). Such systems biology approaches include genetic, epigenetic, transcriptomic, and proteomic analyses. These approaches involve the integration of high-throughput systems-wide measurements (i.e., transcription, translation, metabolism, genetics, and epigenetics), which map causal cellular and molecular events leading to adverse effects, with predictive mathematical models that identify toxicity pathways and mode of action (MOA) of toxicants (Yucesoy and Gallucci, 2018). In conclusion, Yucesoy and Gallucci (2018), noted that systems toxicology approaches could be useful for the extrapolation of early molecular/cellular events to long-term adverse outcomes at the organism level, thereby providing valuable insights to more predictive and accurate risk assessment techniques.

Epidemiological studies in infants and children, may also provide useful information regarding the potential of a compound to affect the developing immune system and may be particularly useful for determining the relevance to humans of findings from studies in animals or generating hypotheses for subsequent targeted toxicological evaluation in animal models. A recent review of the state of the science (Duramad and Holland, 2011) recommended that multiple immune biomarkers be assessed at both the protein and mRNA level in an immune-response pathway-based approach in order to assess effects of exposure to an immunotoxicant in human populations (Genser et al., 2016). Additional endpoints for the assessment of potential developmental immunotoxicity include antibody responses to routine vaccinations, particularly childhood vaccinations; assessment of the incidences of common infectious diseases in exposed versus unexposed populations; and the assessment of the prevalence of diseases related to hypersensitivity (asthma, atopic dermatitis) or autoimmunity (lupus) in exposed versus unexposed populations (Genser et al., 2016).

## 8. Recent assessments of the immunotoxicity of certain widely used FCSs on the rodent immune system

Recently, immunotoxicity studies have been conducted with certain FCSs such as silver and bisphenol A (BPA) to investigate their impact on immunotoxicity in rodents. This section discusses how data on the effects of these FCSs on the immune system were incorporated into FDA's safety assessments.

### 8.1. Silver

As noted in the Babu et al. (2016) publication, silver is widely used due to its antibacterial properties, including its use in FCSs as packaging materials and in water purification filters as a disinfectant coating. The authors indicated that silver was found to be released from storage containers and that limited studies exist to determine the subchronic toxicity effects including immunotoxicity of silver via oral exposure. Therefore, a reproduction study with silver acetate was conducted in rats to assess the effects of long-term low dose exposure to silver acetate on reproduction and development (Babu et al., 2016). In the reproduction study (Babu et al., 2016), the DIT of silver acetate (AgAc) on the F1 offspring of male and female rats orally exposed (via drinking water) to AgAc at dose levels of 0, 0.4, 4, and 40 mg/kg bw/day for 10 weeks prior to and during mating, in addition to during gestation and lactation of the females, was investigated). Flow cytometry was used to assess the changes in immunophenotypic markers (such as $CD4^+$, $CD8^+$ and TCR + cells) of splenic and thymic subsets from F1 generation postnatal (PND) 4 and PND 26 pups of the AgAc-treated rats compared to the control animals. The results of the study indicated that spleens from PND 4 pups had lower percentages of $CD8^+$ T cells in the 4 and 40 mg/kg bw/day dose groups; splenic maturation increased in PND 26 pups compared to PND 4 pups; Con A- and LPS-mediated splenic responses were found to be lower in PND 26 pups of the 40 mg/kg bw/day group without any changes in cytokine (interleukin2; IL-2) release. PND 26 pup splenic NK cell activity was found to be higher in the 0.4 mg/kg bw/day group but unchanged in the 4 and 40 mg/kg bw/day group. In addition, PND 26 pup splenocyte phenotypic marker changes indicated that lower TCR + cells were observed at 4 and 40 mg/kg bw/day group and higher B cell population was found in the 40 mg/kg bw/day group. The authors concluded that maternal exposure to AgAc had a significant impact on the rat splenic development during the early lactation period (Babu et al., 2016). The offspring immunotoxicity no observed adverse effect (NOAEL) and lowest observed adverse effect level (LOAEL) values of 0.4 mg/kg bw/day and 4 mg/kg bw/day, respectively, were derived from the study based on changes in splenic endpoints including altered cell populations with a reduced response to mitogenic stimulus (Babu et al., 2016). It appears that the splenic NK cell activity determination was not a sensitive tool for immunotoxicity assessment in the study since this effect was not dose responsive. Additionally, another part of this developmental immunotoxicity study with AgAc included the toxicogenomic studies. In these studies, whole-genome microassays using gene expression profiling analyses to identify differentially expressed genes (DEGs) in each dose group were conducted to evaluate the effect of silver ions on the developing F1 generation rat thymus at the transcriptional level. The results indicated that no dose-dependent effect was observed on the number of DEGs in the dose groups (Gao et al., 2015).

