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Immunotoxicity testing using human primary leukocytes: An adjunct approach for the evaluation of human risk

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Abstract

Historically, immunotoxicity testing for chemicals, pesticides and pharmaceuticals has relied heavily on animal models to identify effects on the immune system followed by extrapolation to humans. Substantial progress has been made in the past decade on understanding human immune cell regulation, adaptive and innate immune responses and its modulation. The human immune system is complex and there exists diversity within composition, localization, and activation of different immune cell types between individuals. The inherent variation in human populations owing to genetics and environment can have a significant influence on the response of the immune system to infectious agents, drugs, chemicals and other environmental factors. Several recent reports have highlighted that mouse models of sepsis and inflammation are poorly predictive of human disease physiology and pathology. Rodent and human immune cells differ in the expression of cell surface proteins and phenotypes expressed in disease models, which may significantly influence the mechanism of action of xenobiotics and susceptibility yielding a different profile of activity across animal species. In the light of these differences and recent trends toward precision medicine, personalized therapies and the 3Rs (reduce, replace and refine animal use) approaches, the importance of using ‘all human’ model systems cannot be overstated. Hence, this opinion piece aims to discuss new models used to assess the effects of environmental contaminants and immune modulators on the immune response in human cells, the advantages and challenges of using human primary cells in immunotoxicology research and the implication for the future of immunotoxicity testing.

Keywords

Human immunotoxicity; Immunotoxicology; Risk assessment; Primary human leukocytes

1. Historical perspective

In the late-1970's there was a growing recognition that the immune system is a sensitive target organ for chemical-mediated modulation, which led to the establishment of

immunotoxicology as a sub discipline of toxicology. Examples already existed of chemical-mediated hypersensitivity in the occupational setting. In 1915, Prosser White described contact dermatitis in “The Dermatergoses of Occupational Affections of the Skin” (England). An even greater concern was perceived by chemical-mediated immune suppression, which in the worst case could result in impaired immune surveillance of transformed neoplastic cells and an increased incidence of various types of cancers. With funding from the National Toxicology Program (NTP), a testing approach was developed and validated using several already established immunotoxicants [1]. Specifically, the goal of the NTP funded effort was to develop a simple testing battery of immunological tests that could be conducted in combination with traditional toxicology and carcinogenicity studies.

The initial immunotoxicology testing battery consisted of two tiers of assays. The underlying strategy being that if a compound or agent gave negative results in Tier I assays, there was a presumption that the xenobiotic was unlikely to be an immunotoxicant [1]. In the event of a positive Tier I outcome, additional Tier II assays could be performed to obtain more insights concerning immunotoxic potential. Due to the diversity of the cell-types that comprise the immune system and their broad range of functions, Tier I assays included assessments of antigen-induced humoral (anti-sheep red blood cell IgM antibody response) and cell-mediated immunity (mixed lymphocyte response), proliferation induced by polyclonal stimuli and NK cell function. In addition, hematology, lymphoid organ and body weights, cellularity of spleen and thymus, and histopathology of lymphoid organs (spleen, thymus and lymph nodes) were included. Tier II included evaluation of the anti-sheep red blood cell IgG response, delayed type hypersensitivity, cytotoxic T cell response and host resistance to a live pathogen (bacterial, viral or parasitic).

The animal species of choice for conducting the immunotoxicology tier testing battery and which has widely persisted for over four decades has been the mouse. The mouse was initially selected based on a number of pragmatic factors such as the similarity of the mouse immune system to human, especially from a 1970’s perspective. As immunologists embraced the mouse as their model of choice, reagents for studying the mouse immune system continued to be most abundant of any animal species. Also the genetics of the mouse was well characterized with many strains commercially available for experimentation. Conversely, a major drawback of conducting immunotoxicity testing in mice was that the majority of routine toxicity and carcinogenicity testing was and continues to be conducted in the rat. Presently, most of the assays in the original immunotoxicology testing battery have been adapted to the rat.

2. Rationale for immunotoxicology testing in human primary leukocytes

The aim of this opinion piece is not to advocate for replacing the use of rodent models in immunotoxicology with human primary cells, but rather to interject a bit of caution and also to discuss why and under which circumstances utilization of human primary leukocytes could represent a useful adjunct to studies with rodent cells.

