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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*.

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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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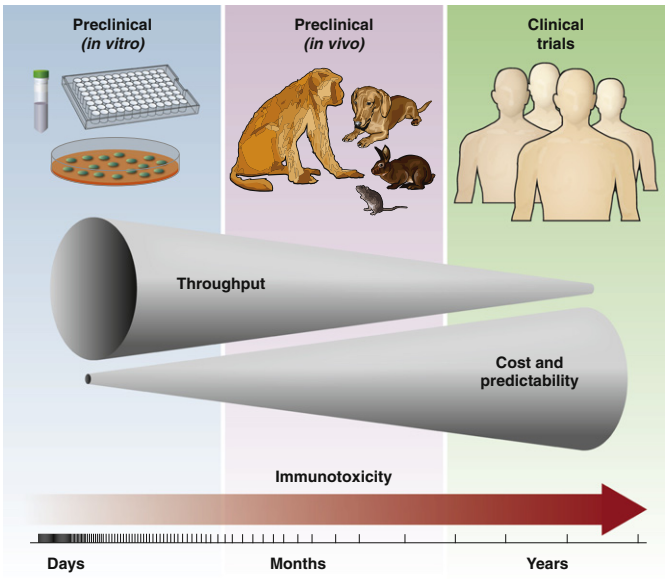

Fig. 1. Challenges in identifying immunotoxicity during nonclinical studies. The likelihood of identifying nanoparticle immunotoxicity increases as a drug product progresses from early *in vitro* models, to preclinical *in vivo* and into clinical phases. However the high cost of *in vivo* tests coupled 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

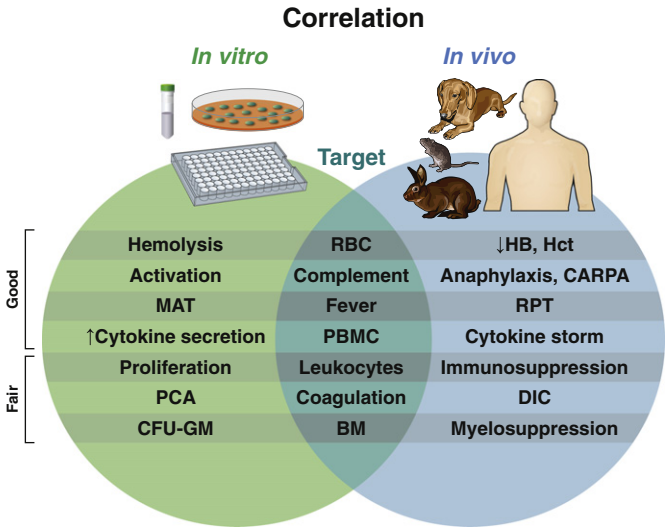

Fig. 2. Correlation between *in vitro* and *in vivo* immunological tests for nanoparticle characterization. This summary highlights the experience of the NCI Nanotechnology Characterization Lab (NCL) through applying an *in vitro* assay cascade (http://ncl.cancer.gov/working_assay-cascade.asp) for preclinical characterization of various types of engineered nanomaterials to more than 280 nanomaterials and comparing this data to the findings of our standard *in vivo* toxicity tests in animals and to clinical data (when available). One limitation of the *in vitro* CFU-GM test is that data interpretation depends on nanoparticle biodistribution, which cannot be tested *in vitro*, interpretation of this test is supported by the PK profile for the given nanoformulation. DIC—disseminated intravascular coagulation; BM—bone marrow; CFU-GM—colony forming unit granulocyte macrophage; PCA—procoagulant activity; PBMC—peripheral blood mononuclear test; MAT—macrophage activation test; RPT—rabbit pyrogen test; RBC—red blood cells; HB—hemoglobin; Hct—hematocrit, downward arrow refers to a decrease, upward arrow refers to an increase.

cause this toxicity in patients, while yielding a negative response for particles that have no reported clinical immunotoxicity. Some of the examples of the assay validation using this approach were reported by us earlier [36]. There is a major caveat to this: this approach makes the potentially inaccurate assumption that all particle types will behave similarly for a given assay. The molecular mechanism for toxicity of a liposome, for example, may be widely different than that of a metal oxide nanoparticle and therefore liposome positive control may not be applicable to other types of nanoparticles. Since all nanomaterials are different, we expect that the spectrum of interferences will be different among the various classes of nanomaterials, but the "positiveness" and "negativeness" in *in vitro* immunoassays can only be estimated by this approach if we have at least one formulation representing a certain class of nanomaterials known to cause a certain type of immunotoxicity in a human or an animal. This raises another very important topic of nanoparticle reference standards, discussion of which is beyond the scope of this review.

Selection of concentration is the second issue commonly applicable to *in vitro* immunoassays. Several approaches have been reported earlier [37,38]. In Box 1, we describe the approach we use for novel nanoparticles for which hematocompatibility has yet to be understood. This approach is based on several assumptions which we will also mention below. If a pharmacokinetic (PK) study has been conducted and the maximum plasma concentration (C_{max}) for the nanoformulation is known, we use the C_{max} as the starting concentration in our *in vitro* assays. When PK data is not available, we rely on the maximum dose expected to be tested *in vivo* or the dose at which the material is expected to reach therapeutic efficacy, and we assume that the entire dose will stay in blood upon injection. To convert the dose information between species, we rely on FDA regulation for the dose selection [39] (see Box 1).

When no information about the dose is known, we test 1 mg/mL as the highest *in vitro* concentration assuming that the test nanomaterials will not be used *in vivo* at a dose exceeding 80 mg/kg (this number is calculated using an average human weight of 70 kg and a blood volume of 5.6 L). When a nanoparticle is used to carry an active pharmaceutical ingredient (API), we perform all calculations based on the API concentration, and use the mass of the nanoparticle as additional metric to correlate with blank nanoparticle control.