Changes in splenic endpoints, altered cell populations, and gene expression profiling analyses could be important in developing biomarkers for determining the effects of a potential immunotoxicant and evaluating a point of departure for risk assessment. However, these studies need to be developed further (with regard to sensitivity of the test to detect dose responsiveness) and combined with well-established immunotoxicity assays and other data in order to fully assess DIT for the determination of a NOAEL from a toxicity study.

### 8.2. BPA

The effects of chronic BPA exposure on leukocyte compositions in the thymus and spleen of Sprague-Dawley rats treated with five doses (2.5, 25, 250, 2,500, and 25,000 μg/kg bw/day) of BPA from GD 6 up to 1 year of age were evaluated using extensive endpoints involving major populations of leukocytes including B cells, T cells, NK cells, granulocytes, monocytes, macrophages, and dendritic cells (Li et al., 2018a). In addition, some rats were evaluated with the vehicle (0.3% aqueous carboxymethylcellulose (CMC) or reference estrogen ethinyl estradiol (EE2 at dose levels of 0.05 or 0.5 μg/kg bw/day). Flow cytometry was used to quantify and analyze the leukocyte composition in spleens and the percentage of $CD4^+$ $CD8^+$ double-positive T cells in thymocytes. The authors concluded that chronic BPA treatment in rats from GD 6 to 1 year of age did not produce significant changes in immune cell composition (Li et al., 2018a).

In another part of the same DIT study with BPA, splenocytes isolated from the 484 BPA-treated rats were evaluated for the immunotoxic effects of BPA, including lymphoproliferation, immunoglobulin (Ig) production by B cells, and cellular activation of T cells, NK cells, monocytes, granulocytes, macrophages, and dendritic cells, following the chronic exposure to the five doses of BPA from GD 6 to 1 year of age (Li et al., 2018b). In this study, the spleens of the tested rats were harvested on PND 90, PND 180 (6 month), or PND 365 (1 year) and the isolated splenocytes were treated with LPS, PWM, or anti-CD3/CD28 for the induction of lymphoproliferation (Li et al., 2018b). For the evaluation of the effects of BPA on IgM responses, splenocytes were stimulated with LPS or PWM to induce IgM responses in B cells and the level of intracellular IgM induced by LPS was quantified by using flow cytometry. To investigate BPA effects on splenic T cell activation, splenocytes were treated with anti-CD3/CD28 to activate splenic T cells and the percentage of $CD25^+$ T cells (indicating anti-CD3/CD28 stimulation) were quantified using flow cytometry. Similarly, to evaluate BPA effects on NK cell activation, the percentage of $CD80^+$ or $CD86^+$ cell populations within activated $CD161a^+$ NK cells from LPS-treated splenocytes was quantified using flow cytometry. The characterization of BPA effects on the cellular activation of spleen-associated myeloid cell lineages involved the use of LPS-treated splenocytes for the activation of spleen-associated myeloid cell lineages such as $CD172\alpha^+$ cells (monocytes/macrophages/granulocytes) and $CD11b/c^+$ cells (macrophages/dendritic cells). Following the LPS stimulation, flow cytometry was again used to quantify the percentage of $CD86^+$or $MHCII^+$ cell populations within $CD172\alpha^+$ cells or $CD11b/c^+$ cells. In conclusion, 35 measurements out of the 630 measurements in BPA-treated rats were found to be statistically different from the vehicle controls, mainly involving increased lymphocyte proliferation when stimulated by LPS or PWM in 1-year old male rats (Li et al., 2018b). Although flow cytometry was useful in quantifying the effects of BPA on lymphocyte proliferation following the stimulation of splenocytes by LPS and PWM, stimulation of splenocytes and thymocytes by mitogens is considered a non-specific measure of adaptive immunity (Johnson et al., 2018) with limited predictive values such that it may not adequately assess DIT. Studies involving stimulation of splenocytes and thymocytes need to be combined with other data to fully assess DIT and determine a NOAEL.

