A porcine model of complement activation-related pseudoallergy to nano-pharmaceuticals: Pros and cons of translation to a preclinical safety test

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Abbreviations:

- API, active pharmaceutical ingredient
- C, complement
- CARPA, C activation-related pseudoallergy
- HSR, hypersensitivity reaction
- MLV, large multilamellar liposomes
- NP, nanoparticle
- PAP, pulmonary arterial blood pressure
- PIM, Pulmonary intravascular macrophage
- SAP, systemic arterial blood pressure

Abstract

Pigs provide a sensitive and quantitative animal model of non-IgE-mediated (pseudoallergic) hypersensitivity reactions (HSRs) caused by liposomes and many other nanoparticulate drugs or drug-carrier nanosystems (nanomedicines). The rapidly arising symptoms, including cardiopulmonary, hemodynamic, hematological, blood chemistry and skin changes, resemble the clinical picture in man undergoing infusion reactions to reactogenic nanoparticles. In addition to summarizing the basic features of the pig CARPA model, the review considers some of the advantages and disadvantages of using the model for preclinical evaluation of nanomedicine safety[ii].

Keywords:

Hypersensitivity reactions, complement, anaphylatoxins, animal models, hemodynamic changes, anaphylaxis, macrophages, mast cells, thromboxane.

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Rationale and Purpose

Nanotechnology has achieved remarkable success in improving the therapeutic index of numerous drugs and agents by using drug-carrier nanosystems (nanocarriers) that carry and, in some cases, target the active pharmaceutical ingredient (API) to its site of action and/or control its absorption, distribution, metabolism, and excretion properties. However, together with these advantageous features, nanoparticulate (non-biological) complex drugs can cause adverse effects that the API alone would not cause. One of such adverse effects is a hypersensitivity reaction (HSR) which may occur typically at the first time of application. These acute and reversible immune reactions, also known as infusion-, or anaphylactic /anaphylactoid-, or idiosyncratic reactions, are not mediated by IgE. Due to this fact, they are commonly called "pseudoallergy," although the European Academy for Allergology and Clinical Immunology suggested to abandon this name (1). Nevertheless, we keep using it partly because it is notable, and partly because no appropriate alternative term was proposed for this type of HSR.

There is a large amount of direct and indirect evidence for the involvement of complement (C) activation in these pseudoallergies, which has led us to call this phenomenon C activation-related pseudoallergy (CARPA) (2). As detailed in previous studies and reviews (3-7), CARPA has been increasingly recognized as a possibly important mechanism of many unpredictable, poorly understood, severe, life-threatening HSRs to various intravenous (IV) drugs and agents, which still arise from time to time despite extensive anti-allergic pre-medications of patients and increasing alertness at the bedside for any sign of HSR. Most recently, HSRs to iron-containing drugs have been associated with CARPA, at least in some of the patients (8, 9).

CARPA was first described in pigs (2), and ever since, pigs have been used as the best animal model to study this phenomenon (3-7). The goals of the present review are to outline the scientific background and essential features, advantages and disadvantages of the model and to provide an update about its use for the prediction of HSRs in man.

Summary of relevant literature

The C system is a phylogenetically ancient, essential part of the immune system consisting of some 30 proteins in serum and cell membranes (10). It represents the humoral arm of innate immunity with several physiological functions, among which first-line antimicrobial defense is the best known. Importantly for us, it has been recognized a long time ago that C activation, i.e., cascadic proteolysis of serum components leading to the formation of anaphylatoxins and other biologically active cleavage products, may play a role in HSRs as well (11). Since liposomes have been known to activate C for nearly half a century (12), association of C activation with HSRs in pigs appeared self-evident when liposome-induced anaphylaxis started to draw attention as a model of drug-induced HSRs (2, 13). Over the past 50 years we learned that liposomes and other NPs can activate C via all known activation pathways, such as classical, alternative and lectin (14–16), although these mechanisms may overlap and even switch, depending on the surface properties of NPs (17). In addition to the physicochemical properties, binding of plasma proteins also plays a significant role in C activation by NPs. This process was described as dynamic and reversible (18,19), lending substantial variation to C3 deposition on NPs in different individuals under different conditions (20).

As for the mechanism of C activation in pigs, the binding of natural IgG and IgM antibodies to reactogenic liposomes points to classical pathway activation (2,21,22). On the other hand, the massive hemodynamic effects of alternative pathway activators zymosan and carboxylated polystyrene NP (PS-NPs) (23) indicate a main role of alternative pathway activation.