A strong argument can be made that during the past 40+ years the immunotoxicology testing battery, as conducted in rodents, has been effective in identifying immunotoxicants and has

successfully protected the general population. In fact, one would be hard pressed to identify agents that gave a negative result in the mouse testing battery and were subsequently found to be human immunotoxicants. The converse to this argument is, how do we know that the mouse testing battery has not either missed agents that possess human immunotoxic activity or yielded false positives? To the later point, in comparative studies of three compounds well established to suppress the mouse IgM response, arsenic, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and benzo[a]pyrene-7,8,-dihydrodiol-9,10-epoxide, one out of three, arsenic, did not suppress the human primary B cell IgM response [2]. In the case of agents possessing weak or even moderate immunotoxic activity, in the absence of testing in a human system, how would immunotoxicity be identified? To build on this line of reasoning, in a not so recent opinion piece, the immunologist Mark Davis [3] noted that with the exception of leukocyte counts there are few clinical metrics, even to this day, that are used to assess human immune competence. In other words there is no common standard that defines a “healthy” human immune system. This is in striking contrast for example to the other organ systems such as the cardiovascular system, renal system and pulmonary system, to name a few.

Over the past several decades, there has been an increasing effort to compare the mouse and human immune systems. In light of the fact that the two species diverged approximately 70 million years ago, it is not surprising, that important differences have been identified with respect to immune development, activation, effector responses and transcriptional programs. Many of these differences have been summarized in a number of recent reviews [4,5]. Likewise, in a recent study genomic responses to acute inflammatory stressors were compared between mouse models and in humans. These comparisons showed a remarkably poor correlation between the two species with the authors noting that the changes in differentially expressed genes in response to inflammatory stimuli in human and murine orthologs was close to random. In a recent study of differentially regulated genes in activated B cells by TCDD across three species, time course studies identified 515 human, 2371 mouse and 712 rat orthologous genes over a 24-h period, respectively. Only 28 orthologs were differentially expressed in response to TCDD in all three species with many of those representing what has typically been recognized as genes within the aryl hydrocarbon receptor (AhR) battery; those largely consisting of drug metabolizing enzymes (e.g., cytochrome P-450 1A1) [6]. The overall conclusion from the study was that despite the conservation of the AhR and its signaling mechanism, TCDD elicits species-specific gene expression changes and that the mechanism of B cell IgM suppression is likely different across these three species [6]. Collectively, the above findings raise questions concerning the reliability of solely depending on rodent models for identifying human immunotoxicants.

3. Current approaches to immunotoxicity testing using primary human leukocytes

Immunology as a field has delved into human immunobiology somewhat reluctantly with the mouse still representing the most widely used model. Yet the application of various new technologies has begun to yield insights into the complex biology of the human immune system. Multi-parametric flow cytometry has allowed for independent detection of a large

number of proteins simultaneously on single cells thereby generating multi-dimensional data. One of the most common applications of flow cytometry in immunotoxicology has been its use in revealing significant alterations in common and rare immune cell populations in the presence of a xenobiotic. Changes in the relative proportions and absolute numbers of immune cells as markers of immunological integrity or significant deviations from the reference ranges upon impact of a potential immunotoxic agent can be very informative of specific target effects on a given population of immune cells. A recent study used a novel flow cytometry based approach, single cell network profiling (SCNP) to simultaneously measure intracellular and extracellular proteins along with changes in intracellular proteins in response to extracellular modulators at the level of individual cells in 60 human donors [7,8]. Systems immunology is indeed state-of-the-art and such approaches are revealing a greater functional understanding of the human immune system. Flow cytometry has become an important tool in measuring human immune cell proliferation, intracellular phosphoprotein and transcription factor levels and intracellular cytokine levels in response to xenobiotics, especially when there are limited numbers of cells available for making evaluations. These multi-functional applications of flow cytometry have provided significant insights into molecular mechanisms while informing about the steady-state levels of proteins, protein turnover after covalent modifications due to a xenobiotic and alteration in signaling pathways by capitalizing on measurements of changes in the phosphorylation of key regulatory proteins. Using 20 color flow cytometers or more recently Mass Cytometry by Time of Flight (CyTOF) [9] with capability of measuring 50+ parameters simultaneously in individual cells along with fluorescent cell barcoding [10], it is now possible to measure multiple signaling events simultaneously thereby allowing for investigations of entire signaling pathways. An analogous quantitative suspension array technology enables the detection of up to 30 proteins including cytokines, chemokines, growth factors and signaling proteins from human blood either through flow cytometry or a luminex platform. These bead-array technologies have greatly expanded the analytical power required for analyzing human clinical samples with lower amounts of starting material (blood or cell culture supernatants) while delivering accurate and reliable end results.