## 9. Discussion

Several endpoints of DIT assays either lack sensitivity or have not been properly validated, although they can provide supporting information for DIT assessment. These assays include evaluation of cytokine levels, cell subtypes by flow cytometry, “-omics” approaches, and total serum immunoglobulin levels (Dietert and Holsapple, 2007; Collinge et al., 2012). Use of these endpoints as biomarkers in humans has not been validated. No models adequately characterize hypersensitivity or induction of autoimmunity. New approaches for the prevention of pediatric- and adult-onset chronic diseases or disorders

may need to be investigated further. Evaluation of immune-based disease patterns beginning in childhood may offer strategies for the prevention of DIT and pediatric immune dysfunction as well as treatment and risk reduction (Dietert et al., 2010).

TDAR and DTH assays have been validated for the assessment of humoral immunity and cell-mediated immunity, respectively (Dietert and Holsapple, 2007; Hessel et al., 2015; Wang et al., 2018), and NK and mitogenic stimulation assays are well characterized for use in various animal species (FDA Redbook, 2000). While significant advances have been made with *in vitro* testing, especially for contact sensitization, existing methods have considerable limitations for replacing animal testing (such as TDAR, KLH and DTH tests). Such limitations include the inability of *in vitro* methods to fully capture the intricate cellular interactions that exist within *in vivo* systems (Boverhof et al., 2014). However, although *in vitro* testing methods have not been validated, they may be useful in hazard identification and could provide important information in the assessment of DIT, particularly when combined with validated immunotoxicity tests (such as TDAR and DTH) and histopathology/immunopathology/clinical pathology. As indicated previously, it appears that *in vitro* test systems are mainly useful in providing more information or direct *in vivo/ex vivo* functional testing (Dietert and Holsapple, 2007), and the development of predictive *in vitro* assays using human cells has been suggested (Lebrec, 2013). The recent immunotoxicity studies on the effects of some FCSs such as AgAc and BPA on immunophenotyping of lymphocytes may provide useful insight into the development of biomarkers for DIT assessment at different stages of development in animals. The studies indicated that flow cytometry was useful in quantifying, analyzing and assessing the effects of AgAc and BPA on changes in immunophenotypic markers of splenocytes and thymocytes and leukocyte composition in spleens. Additionally, flow cytometry was used to quantify the effects of BPA or AgAc on lymphocyte proliferation following the stimulation of splenocytes by mitogens such as Con A, LPS, and PWM. However, as noted by Johnson et al., 2018, stimulation of splenocytes and thymocytes by mitogens is considered a non-specific measure of adaptive immunity and of limited predictive values such that it may not adequately assess DIT. Although the ability to determine the biological relevance of the findings in the study with AgAc is limited due to the study design, the effects observed at 40 mg/kg/d appear to be clearly adverse, as that dosage level is associated with persistent deficits in general mitogenic responses in T- and B-cells and changes in T- and B-cell subpopulations in the spleen. Moreover, due to the lack of testing of adaptive immune function beyond nonspecific mitogenic responses, it is unclear what effect these changes at $\geq$ 4 mg/kg/d would have on the immunocompetence of the adult organism. Given these uncertainties, the default position would be to assume that these changes are adverse until proven otherwise by additional data from appropriately designed studies. These additional studies should, at minimum, dose the F1-generation through achievement of immunocompetence and include at least one functional assessment of immunocompetence, such as TDAR or DTH. Bone marrow cytology and detailed assessments of immune system parameters in lymph nodes, as well as thymus and spleen, would also be helpful. As indicated above, the effect of silver ions at the transcriptional or genomic level was investigated using gene expression profiling analyses for the identification of DEGs of an immune organ such as the thymus. However, such toxicogenomic studies are not yet validated for DIT assessment. In summary, all these studies need to be characterized or developed further and used in combination with immunopathology, clinical pathology, and other well-established immunotoxicology assays in order to fully assess DIT at different stages of the immune system development.

