Chapter 3

Investigative Immunotoxicology

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Abstract

Immunotoxicology is the study of immune system dysfunction that can result from occupational, inadvertent, or therapeutic exposure to a variety of chemical or biologic agents that alter the immune system and affect human health. Immunotoxicology can manifest in a variety of ways, with one of the most prominent effects being immunosuppression. Immunosuppression can be defined as a reduced ability of the immune system to respond to a challenge from a level considered normal, regardless of whether clinical disease results. Although immunosuppression can lead to an increased incidence and severity of infectious and neoplastic disease, interpreting data from experimental immunotoxicology studies, or even epidemiologic studies, for quantitative risk assessment has been a persistent challenge. Decades of research has resulted in the development of specific assays and the identification of sensitive endpoints that measure effects on the immune response, from which many regulatory agencies have developed specific immunotoxicity testing guidelines. However, establishing a direct link between exposure and disease manifestations for immunosuppression in humans is an ongoing challenge due to inherent limitations of epidemiological studies to draw causal conclusions. Efforts have been made to examine the relationships between laboratory measures of immune response and disease resistance in experimental animal models and also in human studies. The identification of sensitive endpoints and the development of experimental assays to identify suspect immunotoxicants are a primary focus of the field of immunotoxicology. This chapter is organized around sections discussing the impact and scientific basis of immunotoxicity testing, predictive immunotoxicity testing strategies, examples of immunotoxicity testing, and key considerations and recent developments related to effective testing strategies for health risk reduction.

Key words Immunotoxicology, Immunosuppression, Host resistance, Xenobiotic exposure, Toxicology, Immunology

1 Introduction

Immunotoxicology is the study of immune system dysfunction that can result from occupational, inadvertent, or therapeutic exposure to a variety of chemical or biologic agents that alter the immune system and affect human health. In general the field has two broad research areas involving studies of the suppression of immunity and studies of enhanced or excessive immune response. Alterations to the immune system due to xenobiotic exposure can potentially increase the risk of infectious or neoplastic disease (immunosuppression), cause inappropriate immune

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responses to common substances (allergy/asthma), or result in responses to self-antigens (autoimmunity) [1]. Immunotoxicity is a growing concern for the chemical/agricultural, occupational, pharmaceutical, and consumer product industries, as well as federal regulatory agencies. Testing guidelines have been established to assess potential immunosuppression, allergic skin sensitization, and autoimmunity, although the most abundant and reliable data available to risk assessors pertain to suppression and hypersensitivity.

This unit will primarily focus on immunosuppression, which can be defined as a reduced ability of the immune system to respond to a challenge from a level considered normal, regardless of whether clinical disease results. In contrast to allergic reactions, which are generally clinically apparent and well-recognized by the general public, the impacts of immunosuppression can range from mild (reduced response to vaccination that does not result in disease) to severe (increased susceptibility to common and opportunistic pathogens and certain cancers) and are more difficult to directly correlate with exposure. Although immunosuppression can lead to an increased incidence and severity of infectious and neoplastic disease, interpreting data from experimental immunotoxicology studies, or even epidemiologic studies, for quantitative risk assessment has been challenging. The earliest classes of immunosuppressive chemicals studied included heavy metals (lead, cadmium, arsenic), halogenated aromatic hydrocarbons (HAH; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls (PCB), polybrominated biphenyls (PBB)), aromatic hydrocarbons (benzene and toluene), pesticides (trimethyl phosphorothioate, carbofuran, chlordane), aromatic amines (benzidine, acetylaminofluorene), and particulates (asbestos, silica, beryllium) [1, 2].

Investigational immunotoxicity requires the use of appropriate tests to assess immunological function in addition to the continual development and validation of these assays. Decades of research have resulted in the development of specific assays that measure effects on humoral immunity, cell-mediated immunity, macrophage function, natural killer (NK) cell cytotoxicity, cytokine activity, and host resistance for the purpose of identifying immunosuppressive agents. From this research, many regulatory agencies have developed specific immunotoxicity testing guidelines. This chapter is focused on discussing the impact and scientific basis of immunotoxicity testing, predictive immunotoxicity testing strategies, circumstances that result in immunotoxicity testing, and key considerations and recent developments related to effective testing strategies for health risk reduction.