Improvement in cell culture approaches; access to human primary cells and a large number of human cytokines, growth factors, and antibodies against human proteins have made mechanistic investigations using human cells feasible. One of the sources of human immune cells is peripheral blood from healthy human volunteers. Advances in cell separation technologies using magnetic beads coupled to antibodies for negative selection allows for routine and rapid isolation of greater than 95% pure cell populations such as naïve B and T lymphocytes and even rarer cell types such as plasma-cytoid dendritic cells from heterogeneous peripheral blood mononuclear cells. More recently, an *in vitro* model using purified human blood-derived B cells cultured in the presence of a cocktail of T cell-derived cytokines including IL-2, IL-6 and IL-10 and CD40 ligand has been used to induce B cell activation, proliferation and subsequent differentiation into IgM secreting cells [2]. The CD40L-mediated primary IgM response mimics Tcell-dependent B cell differentiation and represents a model to confirm B cell immunotoxicants identified in the mouse tier testing approach in human B cells [11–13]. Further mechanistic investigations using, for example the CD40 ligand B cell *in vitro* model are readily feasible and being used given the

availability of antibodies targeting human B cell activation markers (i.e., CD80 and CD86), transcription factors underlying B cell activation and differentiation thereby providing a molecular insight. Mechanistic evaluation of the secondary humoral immune response is likewise feasible using human cells. A recall antibody response to tetanus toxoid or hepatitis vaccine can also be measured effectively using lymphocytes from individuals recently vaccinated with the tetanus or hepatitis B vaccine [14]. Collectively, these assays emerge as putative adjuncts for comprehensive functional analysis of human B cell competence. The effect of different immune modulators on T cell activation, proliferation and differentiation can also be investigated using primary human T cells isolated from peripheral blood. T cells are readily activated *in vitro* using antibodies directed against the CD3 complex and CD28 co-stimulatory receptor on the surface of T cells thereby inducing T cell activation, proliferation and differentiation into cytokine secreting effectors. The status of T cell activation can be quantified by monitoring the magnitude of specific surface proteins such as CD69 by flow cytometry. These approaches for investigating human T cell function are currently being utilized to gain insights into mechanisms of immunotoxicity.

A critical yet under-studied area of investigation in immunotoxicology is the developing immune system. Developmental immunotoxicity can be evaluated using *in vitro* models capitalizing on the availability of human cord blood-derived hematopoietic stem cells. For example, in studying human B cell development, Li and coworkers [15] have used a combination of flow cytometry-based and cell culture-based approaches to show that the environmental contaminant TCDD decreased the total number of hematopoietic stem cells and lineage committed CD19⁺ B cells, suggesting the impairment of human B lymphopoiesis. This study illustrates another novel approach utilizing human primary cells as an adjunct to more traditional rodent based strategies.

As newer techniques emerge facilitating a more in-depth understanding of the human immune system, development and validation of human-immune toxicity testing approaches using primary leukocytes have important potential. In light of the future of human Immunotoxicology, we have proposed approaches for direct immunotoxicity testing using human cells as an adjunct to the NTP tier-testing approach. The first set of tests can be used to screen for lymphocyte phenotypic subsets in peripheral blood namely CD3⁺ (total T cells), CD3⁺CD4⁺ (helper T cells), CD3⁺CD8⁺ (cytotoxic T cells), CD19⁺ (B cells), CD14⁺ (monocytes) and CD3-CD16⁺/CD56⁺ (NK cells) by flow cytometry. Further differentiation into activated phenotypes can be performed by inclusion of markers such as CD80, CD86 or CD69 on the surface of T cells or B cells. These tests can provide information on the immune status, stimulation or impairment. To better characterize the relevance of any changes in immune cell subsets, approaches providing an insight into the mechanism or providing quantification of immune cell function can be utilized. Assays would include the primary antibody-forming response, proliferative responses to mitogenic stimulus such as phytohemagglutinin (PHA), pokeweed mitogen (PWM), mix lymphocyte response and the NK cell cytotoxicity assay to name a few. Such human-based testing platforms when applied selectively would address human relevance and clinical translation thereby bridging the gap between experimental animal models and its extrapolation to predict human risk.