Although established functional tests such as TDAR and KLH tests are commonly conducted in fully immune-function matured or post-weaning rodents (Sewald et al., 2015), it is important to consider adapting these tests for the evaluation of the immune response in juvenile rodents which possess maturing or developing immune system (since an interference with immunological maturation earlier in life may affect the immune response later in life). Considering the use of other animal species, it was noted that there are certain constraints in using other non-rodent species such as dogs or non-human primates in regulatory toxicity testing due to slower rate of development, lower litter sizes, and subsequently lower statistical power in addition to the fact that they require significant resources and time (Sewald et al., 2015).

Limited data are available on DIT in non-human primates, minipigs, and dogs. Most studies use *in utero* exposures only and study designs and protocols have not been thoroughly evaluated (Burns-Naas et al., 2008). However, for biopharmaceuticals, the non-human primates may be the only species for which the agent is biologically active, and DIT testing can be incorporated into embryo-fetal development, pre- and post-natal development, and juvenile studies (Collinge et al., 2012). Generally, similar methods can be used for evaluating DIT in rats and non-human primates, but limited reagents may be available for evaluating cytokine levels and immune cell surface markers in non-rodents.

## 10. Conclusion

Overall, the available information suggests that standard toxicity studies in combination with triggered-based testing approaches (such as hematology changes [altered counts of leukocytes comprising-granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes], histopathology findings, immune organs [such as thymus, spleen, lymph nodes] weight changes, clinical chemistry indicators [altered total serum protein, abnormal A/G ratio]) may not fully detect adverse effects of a toxicant on the immune systems of developing animals. The current immune assays have been generally characterized and validated in the mature immune system of adult animals. Therefore, the DIT assessment is an important aspect to be considered in the evaluation of multiple critical windows or stages during immune system development in juvenile and other immature animals utilized in a toxicity study. The age of the tested animals used to validate the assays and generate reference ranges should be carefully considered for DIT assessment. As indicated in the studies with silver ion and BPA, the effects of compounds on immunophenotyping of lymphocytes in immune organs and tissues may be useful in the development of biomarkers for DIT assessment at different stages of development. Additionally, the developing animal mechanistic studies, -omics, and *in vitro* studies are relevant tools that could become useful in investigating immunotoxicity as well as translating immunotoxicology study findings to human risk assessment (Boverhof et al., 2014; Ruehl-Fehlert et al., 2018). Since DIT may manifest as changes in immune balance or immunosuppression, TDAR complemented with DTH, NK, or CTL, were tests that have been considered to be effective and with good predictability. Additionally, it has been suggested that supporting information for DIT assessment could be obtained from cytokine production, flow cytometric immunophenotyping, and histopathology of immune organs (Dietert and Holsapple, 2007). It appears that the evaluation of cell mediated immunity, apart from humoral immunity, plays an important role in immunotoxicity testing, and DIT assessment should involve a multi-parameter approach (including a robust measure of cell mediated immunity via multiple immunotoxicity assays) to evaluate a point of departure for risk assessment of FCSs. However, the use of all these traditional and new immunotoxicity assays in DIT assessment is a work-in-progress. These assays will need to be developed or explored further and used in combination with other reproductive/developmental toxicity data for the determination of the point of departure for DIT assessment of food contact materials.

## Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We thank Drs. Jason Aungst, Antonia Mattia, and Mary Ditto for their helpful insight, comments and advice during the preparation of this manuscript.