2 Scientific Basis for Immunotoxicology

There is a well-established association between the therapeutic use of chemical immunosuppressants, such as those used in organ transplant therapy or in cancer chemotherapy, and an increased incidence of infections and neoplastic disease in humans [3]. Examples of cancers that are common in immunosuppressed individuals include leukemia and lymphoproliferative disorders, cancers of the skin in transplant patients, as well as Kaposi's sarcoma and Epstein-Barr virus (EBV)-associated B-cell lymphomas in AIDS patients [4]. In addition to immunosuppression induced by pharmaceuticals, there are also data suggesting other xenobiotics have negative impacts on the immune system. Beginning in the early 1970s, evidence began to accumulate demonstrating that exposure to certain environmental chemicals produced immune dysfunction [5]. This was typically evidenced through immune suppression, but in rare cases, stimulation was documented, and the most common health outcomes observed were increased incidences of certain cancers and pathogenic infections [6]. However, establishing a direct link between exposure and disease manifestations for immunosuppression in humans is an ongoing challenge due to inherent limitations of epidemiological studies to draw causal conclusions, particularly for common diseases like respiratory infections. Efforts have been made to examine the relationships between laboratory measures of immune response and disease resistance in experimental animal models as well as human studies. The ongoing need for the identification of sensitive endpoints and experimental assays to identify suspected immunotoxicants is a primary focus of the field of immunotoxicology.

A lack of standardized testing procedures has made it difficult to compare chemical-specific effects and has eventually led to the development of a "tiered" approach with the idea that each subsequent tier provided an opportunity to better define a specific target within the immune system [7]. An interlaboratory validation effort was sponsored by the National Toxicology Program supporting investigations of this tiered testing panel [8] to first establish the selection of a battery of immune assays used to screen potential immunotoxic compounds and then by an analysis using a relatively large database to determine the concordance between the identified immune endpoints and selected assays [9, 10]. These efforts evaluated reproducibility accuracy, as defined by the ability to obtain known or theoretical optimal responses from historical data; assay sensitivity as determined, in part, by obtaining dose-response curves; and predictivity, as judged by correlations with other functional and host resistance tests using known toxicants. Successful fulfillment of these criteria in chemical risk assessment is ultimately necessary for establishing an accurate database of chemical "immunotoxicants" as well as for providing a

grading system for comparison and human risk assessment. These seminal studies were important, not only as a validation exercise for tier testing but for providing a basis for risk assessment using immunotoxicology data.

From these studies it was determined that since the status of the immune system was not measurable until it was challenged, tests that incorporate an antigenic challenge would be the most appropriate. These highlighted several assays including evaluations of humoral and cell-mediated immunity. A decrease in cellular or humoral immune response to vaccination (influenza vaccine, common childhood vaccines, hepatitis antigen) is thought to be a sensitive indicator of immunosuppression in humans and can reflect susceptibility to infectious disease; however, an inadequate response to antigenic challenge may not always represent an "adverse effect." While a significant change in immune function can be considered deleterious, in that it may increase the risk of developing clinical disease, a single alteration in immune function does not necessarily precipitate a disease or clinical health affect. For example, immunocompromised individuals could function normally in the absence of infectious agents. This supported the inclusion of host resistance assays which measure immune responses following administration of low levels of infectious agents or transplantable tumors to help predict the potential for xenobiotics to alter host susceptibility in the human population.

In general, tier 1 is comprised of a series of preliminary in vivo screening assays intended to identify suspect immunotoxicants. This tier includes evaluations of immunopathology, humoral-mediated immunity, cell-mediated immunity, and nonspecific immunity. Tier 2 tests are utilized to identify the specific immune target responsible as well as evaluate effects on host resistance. These include immune cell profiling, more advanced assessment of humoral and cell-mediated immunity, and host resistance challenge models. Tier 3 tests are not as well-defined but are focused on identifying mechanism of action.

Data collected from tiered screening panels have been the basis for several risk assessment guidelines, and most regulatory agencies in the United States (USA), European Union, and Japan have established requirements or guidelines [11]. However, the configurations of these testing panels vary depending upon the agency/organization/program under which they are being conducted. Under chemical regulations, pesticide registrations require the completion of a substantial number of toxicity studies, with a recent additional requirement to conduct specific immunotoxicity assays [12]. In contrast, guidance for pharmaceuticals uses a weight of evidence (WoE) approach that only requires specific immunotoxicity assays if a cause for concern is identified in standard toxicity studies. While originally developed using animal models, these tiered schemes have since been adopted

for human assessment; however neither the sensitivity nor the predictivity of these test panels has been established [8, 13].