4. Assays with "good" *in vitro–in vivo* correlation

4.1. Hemolysis

Refers to damage to red blood cells, which may lead to anemia and other life-threatening conditions. Understanding a nanoparticles hemolytic potential is recognized as an important initial step in assessing biocompatibility. Several studies have reported a good correlation between the results of *in vitro* hemolysis assays and *in vivo* toxicity studies identifying hemolysis as a toxic effect. For example, Lu et al. tested thirteen (13) formulations of metal oxide nanoparticles including CeO_2, NiO, MgO, Co_3O_4, ZnO, and SiO_2, various forms of TiO_2 and Al_2O_3, and carbon black nanoparticles for their ability to damage erythrocytes, and found the *in vitro* assay to be highly predictive [40]. Interestingly, this study also demonstrated that *in vitro* hemolysis data correlates well with *in vivo* data, reporting a proinflammatory potential of 92% (12 out of 13) of the tested particles [40]. This study used freshly drawn human whole blood which had been anticoagulated with sodium citrate for the *in vitro* test and performed the *in vivo* analysis in Wistar rats [40]. Another study testing iron oxide–gold composite nanoparticles *in vitro* using rabbit whole blood anticoagulated with potassium oxalate was performed in conjunction with acute *in vivo* toxicity studies in rats and beagle dogs to understand the biocompatibility of the composite nanoparticles. The authors reported a correlation, in that percent hemolysis *in vitro* was very low (~0.2%) and there was no hemolysis in either species [41]. Several studies have reported that various dendrimers with cationic charge, including G5 and G4 PPI dendrimer [42,43], G4 PAMAM dendrimers [44,45], G3 PAMAM and G3 PPI dendrimers [46] as well as G4 PLL dendrimers [47] resulted in from 14% to 86% hemolysis *in vitro* in whole blood from human donors and various animal species, and that they also led to a decrease in erythrocyte count, hemoglobin and hematocrit values when injected into rodents *in vivo*.

There are a few important considerations regarding the *in vitro* hemolysis test: 1) what anticoagulant to use; 2) what species of origin the blood should be; 3) what protocol to use, and 4) what *in vitro* percentage of hemolysis is a concern. According to the literature and our own experience, the anticoagulant and the species origin of blood are not critical, as no one has yet demonstrated significant differences in assay test results when varying these parameters. This is not the case for other immunoassays. For example, complement activation discussed in the section below is different in various species. The challenge with protocol selection and many other questions regarding nanoparticle hemolytic properties are reviewed in detail by Wildt B. et al. [48]. At the NCL, we use ASTM International protocol E2524-08 [49] to study nanoparticle hemolytic properties. This protocol sets a threshold for *in vitro* hemolysis at 2% [49]. According to the E2524-08 protocol, if the assay result for a test-nanomaterial falls below this level, the material is considered non-hemolytic; hemolysis values between 2 and 5% are interpreted as moderately hemolytic and those above 5% qualify the test-nanomaterial as hemolytic. In all the studies mentioned above, nanoparticles which were not hemolytic *in vivo* resulted in less than 2% hemolysis *in vitro*, and the materials which were hemolytic *in vivo* produced hemolysis values significantly above 5% when tested *in vitro*. In our hands, *in vivo* toxicity studies of nanoparticles with *in vitro* hemolysis of 4 to 5% caused decreases in erythrocyte counts, and hemoglobin and hematocrit values in all treated animals [36]. Nanomaterials with *in vitro* hemolysis above 50% caused immediate animal death when administered intravenously (Fig. 3).

Box 1
Example calculation of nanoparticle concentration for *in vitro* test.

In this example, we assume the mouse dose is known to be 123 mg/kg.

$$human\ \ dose = \frac{mouse\ \ dose}{12.3} = \frac{123\ \ \frac{mg}{kg}}{12.3} = 10\ \ mg/kg$$

(see reference [35]).
Blood volume constitutes approximately 8% of body weight, (e.g. a 70 kg human has approximately 5.6 L (8% of 70) of blood). This allows us to get a very rough estimation of what the maximum blood concentration may be.

$$in\ \ vitro\ \ concentration_{human\ \ matrix} = \frac{human\ \ dose}{human\ \ blood\ \ volume}$$

$$= \frac{70\ \ kg \times 10\ \ \frac{mg}{kg}}{5.6\ \ L} = \frac{700\ \ mg}{5.6\ \ L} = 0.125\ \ mg/mL.$$

We also test two 1:5 dilution as well as 10 ×, and whenever achievable 100 × of this concentration.

Another approach we use to validate *in vitro* assays is to use nanoformulations approved for clinical use as "nanorelevant" controls. We previously reported that the nanoliposome Doxil®, nanoalbumin particle Abraxane® and the nanoemulsion Propofol®, which are all used in clinics, were not hemolytic *in vitro* [50]. Therefore the *in vitro* hemolysis test can be used to screen nanoformulations in order to gauge their erythrocyte damaging potential relative to these agents, and to get an insight into what physicochemical attribute is associated with any hemolytic activity. This information can be used to guide formulation optimization and selection of lead candidates for further development.

4.2. Complement activation

Leads to the release of several complement split products, some of which are highly reactive in promoting inflammatory response (C3a, C4a and C5a) and are known as anaphylatoxins (cytokine-like molecules causing anaphylaxis). Complement Activation Related PseudoAllergy (CARPA) syndrome is a common dose-limiting toxicity of PEGylated-liposomes and other lipid and polymeric nanocarriers [52,53]. Various animal species have different sensitivity to complement activating substances. For example, according to the study by Szebeni et al., the dose of total phospholipid sufficient to trigger complement activation related to hypersensitivity reactions is 0.01–0.2 mg/kg in humans, 0.01–0.3 mg/kg in pigs, 0.05–0.1 mg/kg in dogs and 5–25 mg/kg in rats [53]. This study suggests that pigs and dogs are better *in vivo* models for complement-mediated hypersensitivity reactions than rats. In our hands only human matrix and matrix from non-human primates, when used in *in vitro* complement activation assay, were sensitive to complement activation by engineered nanomaterials, while matrices from rat, mouse, mini-pig and guinea pig were not [54].