## References

Aiba, S., Kimura, Y., 2017. *In vitro* test methods to evaluate the effects of chemicals on innate and adaptive immune responses. Curr. Opin. Toxicol. 5, 6–12.

Babu, U.S., Balan, K.V., Bigley, E., Pereira, M., Black, T., Olejnik, N., Keltner, Z., Sprando, R.L., 2016. Effects of maternal silver acetate exposure on immune biomarkers in a rodent model. Food Chem. Toxicol. 98 (Pt B), 195–200.

Bellamri, N., Morzadec, C., Fardel, O., Vernhet, L., 2018. Arsenic and the immune system. Curr. Opin. Toxicol. 10, 60–68.

Blossom, S.J., Gilbert, K.M., 2018. Epigenetic underpinnings of developmental immunotoxicity and autoimmune disease. Curr. Opin. Toxicol. 10, 23–30.

Boverhof, D.R., Ladics, G., Luebke, B., Botham, J., Corsini, E., Evans, E., Germolec, D., Holsapple, M., Loveless, S.E., Lu, H., van der Laan, J.W., White Jr., K.L., Yang, Y., 2014. Approaches and considerations for the assessment of immunotoxicity for environmental chemicals: a workshop summary. Regul. Toxicol. Pharmacol. 68 (1), 96–107.

Burns-Naas, L.A., Hastings, K.L., Ladics, G.S., Makris, S.L., Parker, G.A., Holsapple, M.P., 2008. What's so special about the developing immune system? Int. J. Toxicol. 27 (2), 223–254.

Collinge, M., Burns-Naas, L.A., Chellman, G.J., Kawabata, T.T., Komocsar, W.J., Piccotti, J.R., Shenton, J., Wierda, D., 2012. Developmental immunotoxicity (DIT) testing of pharmaceuticals: current practices, state of the science, knowledge gaps, and recommendations. J. Immunol. 9 (2), 210–230.

Corsini, E., Roggen, E.l., 2017. Overview of *in vitro* assessment of immunotoxicity. Curr. Opin. Toxicol. 5, 13–18.

DeWitt, J.C., Patisaul, H.B., 2018. Endocrine disruptors and the developing immune system. Curr. Opin. Toxicol. 10, 31–36.

Dietert, R.R., DeWitt, J.C., Germolec, D.R., Zelikoff, J.T., 2010. Breaking patterns of environmentally influenced disease for health risk reduction: immune perspectives. Environ. Health Perspect. 118 (8), 1091–1099.

Dietert, R.R., Holsapple, M.P., 2007. Methodologies for developmental immunotoxicity (DIT) testing. Methods 41 (1), 123–131.

Duramad, P., Holland, N.T., 2011. Biomarkers of immunotoxicity for environmental and public health research. Int. J. Environ. Public Health (8), 1388–1401.

European Food Safety Authority (EFSA), 2017. EFSA Journal Published. John Wiley and Sons Ltd on behalf of European Food Safety Authority.

FDA Redbook, 2000. Guidance for Industry and Other Stakeholders, Toxicological Principles for the Safety Assessment of Food Ingredients; 1993 Draft “Redbook II” Chapter VD. Immunotoxicity Studies.

FDA, 2019. Preparation of Food Contact Notifications for Food Contact Substances in Contact with Infant Formula And/or Human Milk: Guidance for Industry. CFSAN, US FDA, College Park, MD. https://www.fda.gov/media/124714/download.

Gao, X., Yourick, J.J., Topping, V.D., Black, T., Olejnik, N., Keltner, Z., Sprando, R.L., 2015. Toxicogenomic study in rat thymus of F1 generation offspring following maternal exposure to silver ion. Toxicol Rep 2, 341–350.

Genser, B., Fischer, J.E., Figueiredo, C.a., Alcantara-Neves, N., Barreto, M.L., Cooper, P.J., Amorim, L.D., Saemann, M.d., Weichhart, M.D., Rodriguez, L.C., 2016. Applied immune-epidemiological research: an approach for integrating existing knowledge into the statistical analysis of multiple immune markers. BMC Immunol. 17, 11.