Although the exact social and economic impacts related to immunotoxic agent exposure are not known, data suggest that due to the severity and incidence of infectious and neoplastic diseases, even small changes in disease frequency may have major impacts. Mortality and to a lesser extent morbidity, resulting from exposure to common pathogens such as influenza and pneumonia, have been determined and are used in the risk management process. Deaths have the most costly impact on society, and in 2006, the age-adjusted death rate for influenza and pneumonia was 0.3 and 17.5 per 100,000, respectively [14]. Together these infections were ranked as the ninth leading cause of death in the United States for all ages in 2010 [15]. While the impact on the general public is evident, individuals working in the healthcare sector are at an increased risk for exposure to influenza and other pathogens. From an occupational perspective, the Centers for Disease Control and Prevention (CDC) estimates that approximately two million healthcare-associated infections occur annually in the USA and are associated with nearly 100,000 deaths each year. Among eight different reports of nosocomial influenza outbreaks in healthcare settings, the infection rate of staff members ranged from 8 to 63% [16], with the additional economic burden of a single nosocomial influenza outbreak at a hospital estimated to cost $34,179 [17]. These data provide support for the scientific basis and importance of immunotoxicity testing.

3 Predictive Immunotoxicity Testing Strategies for Health Risk Reduction

Understanding of the mechanisms of action of the xenobiotic and the relationship between the effective biologic dose and the various immunologic and adverse effects is critical for the assessment of human risk. Since relatively little is known about the relationship between qualitative and quantitative changes in immune endpoints and development of clinical disease, particularly at the low end of the dose-response curve, the big questions has been “What is the simplest testing configuration that can accurately identify immunotoxicants?” Early efforts were made to establish the most predictive endpoints and assays related to immunotoxicant exposure in animals [9, 10]. It was the general consensus from these studies that functional testing provides the greatest sensitivity for identifying immunosuppression, but before any conclusions can be made, a battery of tests evaluating immunotoxicity must be conducted. Based on the analysis of a series of known immunotoxicants, it was established that evaluation of the primary antibody response and enumeration of lymphocyte populations provided a high frequency of concordance relative to the existing test battery. The humoral immune response including the production, the release, and an

increase in circulating levels of antigen-specific antibodies is important for protection against infectious agents and for prevention or reduction of severity of influenza, respiratory infection, colds, and other diseases. Reduced antibody production is an indication of decreased immune function or immunosuppression that may indicate a greater risk of disease. Antigen-specific IgM to a T-cell-dependent antigen is considered one of the most predictive measures of overall immune function in rodents because cooperation between T-cells, B-cells, and antigen-presenting cells is required to develop an antibody response [9]. This, along with a nonfunctional test, such as thymus weights or lymphocyte enumeration, allowed achieving concordance of well over 90%, with respect to identifying potential immunotoxic agents [9, 10].

Host resistance is typically the standard against which other assays are judged because altered resistance is a biologically plausible effect with clear relevance for potential adverse effects in humans. Host resistance endpoints have evolved from relatively nonspecific (animal morbidity and mortality) to quantitative, such as tumor growth and infiltration, viral titers, or bacterial cell counts, which has increased the sensitivity of the assays. In the seminal studies described by Luster et al., a strong correlation was also identified between changes in immune function and altered host resistance, in that there were no instances when host resistance was altered without affecting immune tests [10]. However, alterations in the immune tests can be observed in the absence of detectable changes in host resistance. The predictive power of commonly used assays for altered host resistance assays varies, although concordance rates can reach 100% when data from multiple assays are combined [9, 10]. While experimental animal models provide an opportunity to collect reliable data from immune assays examining absorption, distribution, metabolism, and excretion, which cannot be collected in human studies, extrapolating these findings across species is a concern. Establishing the quantitative relationship between altered immune responses and frequency or severity of disease in human populations is challenging, as humans are genetically dissimilar and heterogeneous from an environmental and lifestyle standpoint. There are several confounding factors that can influence the translation of the results from animal studies to the human population. These include, but are not limited to, genetics, age, gender, nutritional status, stress, varying degrees and lengths of exposure, and prior disease status. However, human immunotoxicity exposure studies are powerful in that they do provide information about realistic exposure scenarios, such as multiple routes of exposure, and include a much more diverse range of genetic backgrounds than experimental animal models while providing information about potential health outcomes

commonly documented as increased incidences of certain cancers and common infections [18, 19]. Experimental designs can be difficult, and investigators often have to rely on the identification of exposed and unexposed populations to conduct their studies. These studies can range from clinical trials to large, population-based, observational studies. Clinical studies are particularly useful as they can provide data on the frequency of infections or the level of immune response to vaccines. Epidemiological studies can be observational with a small sample size in individuals with transient high-level occupational exposure or large groups with chronic low-level exposures. These are often the result of accidental exposures. The function of the immune system in humans is typically investigated by strictly noninvasive methods that are not always easy to standardize and therefore are often limited to blood collection. Select endpoints have been proposed and utilized to evaluate human immunotoxicity, and these include human antibody titers, natural antibody levels to ubiquitous antigens, secondary antibody response to proteins and polysaccharides, immunophenotyping, NK cell activity or numbers, clinical chemistry, and hematological profiling. While peripheral cell counts and differentials are a common endpoint in human studies, they are not always sensitive markers for immunosuppression [20]. Quantitative functional data (responses to novel antigens) can generally predict resistance to infectious agents and tumor cells providing insight into the potential consequences of suppression. Antibody responses can be examined by measuring antigen-specific antibody levels after vaccination in humans, and this is often conducted with commercial vaccines such as hepatitis antigen [21, 22], influenza vaccine [23], or common childhood vaccines [24].