Several research groups have reported a correlation between *in vitro* complement activation by various engineered nanomaterials using human serum- or plasma-based assays and *in vivo* complement activation in sensitive animal species (pigs and dogs). For example, Merkel et al. used an *in vitro* human serum-based assay and performed an *in vivo* study in a Yorkshire swine model to analyze complement activation and relate it to hypersensitivity reactions in response to poly(ethylenimine) and poly(ethyleneimine)-graft-poly(ethyleneglycol) block copolymers [52]. They showed that polymer formulations which resulted in as low as 2-fold increases above baseline in the terminal complement complex sC5b-9 *in vitro* caused significant hypersensitivity *in vivo*. Another study investigating the biocompatibility of perfluorocarbon-based nanoemulsions showed a good correlation between an *in vitro* complement activation assay conducted in human serum and *in vivo* complement activation in mice [55]. Similarly, several formats of *in vitro* assays using human serum were successfully used to detect complement activation by iron oxide nanoparticles [56], lipid nanocapsules [57], and polymeric nanoparticles [58]. We have reported that Abraxane® (a nanoformulation of the anticancer drug Paclitaxel), which does not cause complement mediated hypersensitivity in patients, did not activate complement *in vitro* in an assay utilizing human plasma [36]. In contrast, a traditional cremophor-EL formulation of the same API, Taxol®, known to cause severe hypersensitivity in patients, also activated complement in the *in vitro* assay [36]. Doxil®, another clinically-approved nanoformulation known to cause CARPA in sensitive individuals [59], also activates complement *in vitro* [60–62].

In Fig. 4 we present a case study where three metal oxide nanoparticle formulations with identical cores and different surface coatings were tested *in vivo* in rats and rabbits and *in vitro* in plasma from these species. *In vivo*, none of these formulations caused reactions in rats, but two caused anaphylaxis in rabbits. *In vitro* (in rat and rabbit plasma), the same formulations causing anaphylaxis *in vivo* caused complement activation, and those that did not produce a response (in rats or rabbits) did not activate complement. Two of these formulations resulted in a ~2-fold increase in complement activation in human plasma *in vitro*, which was comparable to that seen with Doxil®. This data suggest that these formulations may cause complement-mediated hypersensitivity reactions in sensitive human individuals, similar to that observed with Doxil®, so clinical studies using these materials may require immunosupressive medication, as with Doxil®.

Many other methods are available for the analysis of complement activation *in vitro* and are reviewed in details by Morales and Sims [63]. Each method has its own strengths and limitations. Selecting a particular method should depend on the nanoparticles under study, as certain nanomaterials may interfere with an individual assay format, as well as on the availability of reagents and instrumentation in a given lab. As yet there has been no inter-laboratory study comparing the predictability of different formats of *in vitro* complement assays.

4.3. Cytokines

Often used as biomarkers for acute inflammation [20,64–67]. Some of them, e.g. IL-1β and IL-6, are also known markers of pyrogenic response. Nanomaterials may be intentionally engineered to promote the immune response by activating cytokine expression, and this property is gaining attention in the vaccine development field [68]. In contrast, undesirable induction of cytokine response may cause overt immunostimulation, including life-threatening conditions such as DIC and cytokine storm. There are examples where *in vitro* screening of nanoparticle-mediated cytokine response correlates well with *in vivo* cytokine induction [69]. In Fig. 5 we review a case study in which two metal oxide nanoparticles formulations with identical cores and different surface modifications were tested *in vivo* in rats and in rabbits. Both nanoparticles had undetectable endotoxin levels as assessed by the gel-clot LAL assay. Formulation #1 was non-toxic, while Formulation #2 resulted in animal death. Necropsy and histopathology examination revealed congestion in animal spleens and other organs, similar to those seen in septic shock. Analysis of plasma samples from affected animals revealed high levels of inflammatory cytokines IL-1, TNF and IL-8. Formulation #2, which caused a cytokine storm *in vivo*, also activated cytokines *in vitro* in normal human PBMC. This study emphasizes the importance of the cytokine test *in vitro* prior to testing nanomaterials *in vivo*.