Regardless of the particular biochemical pathway of C activation, the HSRs observed in pigs share many symptoms of human HSR reactions, most importantly the cardiovascular distress, which in extreme cases may even be lethal in patients. This critical relevance for safety, together with the sensitivity and reproducibility of the model enabling the analysis of minor amounts of drugs in relatively few animals, motivated several "porcine CARPA" studies over the past 20 years to explore the

reactogenicity of a broad range of NPs and to better understand the mechanisms of these reactions (13,2,24–33).

Discussion

Technical aspects of the model

Figure 1 illustrates the setup of the model with the different measurements and instruments applied. In brief, adolescent pigs (3–4 months old, 25–30 kg) are initially sedated, then anesthetized and undergo endotracheal intubation and catheterization of the pulmonary artery and the femoral artery to measure the pulmonary arterial pressure (PAP), systemic arterial pressure (SAP), and heart rate (HR). The introduction of tested materials and taking of blood samples are performed via venous catheters. The PAP, SAP and HR are monitored and recorded continuously, as well as the EKG and respiratory parameters (respiratory rate, RR) and end-tidal CO_2 (EtCO$_2$).

The symptoms of porcine CARPA and their human relevance

Just as in humans, the symptoms of an HSR in pigs arise within minutes after intravenous adminstration of reactogenic NPs (reactive NPs) and the reactions subside within 15–60 min, depending on severity. Figure 2 illustrates the typical symptoms of severe HSRs in pigs with transient, but major hemodynamic, respiratory and skin changes (28). In this example, after the larger bolus dose of the reaction trigger intravenous lipid emulsion (ILE), the PAP steeply rose to maximal value and the animal stopped breathing spontaneously, controlled ventilation was initiated, and the RR fell to the rate of the respirator and the end-tidal CO_2 dropped, indicating reduced pulmonary perfusion with decreased cardiac output, which are typical of shock. At the same time the plasma TxB$_2$ level rose 30-fold over baseline and the animal displayed flushing and rash (inserts). After about 10 minutes all values started to return to normal.
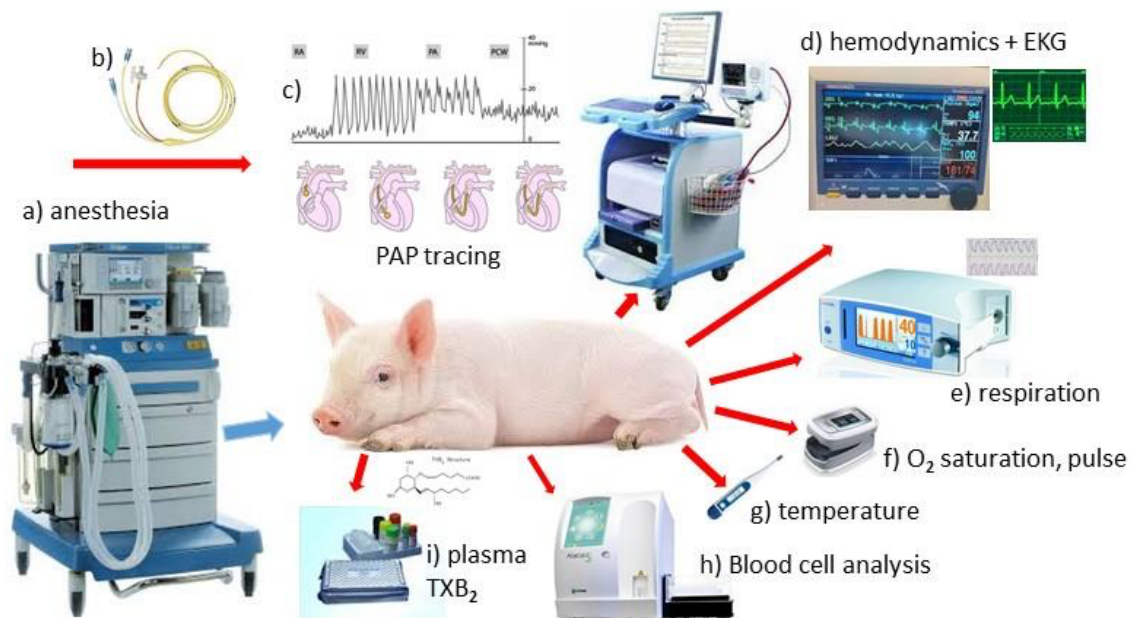