In humans, mild-to-moderate suppression of the immune response is linked to reduced resistance to common community-acquired infections, whereas opportunistic infections, which are very rare in the general population, are common in individuals with severe suppression. Data regarding the incidence and prevalence of acute, chronic, and opportunistic infections provides important information about human risk assessment. However, virulence of the organism, infectious dose, integrity of the host's anatomical and functional barriers, type of pathogen (common, opportunistic, or latent) and its mechanism of pathogenicity, as well as overall immunocompetence of the individual have been shown to influence risk assessment. It may be assumed in the general population that an infectious disease such as influenza may develop in any individual independent of their immune capacity or prior immunization, provided that the quantity or virulence of the challenging agent is sufficient to overwhelm that individual's defense capacity.

4 Examples of Evaluations of Immunotoxicity

While testing for potential immunotoxicity in experimental animals has gained increased acceptance, limited systemic epidemiological immunotoxicological studies have been undertaken due to a number of difficulties in working with human populations. Occasionally accidental exposures occur and thus present unique experimental populations to study. Although the results of investigations into such incidents are open to much criticism, there should be a counterbalance by understanding of reactionary conditions in which results had to be obtained. There have been several studies that have demonstrated agreement of immunotoxicity between human and animal studies. These include but are not limited to investigation of perfluorinated compounds (PFCs), halogenated aromatic hydrocarbons (HAHs), pesticides, heavy metal, and solvents. Select examples of human and animal studies are described in this section.

Recently the PFC, perfluorooctanoic acid (PFOA), has been investigated for suspected immunotoxicity. PFOA is a synthetic, highly stable chemical that is used in manufacturing of protective coatings for carpets, stain- and grease-resistant clothing, paper coatings, and nonstick pans [25]. Because of its high stability and extremely low surface tension, it is used in numerous consumer and industrial applications. While PFOA was identified in all serum samples tested for perfluorinated compounds from the general US population in the 1999 National Health and Nutrition Examination Survey (NHANES 1999–2000) [26], serum PFOA levels for individuals who had occupational exposure were typically found to be ~ 4 to 5 times greater than the general US population [27]. A relatively recent cohort of workers exposed to PFOA at a DuPont chemical plant in Parkersburg, West Virginia, has provided information about the immunotoxicity of this chemical. In addition to occupational exposures, these workers were also exposed to PFOA in drinking water contaminated by production facilities. Reduced antibody titers to influenza vaccine [23] and an increased incidence of kidney and testicular cancer [28] were found to be correlated with increased serum PFOA levels for individuals who worked at this plant. In addition, occupational exposure to PFOA has been linked to health effects such as prostate cancer and liver disease, malignant and nonmalignant renal disease, diabetes mellitus, chronic renal disease, and hypothyroidism [27, 29, 30]. However these findings are not always supported by other studies, and the translation to human health effects has often been controversial [18, 31, 32].

In addition to occupational exposure studies, other human exposure studies have reported immunotoxicity related to PFC exposure. Emmett et al. reported a slight increase in absolute monocyte counts of residents who lived in a water district contaminated with PFOA; however, there was no significant

relationship between serum PFOA and the percentage of monocytes in differential white cell counts [32]. Brieger et al. explored the impact of PFOA and perfluorooctanesulfonic acid (PFOS) on selected functions of human leukocytes in vitro and reported that PFOA and PFOS were associated with reduced NK cell activity and diminished release of the pro-inflammatory cytokine TNF-α following lipopolysaccharide (LPS) stimulation [33]. Two recently published studies indicate that early childhood exposure to PFCs may result in immune consequences [24, 34]. In a prospective study of a birth cohort from the National Hospital in the Faroe Islands involving a total of 587 children, the investigators found that 5- to 7-year-old children with high serum concentrations of PFCs did not respond as well to diphtheria and tetanus immunization compared to children with lower PFC levels. A twofold increase in serum PFC concentrations at 5 years of age was reported to reduce responses to tetanus and diphtheria booster immunizations by half. In a second prospective birth cohort study, investigators found an inverse association between the levels of anti-rubella antibodies in children at age 3 years and maternal plasma concentrations of PFCs; however, no significant associations were identified between the concentrations of PFC and antibody titers to other vaccines [24]. In general, while data on the immunotoxicity of PFCs such as PFOA in humans are limited, they seem to suggest the exposure may be associated with immunosuppressive effects. Supporting the epidemiological findings, data consistently exhibits that PFOA is immunotoxic in animals. For example, decreased spleen and thymus weights and cellularity, along with suppression of the primary antibody response, as determined by antigen-specific IgM antibody production to single challenge with T-cell specific antigens in PFOA exposed mice have been demonstrated [35, 36].