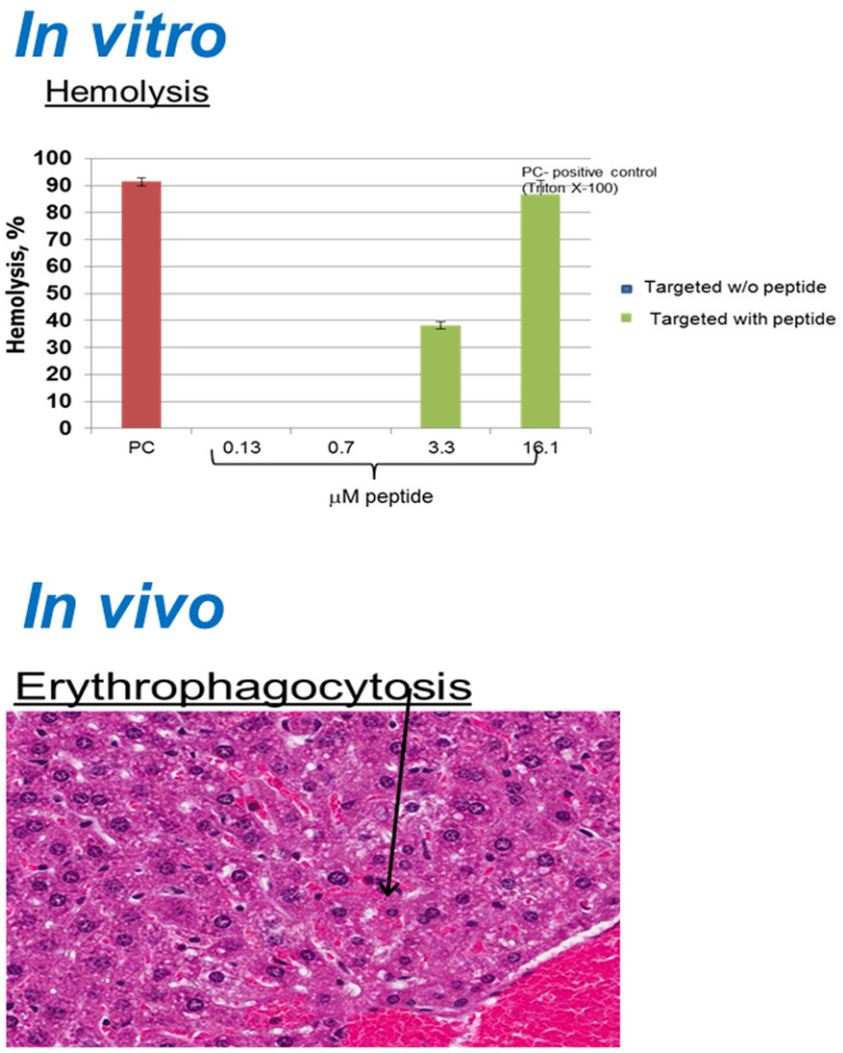

Fig. 3. *In vitro–in vivo* correlation of hemolysis assay results. A nanoformulation containing a cationic membrane perforating peptide was tested *in vitro* (top panel) and *in vivo* (bottom panel). The precursor formulation without the peptide was also tested as a control (data not shown). The peptide-containing formulation resulted in dose-responsive hemolysis *in vitro* (top panel) and caused animal death when injected intravenously. Histopathology evaluation revealed erythrophagocytosis (bottom panel) consistent with hemolysis. Images are adapted from reference [51] with permission.

Since cytokine mediated reactions are common dose-limiting toxicities of nucleic acid-based therapeutic constructs (e.g. oligonucleotides, siRNA and plasmid DNA) [70–72], an *in vitro* cytokine detection assays represent valuable tools for screening nanoformulations. In our experience, whole blood, PBMC and MM-6-cell line-based assays perform equally well for the study of cytokine induction by nanoparticles.

Since two of these cytokines, IL-1and IL-6, are also causative agents in fever and febrile response, several assays utilizing whole blood, fresh or cryopreserved PBMC and MM-6 cell lines have been validated for the screening of endotoxin contamination in drugs. These assays have been proposed as *in vitro* surrogates of the Rabbit Pyrogen Test (RPT) [73–76]. We have tested these assays for various nanomaterials and observed a good correlation between *in vitro* induction of IL-1β and fever response in rabbits by certain nanomaterials ([77], and unpublished data).

4.4. Opsonization and phagocytosis

Opsonization refers to the binding of plasma proteins to a nanoparticle surface, which makes the nanoparticles more "visible" to phagocytes, and aids in removal from the circulation. For nanoparticles, phagocytes utilize multiple routes of elimination from circulation, which include various forms of phagocytosis (complement receptor-, FcγR- and mannose receptor-mediated) and pinocytosis (macropinocytosis, clathrin-, caveolin-dependent and independent) [78–81]. Only proteins aiding in uptake are called opsonins (e.g. complement proteins and immunoglobulins). Nanoparticles with certain surfaces, especially those unprotected by hydrophilic polymers, bind large amounts of proteins [32]. The so-called "protein corona" contains more proteins than just opsonins, however, the biological significance of binding these proteins is poorly understood. There have been some attempts to connect protein coronas to nanoparticle toxicity based on the function of individual proteins detected in the corona [82]. Experimental evidence in support of a link between specific proteins (or protein profiles) in the corona and nanoparticle toxicity has yet to be established. What has been proven experimentally is the significance of the total amount of protein binding to nanoparticle for their biodistribution to the organs of the MPS, circulation time, and in some cases inflammation at the site of nanoparticle retention [30]. Relevant to this review, it is now well-established that *in vitro* protein binding and phagocytosis experiments correlate well with *in vivo* biodistribution studies demonstrating particle retention by the organs of the MPS [30]. For example, *in vitro* protein binding and associated phagocytosis by RAW264.7 murine macrophages were shown to correlate with the accumulation of poly(vinyl-pyrrolidone)-block-poly(D,L-lactide) polymer nanoparticles in the spleens and livers of rats used as a model in an *in vivo* biodistribution study [83]. Likewise,