Figure 1. Parameters measured, and equipment used in the porcine CARPA model. a) anesthesia machine; b) Swan-Ganz balloon catheter, used for the measurement of pulmonary arterial pressure; c) blood pressure wave forms during passage of the tip of the Swan-Ganz catheter via the right atrium, right ventricle, and pulmonary artery until being wedged into the pulmonary capillary bed, d) computerized hemodynamic monitoring system tracing the systemic and pulmonary pressures, heart rate, and the EKG; e) capnograph measuring the respiratory rate (RR) and end-tidal carbon-dioxide (EtCO2; f) pulse oximeter measuring oxygen saturation and pulse rate; g) rectal temperature probe; h) blood cell analyzer; i) Enzyme Linked Immunosorbent Assay for measuring plasma mediators, such as TxB2. The figure was reproduced from Ref. (7) with permission.
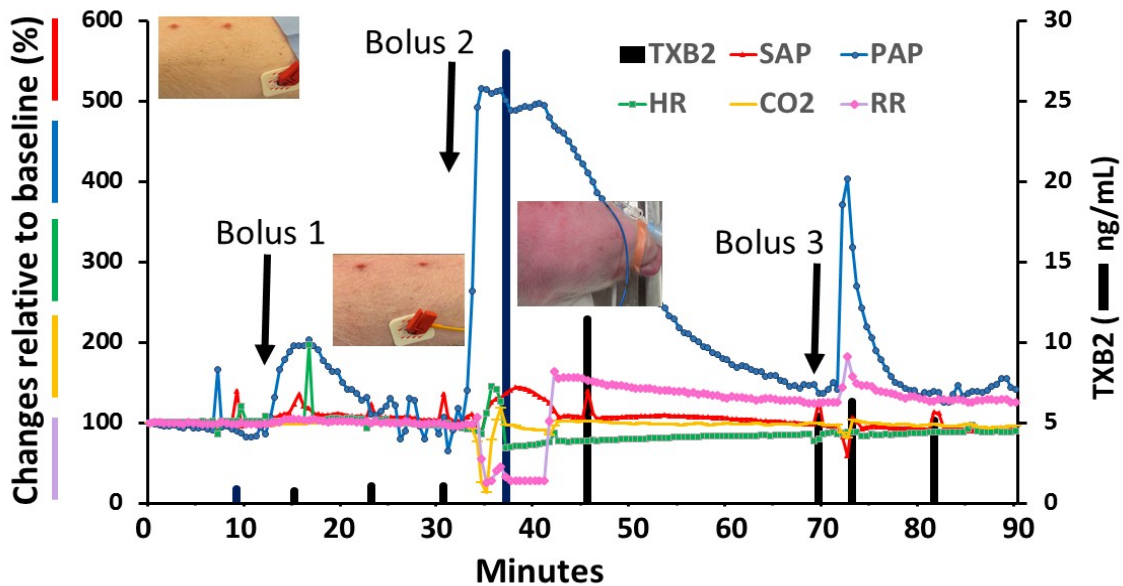
Figure 2. Hypersensitivity reaction of a pig to intravenous (IV) treatment with an IV lipid emulsion (Intralipid 20%, ILE) and zymosan. The arrow shows the timing of IV injections: Bolus 1: 1.5 mL/kg ILE; Bolus 2: 5 mL/kg ILE; Bolus 3: 0.1 mg/kg zymosan. The figure was constructed from published (28) and unpublished data. The inserted skin photos are positioned according to the time of their capture. CO2, = end-tidal CO2.

Figure 3A illustrates yet another universal symptom of pig HSR to nanoparticles: transient blood cell changes. Typically, the white blood cells (WBC) and platelets (PLT) drop immediately after the injection of the reactogenic test substance and then return to baseline in about 15 minutes, while the WBC may later rise over

