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Understanding the correlation between *in vitro* and *in vivo* immunotoxicity tests for nanomedicines

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ABSTRACT

Preclinical characterization of novel nanotechnology-based formulations is often challenged by physicochemical characteristics, sterility/sterilization issues, safety and efficacy. Such challenges are not unique to nanomedicine, as they are common in the development of small and macromolecular drugs. However, due to the lack of a general consensus on critical characterization parameters, a shortage of harmonized protocols to support testing, and the vast variety of engineered nanomaterials, the translation of nanomedicines into clinic is particularly complex. Understanding the immune compatibility of nanoformulations has been identified as one of the important factors in (pre)clinical development and requires reliable *in vitro* and *in vivo* immunotoxicity tests. The generally low sensitivity of standard *in vivo* toxicity tests to immunotoxicities, inter-species variability in the structure and function of the immune system, high costs and relatively low throughput of *in vivo* tests, and ethical concerns about animal use underscore the need for trustworthy *in vitro* assays. Here, we consider the correlation (or lack thereof) between *in vitro* and *in vivo* immunotoxicity tests as a mean to identify useful *in vitro* assays. We review literature examples and case studies from the experience of the NCI Nanotechnology Characterization Lab, and highlight assays where predictability has been demonstrated for a variety of nanomaterials and assays with high potential for predictability *in vivo*.

1. Introduction

The immune system performs multiple functions, which include protecting the host from invading pathogens, as well as patrolling the body to effectively identify and remove dead and damaged cells [1,2]. This immune "surveillance" is critical in maintaining healthy homeostasis. Alterations to the immune system's structure and/or function may lead to various pathophysiological conditions, some of which may be life-threatening [3]. Hence, understanding the impact of various environmental factors, chemicals, cosmetic, household and pharmaceutical products on the immune system has become an area of focus in modern toxicology. Immunotoxicology is a relatively young and rapidly developing area of science which deals primarily with identifying substances affecting the structure and function of the immune system and causing undesirable effects such as immunostimulation, immunosuppression, hypersensitivity reactions and autoimmunity [3–5]. Although most currently available immunotoxicity data come from studies with environmental factors, the immunomodulatory properties of pharmaceutical products (drugs and medical devices) have also received attention in the past decade [3–5]. According to several reports from academia [6], the pharmaceutical industry [7] and the US Food and Drug Administration [8,9], approximately 10–20% of drugs withdrawn from clinical use between 1969 and 2005 were pulled due to immunotoxicity. The range of adverse immune reactions included anaphylaxis, allergy, hypersensitivity, idiosyncratic reactions, and immunosuppression [6–9]. Rigorous assessment of adverse immune effects during preclinical drug evaluation could help to avoid such reactions in patients in the future.

The likelihood of identifying immunotoxicity increases with progression from the preclinical to the clinical phase (Fig. 1).

The common goal of preclinical immunotoxicity studies is to identify potential concerns before a new drug or a medical device is given to patients enrolled into clinical trials. Traditionally, standard *in vivo* toxicological studies include analysis of lymphoid organ weights, histological evaluation of immune organs and tissues, understanding clinical chemistry parameters and hematology in two animal species: a rodent (commonly rat) and a non-rodent (commonly dog) [10]. Extrapolation of findings from these *in vivo* toxicity tests to human patients is often challenging due to the differences in composition, organization and sensitivity to certain agents between the human immune system and that of the animal species used for testing [11–14]. In addition, while these tests detect strong immunosuppression and immunostimulation, their sensitivity to moderate immunotoxicity resulting from immune dysregulation (which often manifests only at the functional level), is relatively low [11–15]. This is why immunotoxicologists supplement standard toxicity studies with immune function tests. These have been found to be very useful for identifying drugs which cause immunotoxicity in humans [11].

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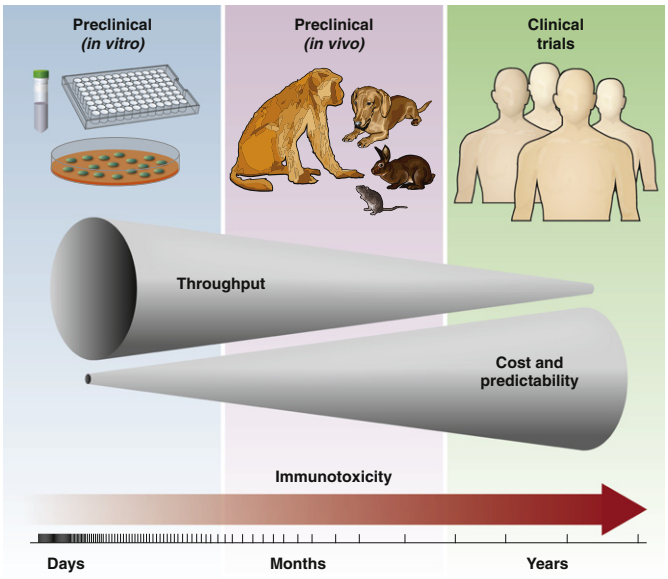

Fig. 1. Challenges in identifying immunotoxicity during nonclinical studies. The likelihood of identifying nanoparticle immunotoxicity increases as a drug product progresses from early *in vitro* models, to preclinical *in vivo* and into clinical phases. However the high cost of *in vivo* tests coupled with increasing ethical concerns regarding animal use often impedes the application of these *in vivo* tests despite their advantages in predictability. In contrast, the high-throughput nature and lower time and resource requirements of preclinical *in vitro* tests makes them an attractive alternative.