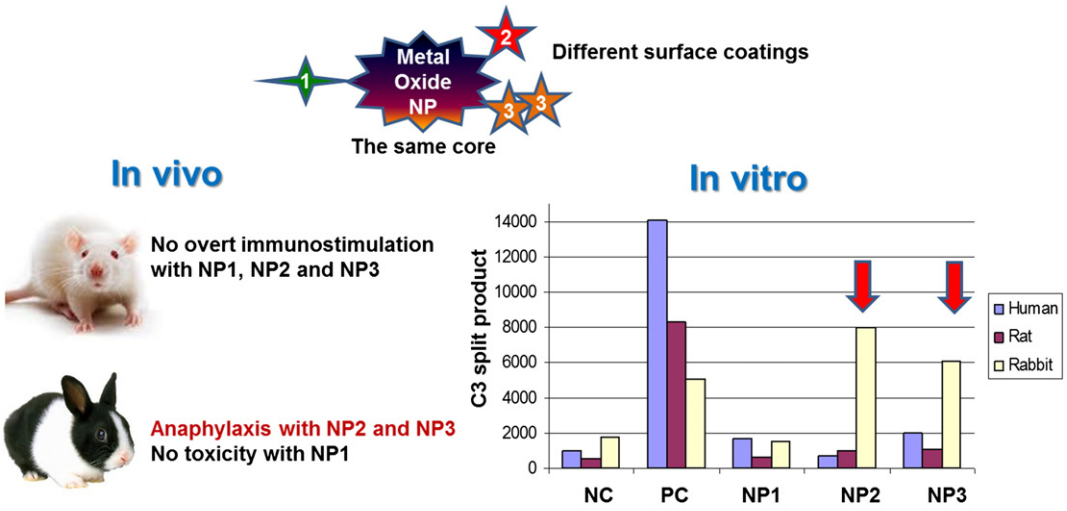

Fig. 4. *In vitro–in vivo* correlation of the complement assay. Three nanoformulations (NP1, NP2, and NP3) of metal oxide nanoparticles with identical cores and different surface chemistries were tested *in vivo* in rats and rabbits and *in vitro* using human, rat and rabbit plasma. PBS was used as a negative control (NC) and cobra venom factor was used as a positive control (PC) *in vitro*. Formulations causing anaphylaxis in rabbits *in vivo* also activated complement in rabbit plasma *in vitro*. Red arrows identify formulations reactive with rabbit complement *in vitro*.

in vitro phagocytosis of cross-linked albumin nanospheres in human U937 monocyte–macrophage cell lines and murine peritoneal macrophages correlated with liver uptake in rats [84]. Nanoparticle capture by the spleens of mice was predicted by an *in vitro* method employing a spleen tissue culture model [85]. *In vitro* phagocytosis of poly(caprolactone) nanoparticles by murine peritoneal macrophages was used to predict the biodistribution of these particles to the livers of mice [58]. A similar correlation was obtained between *in vitro* phagocytosis in THP-1 cells and *in vivo* liver accumulation of lipid nanocapsules [57] and iron oxide nanoparticles [56].

Within our portfolio here at the NCL, uptake of colloidal gold nanoparticle by macrophages *in vitro* correlates well with MPS capture *in vivo*. PEGylated versions of the same nanoparticles are not taken up by macrophages *in vitro* and there is no uptake *in vivo* (Fig. 6A). Abundant protein binding detected *in vitro* (Fig. 6B), correlates with HL-60 monocyte–macrophage cell line uptake *in vitro* (data not shown) and with nanoparticle accumulation in lung-resident macrophages and inflammation-mediated toxicity *in vivo* (Fig. 6B).

In summary, regardless of what *in vitro* model was used (primary cell or cell lines) and irrespective of the type of cells (macrophages, monocytes, or monocyte–macrophages), nanoparticle uptake *in vitro* is an appropriate surrogate for predicting MPS capture *in vivo*. Therefore these *in vitro* assays may be used in preclinical characterization to screen multiple formulations to select lead candidate(s) with low/no retention for indications where MPS capture is undesirable (e.g. drug delivery). Conversely, this method can also identify

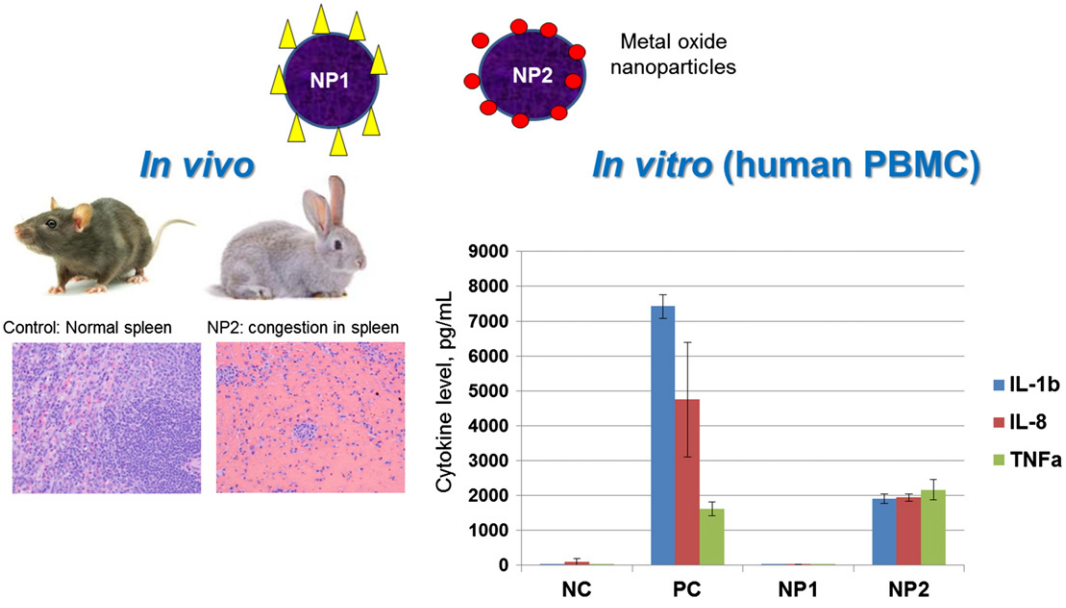

Fig. 5. *In vitro–in vivo* correlation of the cytokine assay. Two nanoformulations (NP1 and NP2) of metal oxide nanoparticles with identical cores and different surface chemistries were tested *in vivo* in rats and rabbits and *in vitro* using normal human peripheral blood mononuclear cells. PBS was used as a negative control (NC) and 10 ng/mL K12 *E. coli* endotoxin was used as a positive control (PC) *in vitro*. Formulation 2 caused cytokine induction and related toxicity (congestion) in animals and induced inflammatory cytokines *in vitro* in human PBMC.

particles with high retention, for applications where capture by the immune cells is advantageous (e.g. vaccine delivery or liver/spleen imaging).