Probably the most extensively studied class of environmental pollutants are halogenated aromatic hydrocarbons (HAHs), including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins, dibenzofurans, and polybrominated biphenyls (PBBs) [37]. These compounds, many of which are widespread in the environment, are primarily used in commercial production of industrial chemicals, pesticides, flame retardants, and heat conductors.

An accidental poisoning in Taiwan in 1979 involving over 2000 people was a result of PCB exposure. The source of poisoning was a particular brand of rice-bran oil that was accidently contaminated by PCBs. Examination of the immune system function in the patients at 1 year postexposure revealed decreased concentration of IgM and IgA but not IgG; decreased percentage of total T-cells, active T-cells, and helper T-cells; enhancement of spontaneous lymphocyte proliferation; and enhancement of lymphocyte proliferation with phytohemagglutinin (PHA), pokeweed mitogen (PWM), and tuberculin (PPD) stimulation but not concanavalin A (ConA) [38].

Several birth cohort studies have subsequently investigated PCB exposure and immune responses. In one study, data from families participating in a prospective birth cohort in eastern Slovakia was examined to determine if early life exposure to PCB influenced the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine [39]. At birth, maternal and cord blood were collected for chemical analyses, and infants were immunized with BCG. Six months later, blood was collected from infants for chemical analyses and to determine BCG-specific IgG and IgA levels. The association suggests that PCB exposures result in decreased responses to BCG vaccine. Healthy mother-infant pairs were evaluated for PCB exposure in maternal plasma, cord plasma, and breast milk, and results indicate that prenatal exposure to PCBs was also associated with changes in the T-cell lymphocyte population in health Dutch infants [22]. Prenatal PCB exposure was associated with an increased number of total lymphocytes, T-cells, and lower antibody levels to mumps and measles at preschool age along with a higher prevalence of recurrent middle-ear infections [22, 40]. In addition, select occupational exposure and epidemiologically cohorts have reported associations between PCB exposure certain cancers including respiratory cancers [41], breast cancer [42], prostate cancer [43], and non-Hodgkin lymphoma [19]. While these studies collectively support a PCB exposure-related functional impact on the human immune system, mixed findings have been reported in animal studies. The only immune parameter that was consistently altered in animal studies was the antibody response to SRBC, where significant dose-related suppression was noted in both the primary IgM response and the secondary IgG response [44]. Although not as consistently observed, enhanced lymphocyte proliferation, decreased thymic weights, and suppression of NK cell activity have also been reported in animal models [45].

While not as well-characterized as PCBs, PBBs have also been investigated for immunotoxicity. The Michigan PBB example is one of the most well-investigated accidental exposure studies. A commercial preparation of PBB was accidently used in place of an inorganic ingredient in preparing a feed supplement for lactating cows [46], and subsequently, PBB was inadvertently used in agriculture throughout Michigan in 1973 and 1974. Besides exposure to livestock, this accident resulted in human exposure to the PBB because the agent contaminated not only beef and dairy cow products but also poultry and eggs. Identification of PBB as the toxic ingredient did not occur until 9 months after the contamination, by which time the PBB had become widely distributed throughout the state. Elevated levels of PBB were identified in serum and adipose tissues of exposed individuals at least through 1980 [47]. Although the original study had statistical flaws, the authors concluded that in at least 18 to 45 Michigan residents sampled, a statistically significant decrease in absolute number of T-cells as well as decreased mitogenic response to standard T-cell mitogens was demonstrated. Interestingly, a persistent increase in NK cells in