Germolec, D., Luebke, R., Rooney, A., Shipkowski, K., Vandebriel, R., van Loveren, H., 2017. Immunotoxicology: a brief history, current status, and strategies for future immunotoxicity assessment. Curr. Opin. Toxicol. 5, 55–59.

Hessel, E.V., Tonk, E.C., Bos, P.M., van Loveren, H., Piersma, A.H., 2015. Developmental immunotoxicity of chemicals in rodents and its possible regulatory impact. Crit. Rev. Toxicol. 45 (1), 68–82.

Holsapple, M.P., 2003. Developmental immunotoxicity testing: a review. Toxicology 185, 193–203.

Holsapple, M., Bach, A., Blevins, L., Crawford, R., Li, J., Kaminski, N., 2017. The challenges ahead in immunotoxicity asessment: an in vitro model of human leukopoesis. Curr. Opin. Toxicol. 5, 28–32.

Holsapple, M., Prell, R., Costock, S., 2018. Developmental immunotoxicology testing (DIT). In: third ed. In: McQueen, Charlene A. (Ed.), Comprehensive Toxicology, vol. 11. Elsevier, pp. 467–497.

Johnson, V.J., Germolec, D.R., Luebke, R.W., Luster, M.I., 2018. Immunotoxicity studies. In: third ed. In: McQueen, Charlene A. (Ed.), Comprehensive Toxicology, vol. 9. Elsevier, pp. 255–270.

Johnston, H.J., Verdon, R., Gillies, S., Brown, D.M., Fernandes, T.F., Henry, T.B., Rossi, A.G., Tran, L., Tucker, C., Tyler, C.R., Stone, V., 2017. Adoption of *in vitro* systems and zebrafish as alternative models for reducing rodent use in assessments of immunological and oxidative stress responses to nanomaterials. Crit. Rev. Toxicol. 48, 252–271.

Lawrence, D.A., 2010. Overview of the immune system and immunotoxicology. In: second ed. In: Charlene, A. McQueen (Ed.), Comprehensive Toxicology, vol. 5. Elsevier, pp. 1–34.

Lawrence, D.A., 2018. Overview of the immune system and immunotoxicology. In: third ed. In: Charlene, A. McQueen (Ed.), Comprehensive Toxicology, vol. 11. Elsevier, pp. 1–28.

Ladics, G.S., 2007. Use of SRBC antibody responses for immunotoxicity testing. Methods 41 (1), 9–19.

Lebrec, H.N., 2013. Regulatory forum opinion piece*: immunotoxicology assessments in nonhuman primates–challenges and opportunities. Toxicol. Pathol. 41 (3), 548–551.

Li, J., Bach, A., Crawford, R.B., Phadnis-Moghe, A.S., Chen, W., D'Ingillo, S., Kovalova, N., Suarez-Martinez, J.E., Zhou, J., Kaplan, B.L.F., Kaminski, N.E., 2018a. CLARITY-BPA: effects of chronic Bisphenol A exposure on the immune system: Part 1 – quantification of the relative number and proportion of leukocyte populations in the spleen and thymus. Toxicology 396–397, 46–53.

Li, J., Bach, A., Crawford, R.B., Phadnis-Moghe, A.S., Chen, W., D'Ingillo, S., Kovalova, N., Suarez-Martinez, J.E., Zhou, J., Kaplan, B.L.F., Kaminski, N.E., 2018b. CLARITY-BPA: effects of chronic bisphenol A exposure on the immune system: Part 2 - characterization of lymphoproliferative and immune effector responses by splenic leukocytes. Toxicology 396– 397, 54–67.

Loberg, L.I., Archer, K.A., 2018. 11.16 - Assessment of Innate Immunity. Comprehensive Toxicology, third ed. Elsevier, C. A. McQueen. Oxford, pp. 343–360.