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baseline (not shown in Fig 3A), a reflection of overcompensation. The WBC changes include changes of granulocytes (Gr) and lymphocytes (Ly) in variable ratios. The most severe reactions in pigs, rapidly progressing into shock (Figure 3B), resemble the cardiovascular collapse underlying the lethal anaphylaxis in man.
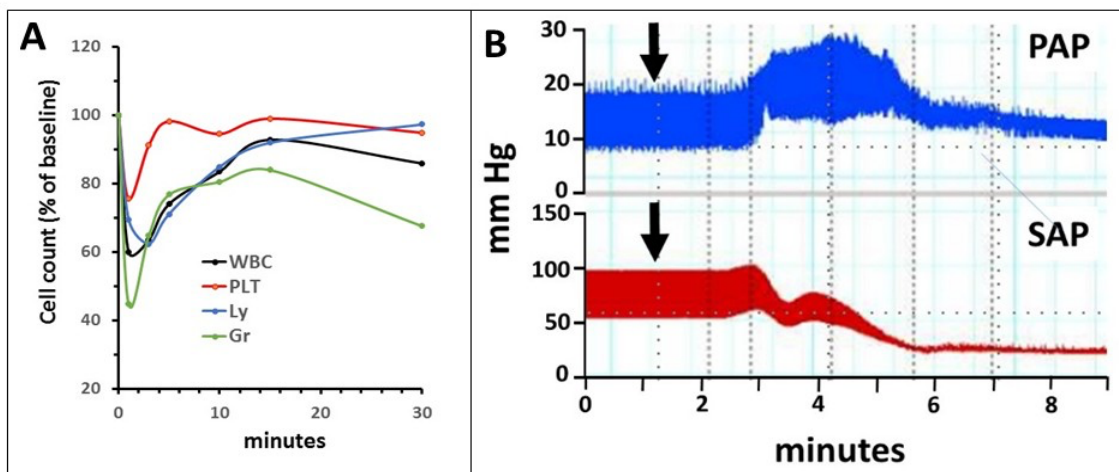

Figure 3. Blood cell changes and shock in severe HSR caused by liposomes in a pig. The arrow shows the timing of intravenous injection of PEGylated liposomal doxorubicin (Doxil, 0.1 mg/kg phospholipid), followed by cardiovascular collapse within 4–5 minutes. Real-time recordings of A) pulmonary arterial pressure (PAP) and B) systemic arterial pressure (SAP). Gr = granulocytes; Ly = lymphocytes; PLT = platelets; WBC = white blood cells

In addition to reproducing the severe, life-threatening cardiovascular symptoms and skin alterations, what makes the pig model relevant to human HSRs is that the dose eliciting the reaction is identical or very similar to the doses that trigger life-threatening reactions in man. This statement is based on calculations that the bolus dose of PEGylated liposomal doxorubicin (Doxil) triggering pulmonary hypertension in pigs is identical to the amount of Doxil reaching the circulation of reactive patients within the first minutes of infusion, when the symptoms start (34). Although similar calculations have not been made for other reactive NPs, the reactogenic dose of phospholipid-containing reactive NPs is in the 0.01–0.1 mg/kg range on phospholipid weight/pig weight basis, a value that may guide similar calculations for other liposomal drugs. In rats and mice hemodynamic changes are triggered only by doses that are orders of magnitude higher (35).

Hemodynamic alterations and their mechanism

Focusing on the initial hemodynamic changes and their likely mechanism, the transient, but significant rise of PAP is the most reproducible measure of adverse immuno-circulatory response to reactogenic NPs, which we quantify as the primary endpoint of HSRs. Interestingly, while the PAP almost always rises, the extent and direction of changes of SAP are highly variable. The HR usually increases, or it does not change, while the most intense reactions may entail paradoxical bradycardia (26).

The pulmonary hypertension is most likely due to the release of TxA2, a known pulmonary vasoconstrictor eicosanoid. This assumption is supported by the remarkable parallelism between the rises of PAP and TxB2, the stable metabolite of TxA_2 (Figure 2), and the observation that indomethacin, a cyclooxygenase blocker of TxA_2 release, inhibits both processes (2, 32).

As for the source of TxA_2, the primary suspects are pulmonary intravascular macrophages (PIM cells), which are resident macrophages adhered to the endothelium of pulmonary capillaries. The abundant presence of PIMs is observed only in a few species, such as sheep, cattle, horse, and cat (36). Their function is to screen the blood from particulate pathogens

(37–41). PIM cells express anaphylatoxin C5a receptor (ATR, C5aR) on their surface as well as Fc, Toll like- and C receptors (CR1, CR3 and CR4), and can secrete vasoactive mediators including TxA_2, histamine, leukotrienes, PAF, and IL-6, IL-8 and IL-1β). The combination of different vasoactivity of all secretion products explains the versatility of systemic blood pressure changes in CARPA (40). However, TxA_2 and many of these mediators may also be released by other ATR+ cells, including mast cells, leukocytes and activated platelets (42). The relative contribution of these cells to TxA_2 production in pig CARPA is not yet clarified.