5. Assays with "fair" *in vitro–in vivo* correlation

The assays placed in this category are those to assess the thrombogenic and immunosuppressive potential of nanoparticles. Although good *in vitro–in vivo* correlation has been established for individual thrombogenicity and immunosuppression assays as will be discussed below, the overall amount of data regarding application of the assays for various types of engineered nanomaterials is not as high as for the assays listed in the previous category (i.e. "good" *in vitro–in vivo* correlation). Another challenge with these assays is their multi-component nature, which stems from the physiological complexity of the toxicity they are intended to identify. For example, activation of complement and hemolysis target single end-points, complement proteins and erythrocytes, respectively, while thrombogenicity involves a complex biochemical system of plasma coagulation factors and various cell types including platelets, leukocytes, endothelial cells and some cancer cells. Since various mechanisms are involved in thrombogenic response to nanomaterials, and since various nanomaterials may affect one or more components of the coagulation system, there is no single assay or test which can accomplish the goal. As such, several assays addressing multiple end-points are needed to screen for thrombogenicity. The same is true for immunosuppression, because in this case multiple cell types (macrophages, dendritic cells, bone marrow stem cells, and leukocytes) and multiple end-points (proliferation, expression of activation markers, differentiation etc.) are involved.

5.1. Thromobogenicity

A single definition referring to multiple pathologies which involve one or more components of the blood coagulation system. The mechanisms of thrombogenicity are often complex and involve multiple cell types (thrombocytes, leukocytes, endothelial cells) and plasma coagulation factors. As such, there is no single assay which can be used to assess nanoparticle thrombogenic potential. Nevertheless, most researchers have found *in vitro* assays targeting platelets and three plasma coagulation pathways (extrinsic, intrinsic and common) useful for estimating nanoparticle pro- and anti-coagulant properties. For example, using perfluorocarbon nanoparticles, Myerson et al. reported that plasma coagulation in activated partial thromboplastic time (APTT) and thrombin-generation assays *in vitro* was predictive of changes leading to arterial occlusion *in vivo* [88]. Another recent study reported a correlation between the results of an *in vitro* blood clotting time assay and *in vivo* blood coagulation in response to small silica oxide nanoparticles [89]. Activation and cleavage of mouse plasma kallikrein by superparamagnetic iron oxide nanoparticles *in vitro* were shown to be related to kallikrein activation *in vivo* [90]. A study of five nanomaterials (carbon black, silicon dioxide, silicon carbide, titanium carbide and copper oxide) for which various degrees of thrombogenicity had been demonstrated *in vivo* by previous studies revealed corresponding procoagulant activity *in vitro* in APTT and calibrated thrombin generation assays [91]. This study also concluded that although both assays detected procoagulant activity of the tested nanomaterials, the calibrated thrombin generation assay appeared to be more sensitive [91].

A correlation between an *in vitro* platelet aggregation assay and vascular thrombosis *in vivo* has been demonstrated for carbon-based nanomaterials, including single- and multi-wall carbon nanotubes, C60 fullerene derivatives and amorphous carbon particulates [92]. Likewise, a correlation between *in vitro* platelet aggregation and *in vivo* thrombosis was reported in a study investigating several quantum dots with various surface coatings [93], S-purified single wall carbon nanotubes [94], and metallic silver nanoparticles [95]. Certain types of PAMAM dendrimers and metal oxide nanoparticles causing DIC and

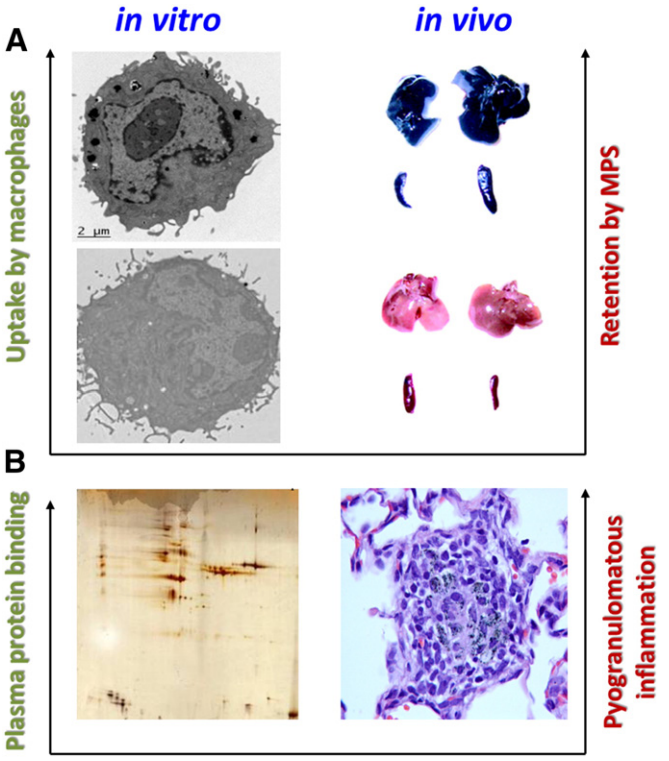

Fig. 6. *In vitro–in vivo* correlation of phagocytosis and protein binding. A. Nanoparticles with surfaces unprotected by hydrophilic polymers are readily taken up by macrophages *in vitro* and *in vivo*. An *in vitro* phagocytosis assay and electron microscopy were used to detect uptake of citrate stabilized colloidal gold nanoparticles (top panel, left) and PEGylated colloidal gold nanoparticles (bottom panel, left). Nanoparticles taken up by macrophages *in vitro* were also retained by MPS organs *in vivo* (top panel, right). B. Gold shell nanoparticles showing abundant binding of plasma proteins (bottom, left) were retained by macrophages *in vitro* and resulted in inflammation-mediated toxicity *in vivo* (bottom, right). Their counterparts which did not bind proteins were not taken up by cells and were not toxic.
Panel A: *In vivo* MPS retention image was adopted from reference [86] with permissions.
Panel B: Adopted from reference [87] with permission.