Luster, M.I., Johnson, V.J., Yucesoy, B., Simeonova, P.P., 2005. Biomarkers to assess potential developmental immunotoxicity in children. Toxicol. Appl. Pharmacol. 206 (2), 229–236.

Luster, M.I., Portier, C., Pait, D.G., White, K.L., Gennings, C., Munsen, A.E., Rosenthal, G.J., 1992. Risk assessment in Immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam. Appl. Toxicol. 18, 200–210.

Manley, K., Han, W., Zelin, G., Lawrence, D.A., 2018. Crosstalk between the immune, endocrine, and nervous systems in immunotoxicology. Curr. Opin. Toxicol. 10, 37–45.

Murphy, K., Travers, P., Walport, M., 2008. Janeway's Immunobiology, seventh ed. .

Nakayama, T., 2016. An inflammatory response is essential for the development of adaptive immunity-immunogenicity and immunotoxicity. Vaccine 34 (47), 5815–5818.

Neal-Kluever, A., Aungst, J., Gu, Y., Hatwell, K., Muldoon-Jacobs, K., Liem, A., Ogungbesan, A., Shackelford, M., 2014. Infant toxicology: state of the science and considerations in evaluation of safety. Food Chem Toxicol. 2014 70, 68–83.

Parker, G.A., Picut, C.A., Swanson, C., Toot, J.D., 2015. Histologic features of Postnatal Development of immune system organs in the sprague-dawley rat. Toxicol. Pathol. 43 (6), 794–815.

Phadnis-Moghe, A.S., Kaminski, N.E., 2017. Immunotoxicity testing using human primary leukocytes: an adjunct approach for the evaluation of human risk. Curr. Opin. Toxicol. 3, 25–29.

Piersma, A.H., Tonk, E.C., Makris, S.L., Crofton, K.M., Dietert, R.R., van Loveren, H., 2012. Juvenile toxicity testing protocols for chemicals. Reprod. Toxicol. 34 (3), 482–486.

Planchart, A., Mattingly, C.J., Allen, D., Cegar, P., Casey, W., Hinton, D., Kanungo, J., Kullamn, S.W., Tal, T., Bondesson, M., Burgess, S.M., Sullivan, C., Kim, C., Behl, M., Padilla, S., Reif, D.M., Tanguay, R.L., Hamm, J., 2016. Advancing toxicology research using *in vivo* high throughput toxicology with small fish models. ALTEX 33, 435–452.

Quinones-Parra, S., Grant, E., Loh, L., Nguyen, T.H., Campbell, K.A., Tong, S.Y., Miller, A., Doherty, P.C., Vijaykrishna, D., Rossjohn, J., Gras, S., Kedzierska, K., 2014. Preexisting CD8+ T-cell immunity to the H7N9 influenza A virus varies across ethnicities. Proc. Natl. Acad. Sci. U. S. A. 111 (3), 1049–1054.

Ruehl-Fehlert, C., Parker, G.A., Elmore, S.A., Kuper, C.F., 2018. Immune System. Fundamentals of Toxicologic Pathology, third ed. pp. 273–313 (Chapter 12).

Settivari, R.S., Krieger, S.M., Hindle, S., Gehen, S.C., Hollnagel, H.M., Boverhof, D.R., 2017. Current status of alternative methods for assessing immunotoxicity: a chemical industry perspective. Curr. Opin. Toxicol. 5, 19–27.

Sewald, K., Mueller, M., Buschmann, J., Hansen, T., Lewin, G., 2015. Development of hematological and immunological characteristics in neonatal rats. Reprod. Toxicol. 56, 109–117.

Wang, X., Coppi, A., Lebrec, H., 2018. T-Cell-Dependent antibody response assay: biology, methods, and application, third ed. McQueen Charlene A., Comprehensive Toxicology, vol. 11. Elsevier, pp. 376–398.

Yucesoy, B., Gallucci, R., 2018. 11.25 - Systems Biology in Immunotoxicology. Comprehensive Toxicology, third ed. Elsevier, C. A. McQueen. Oxford, pp. 559–581.