The key role of macrophages in the cardiopulmonary distress of pigs following reactogenic NP administration was supported by a recent study showing close parallelism of the time courses of pulmonary hypertension caused by spherical polystyrene NPs (PS-NPs), their clearance from blood in mice and their uptake by cultured macrophages (32). Although the rapid phagocytosis of PS-NPs by PIM cells was suggested to be the main mechanism of the pigs' pulmonary response independent of C activation (32, 41), a follow-up review (43) and a recent study (23) argued against premature exclusion of the role of C. It was pointed out that the *in vitro* ELISA results conducted in whole blood (32) could not provide adequate evidence for the absence of C activation *in vivo*, so the question needs to be further studied. In doing so, we found that other methodical approaches, namely FACS analysis of NP-coated C fragments (C5b-9 and iC3b) and Western blot detection of C3 degradation did indicate C activation in pig serum by PS-NPs *in vitro* (23). Since the detected iC3b, C3d, and C3dg are known opsonins, and opsonization is a well-known trigger for enhanced phagocytic uptake (44–46), it is very likely that C activation played a role in the rapid clearance and "robust" uptake of PS-NPs by macrophages (32) via its opsonic ability. Whether or not opsonization of NPs by C3b and its byproducts is "complemented" by concurrent C3a/C5a production and stimulation of cells via anaphylatoxin receptors is not yet clear. In any case, these data emphasize the complexity of CARPA, and the existence of two or more activation mechanisms ("double hit") (5, 27)

rather than rapid phagocytosis representing an alternative mechanism of HSRs, competing with the CARPA concept (32, 41). On top of advancing this academic debate, the latter study (23) highlighted that 500 nm PS-NPs are the most potent inducers of HSR in pigs studied to date, possibly due to their high negative surface charge and hydrophobicity. Also, despite the difficulties in projecting *in* vitro C assay data to *in vivo* physiological changes (43), the study showed significant correlation between C activation by different sized PS-NPs in human serum and pulmonary hypertensive effect in pigs providing strong support for the CARPA background of PS-NP reactions.

Use of the pig model for safety screening

The European Medicines Agency's latest guidance on generic liposome development (47) recommends "*the use of in vitro and in vivo immune reactogenicity assays such as complement (and/or macrophage/basophil activation assays) and testing for complement activation-related pseudoallergy (CARPA) in sensitive animal model to evaluate the extent of potential adverse event*". Accordingly, *in vitro* C assays and the porcine CARPA model have been increasingly applied for preclinical safety screening of nanodrug or contrast agent candidates. Recent examples of using these tests for safety assessment include the studies on nitroglycerin-loaded shear-responsive liposomes (30) and dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs) developed for magnetic resonance imaging (MRI) (31, 48).

A recent study suggested association between the pulmonary hypertensive effect in pigs and historic data on HSRs in man following IV use of the MRI agents Sinerem® and Resovist®. These are SPIONs and Sinerem, which was reactive in pigs, had been withdrawn from the market because of HSRs (33).

The pig model may be uniquely applicable for the prediction of HSRs due to the fact that the most sensitive endpoint of HSRs, the rise of PAP, is also the most reproducible physiological response to reactive NP exposure. This reproducibility is demonstrated in Table 1, showing the inter-experimental variation of pulmonary hypertensive responses to (first) bolus administration of reactogenic liposomal nanomedicines and zymosan.

Table 1. Pulmonary Hypertensive Effects of Complement Activators: Data Collected From "n" Different Experiments Performed Over > 6 Years. Reproduced from (7) with permission.

	1st PAP change (% of baseline) +/- SEM	n
Zymosan	$368,9 \pm 57,32$	15
Ambisome	$236,81 \pm 100,91$	7
Doxil	$233,11 \pm 91,79$	12

Similar analysis performed on the changes of PAP in pigs injected with multilamellar liposomes (MLV) for the first time gave a 79 ± 9 % increase (mean $\pm$ SEM, n=18) (2). Likewise, injection of the same MLVs in one animal 8 times over 8 hours led to a remarkably small (2.56%) coefficient of variation of PAP ($199 \pm 0.5\%$, mean $\pm$ SEM (2).