Widespread application of these function tests is often hindered by high costs and their relatively low throughput. More commonly, formulations shown to cause adverse effects *in vivo* are then further tested *in vitro* to verify findings and to attempt to understand the mechanism(s) of the observed immunotoxicity. This strategy is intuitively the reverse of traditional biological preclinical evaluation, but now has an established record of use in the pharmaceutical industry. It is the general consensus of scientists in the nanomedicine field that this strategy should also be applicable to engineered nanomaterials since no novel immunotoxicity has been described to-date which is specific to nanoparticles [16–18]. However, the complexity of many nanomedicine formulations requires a broad spectrum of rigorous tests to characterize the physicochemical properties which may contribute to immunotoxicity. There is therefore a growing recognition of the need for rapid screening methods to identify what nanomaterial physicochemical parameters contribute to immunotoxicity which can be used early in the preclinical stage. A cascade of validated, reliable and predictive *in vitro* assays would address this need. One of the critical factors necessary for the compilation of such a testing cascade is a firmly established correlation between *in vitro* assays and their *in vivo* counterparts addressing the same immunological parameters.

In this review, we summarize the literature reports comparing performance of *in vitro* and *in vivo* immunotoxicity tests, and share the Nanotechnology Characterization Lab's (NCL's) experience with *in vitro* and *in vivo* assessment of engineered nanomaterials with respect to immunotoxicity. NCL has been in operation since 2004, during which time we have tested more than 280 formulations representing the majority of engineered nanomaterial classes. The purpose of this review is to discuss *in vitro* assays and their correlation to corresponding *in vivo* immunotoxicities. It is well established now-a-days that nanoparticle physicochemical properties such as size, charge, hydrophobicity and surface chemistries determine nanoparticle interactions with the immune system. These structure–activity relationship findings are reviewed in depth by other reports [19–29] and thus are omitted from this review. Herein we will focus on assays, which can be utilized to understand nanoparticle interactions with various components of the immune system and their utility in development of safe nanomedicines. We offer our selection of *in vitro* immunoassays with high potential to be predictable of immunotoxicity *in vivo*, and address the strengths and limitations of other methods.

2. Selecting *in vitro* immunotoxicity tests for nanomaterials

The aim of *in vitro* tests is to rapidly evaluate the formulation's potential to cause acute reactions *in vivo*. With respect to immunotoxicity of nanomaterials, it is now generally accepted that a nanomaterial that comes into contact with the blood should be evaluated for its effects on erythrocytes and the complement system, to identify severe acute toxicities, such as hemolysis and anaphylaxis, respectively. This generalization applies whether nanoparticles are used as components of a medical device, as drug carriers, drugs or imaging agents [19–25]. Assessment of the particle's thrombogenic potential is also important to address an increasing concern regarding nanoparticle propensity to cause vascular thrombosis and disseminated intravascular coagulation (DIC)-like toxicities [32]. Preclinical screening for this toxicity is complicated, as it involves multiple end-points: platelets, coagulation factors, leukocytes and endothelial cells. A nanoparticles plasma protein binding is now widely accepted as an indicator of the speed with which the particle will be cleared from the circulation and of distribution to the cells of the mononuclear phagocytic system (MPS) [30–35]. Additionally, induction of proinflammatory cytokines is considered as a surrogate for cytokine-associated toxicities including, but not limited to: DIC, pyrogenicity, and hypercytokinemia. Thus, common markers for nanoparticle acute toxicities are: hemolysis, complement activation, thrombogenicity, phagocytosis, pyrogenicity and cytokine induction. Most of these toxicities can be rapidly assessed *in vitro* prior to more resource- and time-consuming *in vivo* studies (Fig. 2). Immunosuppression is another important toxicity, which initially can be assessed through assays targeting multiple immunological end-points, with phagocytosis and leukocyte function being the most widely used.

There are several major challenges in the *in vitro* testing of nanoparticle immunotoxicity: 1) selection of a model; 2) selection of an end-point, 3) selection of relevant positive and negative controls; 4) nanoparticle interference with *in vitro* assays, and 5) understanding assay predictability of corresponding immunotoxicities *in vivo*. For the purposes of this review we will skip the first four challenges, as they have been reviewed earlier [36]. Below, we will focus on the fifth challenge and we will use “markers” of acute toxicities and immunosuppression highlighted above to evaluate the predictability of the *in vitro* tests. When available, we reference nanoparticles actual clinical data for comparison.

3. Considerations for selecting controls and nanoparticle concentrations

Two important general issues commonly arise regarding *in vitro* immunoassays: 1) the *in vitro* immunoassay's sensitivity to nanoparticle-mediated toxicity, and 2) selecting an appropriate nanoparticle concentration so that *in vitro* test results are predictive of *in vivo* toxicity. Here we share the approach we used to validate NCL's *in vitro* assay cascade. We first identified nanoformulations which are approved for clinical use and are associated with certain types of immunotoxicity. For example, the PEGylated nanoliposome formulation of doxorubicin, Doxil®, and nanoemulsion formulation of Paclitaxel, Taxol®, have been shown to cause hypersensitivity reactions related to complement activation in patients. Of course, there are also nanoformulations which do not cause this type of immunotoxicity, for example Abraxane®, the nanoalbumin (“nab”) formulation Paclitaxel, does not cause complement activation. We then use these particles in *in vitro* assays as positive and negative controls, respectively.

By definition, an assay has “good” *in vitro* to *in vivo* correlation if it is able to detect immunotoxicity for a nanoformulation known to