4. Benefits and challenges

Clearly, the primary goal of immunotoxicology testing is to identify agents that can adversely alter the human immune system. As discussed above, utilizing human primary leukocytes in a limited and selective manner in well-defined and validated assays could serve as an important adjunct to the currently used rodent-based immunotoxicology testing battery to confirm effects, or lack of, on the human immune system. Many if not most of the current rodent-based immune function assays in the tier testing approach can also be performed using isolated human primary leukocytes. Moreover, in addition to routine screening, a strong case can be made for employing human primary leukocytes for mechanistic studies. In fact, lessons can be learned from the pharmaceutical industry. Although, there have been important recent advances in immune-based therapeutic strategies from findings initially made in the rodent and then translated to the human, with a number of currently developed biologics being a case in point (e.g., check-point inhibitors in the treatment of cancer), there also exist many examples in which therapeutic strategies effective in the mouse were found to be disappointingly unsuccessful in humans. Conversely, compounds and agents with societal benefit or those that can promote technologic advancements should not be dismissed strictly on the outcome from rodent immunotoxicity testing.

In spite of the benefits that can be derived from utilizing human primary leukocytes for assessing immunotoxicity, there also exist a number of critical challenges. Primary resting leukocytes are short-lived and cannot be propagated for an extended time period in culture; therefore they must be freshly isolated shortly prior to use, typically within 24 h. Historically, human leukocytes from lymphoid tissues, primarily tonsils or spleen have on occasion been used but do not represent a reliable source of cells as tonsils usually are not removed from healthy individuals and human spleen is most often obtained from accident victims. The most reliable source of human leukocytes is peripheral blood of which a unit of blood is the maximum that can be obtained from a donor. Depending on the donor, 1 mL of blood yields 4300–10,800 of white cells, with approximately 50–70% neutrophils, 20% lymphocytes, 4% monocytes, 2–4% eosinophils and 1% basophils. Leukocyte packs, which are the white cells remaining from platelet donors, represent a common and convenient source of human peripheral blood leukocytes for experimentation, providing $1-5 \times 10^9$ leukocytes. Importantly, each purification step for obtaining specific cell populations results in approximately a 50% loss of cells. Therefore, the number of cells obtained from any given donor is limited and, unlike in inbred rodent strains, cannot be pooled across donors as doing so will induce a mixed lymphocyte response. For most assays, miniaturization into a 96-well format is a requirement. Donor-to-donor variability due to differences in genetics, lifestyles, nutrition, to mention only a few, represents another challenge. Since studies using human leukocytes are conducted *in vitro* with direct addition of the agent to cultured cells, a partial work around is that every donor can serve as their own control. Hence, the effect of xenobiotic treatment for each donor's cells can be normalized as the percent change from the control response induced by an immune stimulus (e.g.; percent change from control response to mitogen versus response to mitogen plus test agent) for comparison across donors. Our experience using this approach has shown surprising consistency when

comparing treatment effects across donors. In addition, lab worker safety is an important consideration. All human tissues and samples have the potential of being contaminated with pathogens of which hepatitis and HIV are of greatest concern. Finally, a major limitation in using any *in vitro* approach for assessing immunotoxicity, in any species, is the lack of drug metabolizing capability by leukocytes. The absence of metabolism is a major obstacle to most *in vitro* approaches in toxicology and is the focus of significant attention, especially with an increasing emphasis on *in vitro* toxicity testing as a replacement for animal testing.

5. Concluding remarks

An important objective of toxicity testing is to understand the risk posed by xenobiotics to human health. Although rodent-based models have been notably successful in the determining adverse effects or toxicity, there exist limitations associated with extrapolation of the risk posed to humans. Hence, in the coming years it is important to fully recognize that potential and generate impetus for studies using human primary leukocytes thereby generating a rich resource of immunotoxicology data using human primary leukocytes to facilitate rational decision making for the betterment of human health.

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