Ambiguities of the porcine model: the sensitivity issue and tachyphylaxis

Despite the above-mentioned advantages, the use of pigs for preclinical testing of the safety of NPs has been questioned for the same reason that lends uniqueness to the model: its high sensitivity (41, 49). The reaction rate to most reactogenic NPs is practically 100% in pigs, while in men the incidence of most HSRs to reactive NPs is in the 1–10% range. This implies that the porcine test has large bias to false positivity. However, because of the PIM cells, pigs may be perceived as animals with born hypersensitivity, and, hence, the model is a disease model and not that of normal human responses to NP exposure. To our best knowledge, no rodent, or any other large animal model would serve this purpose, i.e. enabling experimentation on a small number of animals and getting statistically valid information on possible side effects and their prevention. If the model had the same incidence rate of reactions as in humans, hundreds of pigs would be needed to obtain statistically evaluable numbers of reactive animals.

Another unique property of the pig model is tachyphylaxis, i.e., self-induced tolerance arising to certain reactogenic NPs after repetitive

administration. Typically, the reactions caused by PEGylated liposomes are tachyphylactic (27), while those caused by multilamellar DMPG-containing liposomes (2), or AmBisome (50), are not. This implies that when testing tachyphylactic drugs, only the first administration will reflect the response of a hypersensitive individual, the rest of the injections will underestimate the drug's reactivity. In contrast, if the agent is non-tachyphylactic, the model enables quantitation of multiple injections over hours, enabling dose-effect relationship and inhibition studies in individual animals.

Theories on mechanism

The mechanism underlying CARPA, in general, and tachyphylaxis, in particular, are poorly understood. The "double hit" theory (5, 27, 40) mentioned above in the context of PIM cells, postulates that NPs can induce HSRs by simultaneous stimulation of anaphylatoxin receptors (mainly C5aR, CD88) and other surface receptors on PIM cells, which can be linked to release reactions directly or indirectly. Potential receptors include pathogen recognition receptors, also known as pathogen-associated molecular pattern receptors (51,52), mannose-binding lectin receptor (53, 54), C receptors (CR2 and CR3 and Fc receptors) (55) and many others typically present on the surface of mast cells (56, 57). Among these, the human Mas-related G-protein-coupled receptor member X2 (MRGPRX2) was claimed to be crucial for pseudoallergic drug reactions (56, 58).

These different activation pathways are likely to lead to different degrees of stimulation, and exhaustion of one or more pathways upon repetitive exposures might explain tachyphylaxis. It also follows from the above "multiple hit" concept that if one or the other activation pathway is sufficiently strong, or dominates, they alone might trigger the release reaction. For example, in case of very strong C activation, the C5aR (or C3aR)-mediated anaphylatoxin "pathway" might dominate, while direct stimulants of mast cells, such as by opioids, neuromuscular blocking agents, quinolones, compound 48/80 (56), or physical stimuli of cold and trauma (59) might induce pseudoallergy directly, without C activation. Such reactions can be referred as C-independent pseudoallergies (CIPA) (43).

Conclusions

The mechanism of HSR reactions to nanomedicines is poorly understood at this time, and the porcine CARPA model may help disentangle a very complex chain of immuno-hemodynamic changes. A unique feature of the model is that the most sensitive endpoint of HSRs, the rise of PAP, is also the most constant and reproducible physiological response, and it is a direct predictor of the most dangerous consequence of these reactions: anaphylactic shock. Regarding the mechanism of HSRs in this model, we believe the "double hit hypothesis" (5), advanced to "multiple hit hypothesis" (43), represents a more comprehensive explanation of these reactions than the recently proposed "rapid phagocytosis response" hypothesis (41), as it reconciles C-dependent and C-independent immune activations as simultaneous, independent, yet highly coordinated pathways of a complex activation cascade. The key role of secretory macrophages, such as PIM cells, is important to keep in mind, to inspire novel approaches for the prediction and prevention of HSRs (32). Because of its high sensitivity, the porcine CARPA model may serve as an efficient preclinical safety screening test for reactogenicity of NP-based drugs and other agents. However, the data obtained in this model should not be directly extrapolated to man. When considered to be a disease model in healthy animals, the concerns about false positive results are no longer relevant.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose. For signed statements contact the journal office editor@precisionnanomedicine.com.

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