DIC-like toxicities *in vivo* have also been reported to activate the plasma coagulation cascade and platelets, as well as induce platelet aggregation and leukocyte procoagulant activity *in vitro* [89,96–100]. It is worth mentioning that some nanomaterials causing DIC are positive in all *in vitro* coagulation assays, including those for leukocyte procoagulant activity, plasma coagulation time, platelet activation and aggregation tests, while other nanoparticles are reactive only in some of these *in vitro* assays (Dobrovolskaia et al., unpublished observation). At NCL, we did not observe a correlation between the number of *in vitro* coagulation tests in which a nanomaterial was shown to be procoagulant and its thrombigenic "potency" *in vivo*. Therefore, positive reactivity in even one *in vitro* coagulation test is a cause for a careful scrutiny of the *in vivo* characterization phase.

5.2. Leukocyte proliferation

Occurs in response to agents stimulating cell division (mitogens) and may also be caused by specific substances (antigens) that cells were exposed to previously and have retained in their "immunological memory". Leukocyte proliferation assists in the host response to various immunogenic substances. Inhibiting this response may lower the body's protection against invading pathogens and cancer. Leukocyte proliferation tests can be used to screen for nanoparticle immunostimulatory properties, i.e. when nanoparticles act as mitogens or antigens. This latter test has a reciprocal application in that it can be used to identify immunosuppressive nanomaterials. In this case, the cells are exposed to a known mitogen (e.g. PHA-M or LPS)

or an antigen (e.g. a virus) after exposure to a non-cytotoxic concentration of a nanomaterial. Inhibition of the mitogen and/or antigen mediated proliferation by the test nanomaterial is interpreted as immunosuppression. Recently, Moon et al. have described a strong association between leukocyte proliferation *in vitro* and *in vivo* in response to known mitogens (bacterial LPS and Con A), and inhibition of this proliferation by TiO_2 nanoparticles [101].

In the NCL case study presented below, we tested a PEGylated micelle nanocarrier. This formulation did not contain a cytotoxic agent and itself was not cytotoxic *in vitro*. When leukocytes purified from the whole blood of healthy donor volunteers were challenged by mitogen (PHA-M) or antigen (flu virus) after exposure to this nanoformulation, we observed a dose-dependent inhibition of cell proliferation over control cells (Fig. 7). When the same formulation was tested *in vivo*, a treatment-related decrease in leukocyte counts was observed in animals. This was not related to suppression of the bone marrow, but was due to a direct effect on cells in the blood stream.

5.3. Colony Forming Unit—Granulocyte Macrophage (CFU-GM)

An assay for evaluating the growth and differentiation of bone marrow, derived pluripotent stem cells, into macrophages and granulocytes. This assay is widely used in anti-cancer drug screening to assess the immunosuppressive potential of cytotoxic drugs for which myelosuppression is a common dose-limiting toxicity [102]. Multiple studies have established the applicability of *in vitro* CFU-GM methods employing human or canine bone marrow cells to predict the maximum tolerated dose of cytotoxic drugs in humans [102–108]. The *in vitro* version of the assay employing human bone marrow cells was validated by the European Committee for Validation of Alternative Test Methods as a predictive tool to screen for myelosuppression [102]. This *in vitro* assay was successfully used by many researchers to assess the bone marrow toxicity of both nano- and non-nano-formulations, including camptothecin derivatives [109], eighteen oncology formulations known to cause myelosuppression in patients [105], novel compounds [110,111], metal oxide nanoparticles [112], polymeric nanoparticles loaded with cytotoxic oncology drugs [113,114], and magnetic polymeric nanospheres [115]. This assay is very helpful in selecting nanoformulations of traditional cytotoxic oncology drugs with reduced myelosuppresive potential. We have successfully used this assay for 8 years to understand the benefits of reformulating small molecule drugs onto nanotechnology platforms [36]. It is also helpful in understanding the myelosuppressive potential of nanoparticle carriers.

This assay is available in two formats—*in vitro* and *ex vivo* [36]. As with many *in vitro* assays, the *in vitro* version of the assay does not account for nanoparticle biodistribution. Therefore its results have to be considered in the context of data from a PK study demonstrating distribution to bone marrow. There are two other important considerations for this assay. One comes from the study by Erickson-Miller et al. demonstrating interspecies differences in sensitivity to myelosuppresion, in that canine and human bone marrow are more sensitive to materials with certain chemical compositions than in rodent bone marrow [104]. Another important consideration was proposed by Bregoli et al. who evaluated the myelosuppresion of seven nanoformulations representing different types of engineered nanomaterials [112]. The authors concluded that primary bone marrow cells are more sensitive and thus more predictive of nanomaterial toxicity to bone marrow than