many of the same subjects was still observed when retested 5 years later [48]. Additional reported abnormalities included hypergammaglobulinemia, exaggerated hypersensitivity response to streptococci, and increased number of lymphocytes. The circulating blood lymphocytes of these residents also showed significant abnormalities including decreases in the numbers and percentages of peripheral blood lymphocytes that form rosettes with either sheep red blood cells (SRBC) alone or with SRBC sensitized with antibody and complement. Significant reduction of in vitro immune function was noted in 20–25% of the farm residents who had eaten food containing PBB. The decreased immune function detected among the PBB-exposed farm residents tended to affect families as a unit and was independent of exposed individuals age or gender, suggesting the possibility of genetic predisposition [47, 49–51]. Animal studies further support the evidence that exposure to PBBs are immunotoxic. Luster et al. observed decreased thymic weights in rats and splenic weights in mice and suppression of mitogen-stimulated lymphoproliferation in both species following 30 days of oral exposure in mice [52]. In addition, suppression of the antibody response was noted in mice exposed to high concentrations. Other studies have also confirmed suppression of the antibody response to SRBC in mice exposed to PBBs in the diet [23, 53].

The immunotoxicity of 2-,3-,7-,8-tetrachlorodibenzo-p-dioxin (TCDD) has been well-characterized, and based on studies in mice, it is one of the most potent immunosuppressive chemicals known [54]. TCDD is formed during combustion processes such as waste incineration, forest fires, and backyard trash burning. Historically, significant quantities were also produced during the manufacture of certain herbicides (e.g., Agent Orange). A limited number of human studies examined cohorts exposed to TCDD either occupationally or as a result of residing in a TCDD-contaminated area and have reported small but statistically significant changes in various immune parameters in humans exposed to TCDD. The effect of TCDD on immune competence in humans has been difficult to measure, owing in some cases to the lack of documented exposure levels and in other cases to the low level of exposure. An assessment of Vietnam War veterans characterized immune system changes in relation to their operation in various areas of Vietnam [55]. Study subjects were classified into groups based on their Agent Orange exposure history and their current health status. In vitro assessment of peripheral blood cells showed significantly decreased plasma IgG1 levels, decreased production of IFN-γ, and increased production of IL-4 in cells from the Agent Orange exposed veterans. Another accidental exposure occurred during an explosion at a pesticide plant in Seveso, Italy and resulted in TCDD exposure. An epidemiological study of 44 children conducted within 2 years of the accident, reported no changes in immune status [56–58]; however, another study conducted 6 years later found a significant increase in complement levels, as well as increased number of peripheral blood lymphocyte and increased

lymphoproliferative responses [59]. Baccarelli reported decreased IgG levels in relation to increasing plasma levels of TCDD 22 years following the accident [60]. While the clinical significance of this finding is unknown, the authors speculated that the finding may reflect a broad alteration of the immune system that could be revealed with more sensitive markers. Animal studies on TCDD have also been well-documented. Exposure to a single low dose of TCDD impairs host resistance to infectious agents and suppresses lymphocyte responses to a variety of antigens in both developing and adult rodents [61]. The antibody response is the most sensitive immune functional endpoint identified as a result of TCDD exposure in adult mice [62]. Because of the magnitude of immunosuppression induced by TCDD, it has been used extensively as a prototypical immunotoxicant for mechanistic studies, and the majority of experimental animal data suggests that the toxicity is dependent on the activation of the aryl hydrocarbon receptor (AhR) [63].

5 The Future and Needs of Immunotoxicity Testing

Although immunotoxicology is continually evolving, scientists in the field recognize the need to identify the most efficient and sensitive approaches for advancement. One concern has been the realization that the traditional screening methods require a very large number of animals and are inadequate to handle the number of chemicals that may need to be screened. Due to these issues, along with the expense, time, animal welfare concerns, and general push for the reduction of animal use, a recent focus has been placed on the application of new approaches and technologies that would provide sensitive assays while reducing or replacing animal use. This effort has led to the development of more advanced in vitro methods to study immunotoxicology [64]. A major advantage of in vitro systems is that human cell lines can potentially be used for hazard identification of immunosuppressive chemicals and therefore may be more directly applicable to clinical observations. While challenging due to the lack of immunological complexity, in vitro methods have been utilized to evaluate selected steps in particular mechanistic pathways. Models have been described and tested to assess the supply of cells and markers of reduced or modified cellular function, including changes in gene expression, cytokine production, or surface phenotype indicative of cell maturation or function [65]. One proposed assay involves evaluations of myelotoxicity since compounds that are capable of damaging or destroying the bone marrow can have a profound immunotoxic effect on effector cells. The methodology for evaluating myelotoxicity in vitro using bone marrow culture systems is well-characterized [66]. Additional approaches have proposed determination of lymphocytotoxicity by evaluating cell death by

necrosis or apoptosis, evaluation of cytokine expression, and effects on NK cells [65]. In addition to in vitro systems, ex vivo systems evaluating multiple immune functions from human PBMCs have shown promise in determining immunotoxicity and may provide direct relevance to human disease [67]. While there has been a push for sensitive in vitro assays to replace animal studies, it is important to keep in mind that the immune system is extremely sophisticated and is both intraregulated and interregulated. The development of immune responses involve complex networks of innate and adaptive immune cells that migrate throughout the body and differentiate within tissues, a process that we cannot fully replicate in vitro. While in vitro methods may provide information about hazard identification and provide an initial screening mechanism, a true understanding of the mechanism of immunotoxicity may require animal models.

While the application and understanding of in vitro assays has recently increased, our understanding of immunology has also grown immensely in the past two decades due to the use of transgenic, knockout, and mutant rodent models. For example, evolving science has allowed for the utilization of animals models where the production of cytokines can be tracked with a fluorescent marker (IL-4/GFP-enhanced transcript mice) assessing the function of immune cells in vivo, without restimulating the cells ex vivo providing a valuable tool for research of cytokine function [68]. In addition, transgenic mice that have restricted rearranged T-cell receptors (DO11.10 mice) can allow us to assess antigen-specific T-cell responses to particular pathogens or to model antigens such as ovalbumin (OVA) and are useful in examining the ability to generate a functional adaptive immune response. Shepherd et al. utilized this model to further evaluate the mechanism of TCDD immunotoxicity and demonstrated that TDCC suppressed the production of OVA-specific antibodies along with production of IL-2 and IL-10 in a dose-dependent manner in adoptively transferred mice exposed to TCDD [69]. Other studies have utilized knockout mice to explore mechanisms of immunotoxicity. In mice, PFOA immunosuppression is mediated, at least in part, through the peroxisome proliferator-activated receptor alpha (PPARα). In contrast, humans only express low levels of this receptor. Studies in knockout animals lacking PPARα showed that there are other mechanisms of immune suppression, which may be more applicable to human disease [70]. Emerging technologies, such as CRISPR/Cas9 which allows for simplified and faster gene editing in mice, have made investigations into mechanism of immunotoxicology more achievable [71]. In addition, the use of monoclonal antibodies against specific cells or receptors can be used for depletion and has also proved to be a valuable tool to study immunotoxicological mechanisms [72, 73]. Use of these models and methods has helped to expand our basic understanding of chemically induced disease processes and gain a further

understanding of how animal models are applicable to human disease.

As our understanding of immunology has advanced, the screening paradigms for immunotoxicology are continually being updated to include additional relevant and sensitive endpoints, such as enhanced histopathology and routine enumeration of lymphocyte subsets. Advances in multicolor flow cytometry and monoclonal antibody production along with the development of new fluorophores have made it easier to assess total lymphocyte populations, as well as subsets of cell populations and function, all within a single experiment. Traditionally based on the tiered approach, lymphocyte characterization was limited to CD4+ and CD8+ T-cells. The advancements in this technology allow for easy assessment of multiple parameters in a single experiment and for the collection of more detailed phenotypic data, such as the activation status of CD4+ T-cells, CD8+ T-cells, B-cells, and dendritic cells [74]. Additionally, intracellular staining of transcription factors can delineate subsets of CD4+ T-cells such as Th1 cells, Th2 cells, Th17 cells, and regulatory T-cells [72], which may indicate changes in immune responses that would not be observed if looking at CD4+ T-cells alone.

Toxicogenomics is another potentially valuable tool in our quest to better understand immunotoxicological processes, as well as identify biomarkers for diseases. A genomics-based approach utilizing methods such as microarray, RNA-seq (high-throughput RNA sequencing), and qRT-PCR to screen chemicals for immunotox potential or to generate data for risk assessment has shown promise in recent studies [75]. The use of these technologies gives researchers the ability to quantify the transcriptome of either purified or mixed cell populations using an unbiased approach to investigate the effect of certain chemicals, at a relatively low cost and reduced experimental time. However, these studies generate large data sets that can be difficult to interpret, and knowledge of bioinformatics is essential to ensure that the data is analyzed properly. In a birth cohort study by Hochstenbach et al., whole genome gene expression in cord blood was evaluated in response to ex vivo exposure to a range of immunotoxic chemicals [76]. The investigators identified genes that significantly correlated with both TCDD and PCB exposure as well as with measles vaccination response. In addition, genes correlating negatively with exposure in general showed positive correlations with antibody levels and vice versa. In an earlier study, the investigators evaluated human peripheral blood mononuclear cells exposed to a variety of immunotoxicants and identified numerous genes that distinguished immunotoxic from the non-immunotoxic compounds [77]. Furthermore, a toxicogenomics approach using microarray and qRT-PCR was applied in which gene expression changes in human Jurkat lymphoblastic T-cells were investigated in response to a wide variety of immunotoxic agents [78]. Similarly, they reported consistent changes in gene expression due to