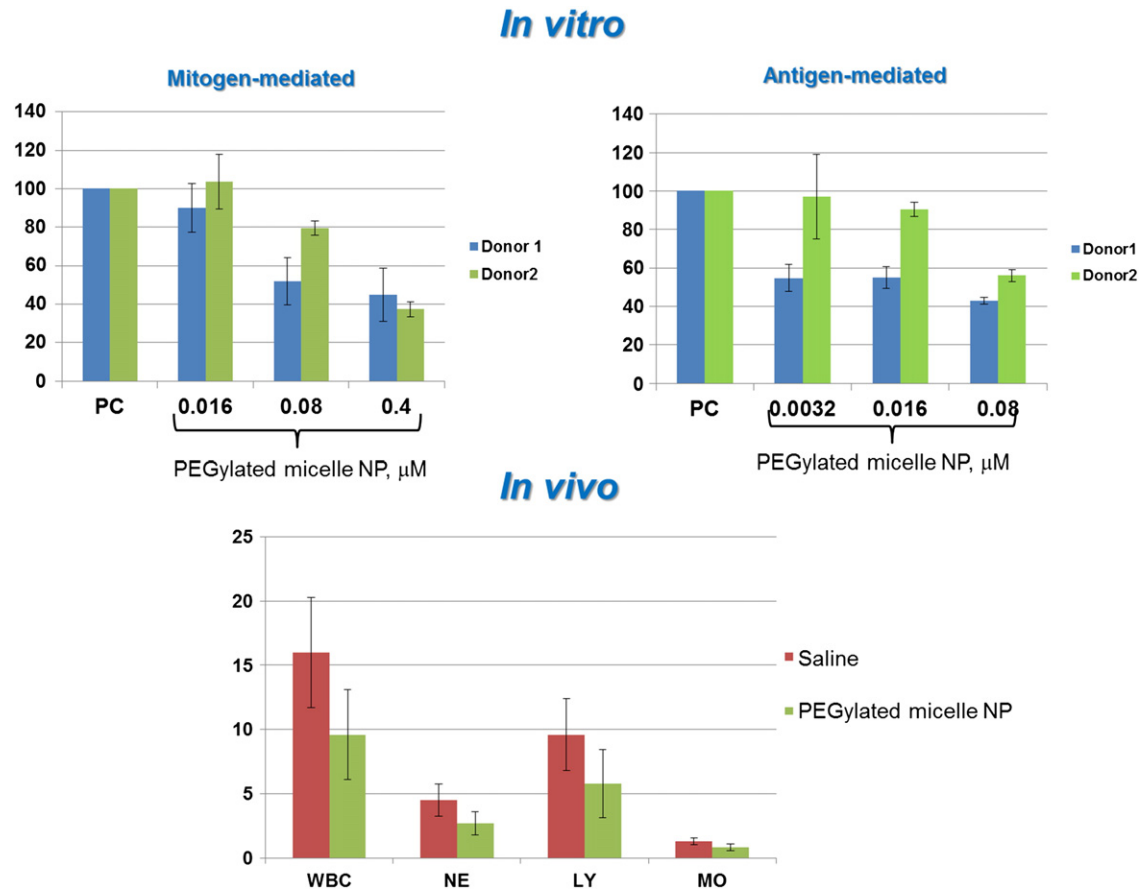

Fig. 7. *In vitro–in vivo* correlation of the leukocyte proliferation assay. A PEGylated micelle nanoparticle was tested at non-cytotoxic concentrations in a leukocyte proliferation test *in vitro* and resulted in inhibition of both mitogen- and antigen-specific proliferation. Human PBMC used *in vitro* and the *in vivo* study was performed in rats. PC = positive control, NP = nanoparticle, WBC = white blood cells, NE = neutrophils, LY = leucocytes, MO = monocytes, PBMC = Peripheral Blood Mononuclear Cells.

hematological cancer cell lines (K562, HL-60, CEM, CEM-R, THP-1, Jurkat and MOLT-4) [112].

6. Conclusions

Although traditional toxicity studies are helpful in identifying acute toxicity to the immune system, they are often insensitive to immunotoxicity resulting from dysregulation of the immune system's function. This is why immunotoxicologists have established a battery of immune function tests, some of which have been proven to be highly predictive of the immunotoxicity in humans. The battery of traditional standard and immune function tests is generally applicable to engineered nanomaterials, but each nanoformulation is unique, and changing one physicochemical attribute can lead to dramatic changes in biocompatibility. These properties can be tuned to achieve desirable efficacy and safety profiles, including immunocompatibility [20–22,116]. Screening nanoparticle formulations *in vitro* allows for rapid and cost-efficient evaluation compared to legacy *in vivo* models.

Valid concerns regarding *in vitro* methods still remain, as multiple parameters influence their *in vivo* predictability. These concerns primarily surround 1) dose-selection, 2) dose-metrics, 3) assay format, 4) species of cells and matrices, and 5) frequently, lack of nano-relevant controls.

The last 10 years of immunocompatibility studies of engineered nanomaterials have improved our current understanding of the subject and helped to identify *in vitro* immunoassays which are predictive of *in vivo* toxicities. *In vitro* hemolysis, complement activation, opsonization and phagocytosis, and cytokine secretion assays can be named among the predictive *in vitro* tests. Identifying changes in immune system function is more complex and requires approaches combining multiple tests. Nevertheless, several assays, including immunosuppression tests (CFU-GM and leukocyte proliferation), and thrombogenicity assays (platelet aggregation, leukocyte PCA and various plasma coagulation tests) have been found to be generally predictive. More studies analyzing various types of nanomaterials in *in vitro* and corresponding *in vivo* tests will further help to establish the correlation and to improve understanding of *in vitro* predictability. Current gaps include the lack of a harmonized dose metric for nanomaterials, relevant nanoparticle positive and negative controls for various *in vitro* immunoassays, and the lack of harmonized approaches for the selection of concentrations for *in vitro* studies. Future work should focus on development and validation of new methods for assessing immune system functionality, qualifying existing methods for various types of engineered nanomaterials, and inter-laboratory studies to examine the robustness of these tests and standardization of *in vitro* assays with good *in vivo* predictability.

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