immunotoxicant exposure that could be categorized into distinct functional subclasses. These studies show promise for the use of toxicogenomics to evaluate immunotoxicity while providing links between short-term animal models and human exposure. While technology is advancing, there is still a need to identify sensitive biomarkers for human immunotoxicity. New techniques are continuously being considered and evaluated for their utility as predictors of potential toxicity to the immune system. While autoantibodies are often used as biomarkers to predict autoimmune disorders [79], biomarkers for immunosuppression are not as well-defined. Although not standardized or validated, several biomarkers for immunosuppression have been proposed. These include cell surface markers, immunoglobulin levels, vaccine responses, thymic output and T-cell receptor rearrangement excision circles (TRECs), and cytokines [80]. Alterations in cytokine profiles have been suggested to be a major risk factor to infection susceptibility. Bead-based multiplex assays (Luminex) allow the detection and quantification of up to 80 different protein targets from a single of sample obtained from plasma, serum, cell culture supernatant, or other bodily fluids. The small amount of sample size needed (typically 25–50 μL) and large amount of data obtained make this an appealing method to use, in combination with other endpoints, to evaluate known and suspected immunotoxicants as well as to discover potential biomarkers. However, issues such as sensitivity and variability are often complicating factors.

An emerging class of epigenetic regulatory elements that have been the subject of recent scientific focus are microRNAs (miRNAs). These molecules are single-stranded, noncoding RNA structures that are approximately 19–23 nucleotides long [81]. MiRNAs exhibit functional significance though posttranscriptional gene regulation due to their ability to bind to target messenger RNA (mRNA) and destabilize and decrease protein translation. Recently, it has been shown that miRNAs play a major role in a variety of immune responses, and they are increasingly being used as biomarkers for certain cancers [82–85]. While their specific role in immunological diseases is still being defined, upregulation of miRNAs has been identified in chemical hypersensitivity [86] suggesting potential utility as biomarkers for immunotoxicity. The assessment of multiple endpoints with a single toxicity study would help to reduce the number of animals used while enhancing the throughput of the experiment [20]. In addition to potential use as biomarkers, the abovementioned endpoints could provide additional information when incorporated into standard immunotoxicity assays.

While host defense assays are considered the gold standard for evaluations of immunosuppression, they are not commonly employed due to concerns such as animal welfare, time, and difficulty. In addition, these approaches have yet to be fully standardized. Although different pathogens are used depending upon the assay, it is important to consider that a different immune response may be stimulated. A variety of host resistance models are used to test

immunotoxicity [87]; however, the question still remains, "What immune tests and models of immune function and impairment are the most predicative and appropriate for predicting clinical disease?" Immunosuppression can manifest in a variety of ways and is not limited to the numerical reduction of antibodies or immune cells. Immunosuppression can also occur due to *shifts* in populations of antibodies or cells from a more protective subset to less protective subsets. For example, the ratio of IgG1/IgG2a produced, rather than total IgG, is indicative of protection against various infections, and the type and location of T-cell subsets are more important in determining immunity to influenza infection than total number of lymphocytes or antibody responses. Therefore, the use of new technologies to understand more specific immune responses may be crucial to understanding the effect of an immunosuppressant.

There is an ongoing need in immunotoxicology testing to develop screening tests to identify adverse health consequences from xenobiotics that produce immunostimulation or modulate inflammatory responses. Since the immune system represents a vast network of regulatory loops, altering the production or expression of one regulatory immune mediator to treat a disease would likely influence other mediators, the consequence of which may have adverse effects that outweigh the benefits of intended use. The discipline continues to evolve through the continuation of basic science in an effort to improve human risk assessment. It is essential to understand the fact that immunotoxicology represents the study of a number of distinct diseases associated with perturbations of the immune system, and that there is a critical need to develop standardized and validated screening tests for these immunotoxicities. Unfortunately validation of the proposed testing schemes to detect immunotoxic effects in humans has yet to be fully established, which is in part due to the lack of large-scale epidemiological studies in populations with mild-to-moderate immunodeficiency. In the future, the field of human immunotoxicology will greatly benefit from the widespread use of recognized testing protocols along with the use of sensitive biomarkers and clinical endpoints in humans. There is no doubt that the field of immunotoxicology will continue to advance as a science and will continue to benefit from the advancement of other disciplines. Guidelines and regulations will need to evolve within the field to reflect our current knowledge and provide the best predictions to reduce risk to human health.

Disclaimer

The findings and conclusion in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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