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# Comparison of complement activation-related pseudoallergy in miniature and domestic pigs: foundation of a validatable immune toxicity model

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**Abstract**

Complement activation-related pseudoallergy (CARPA) is an acute adverse immune reaction caused by many nanomedicines. There is a regulatory need for a sensitive and standardizable *in vivo* predictive assay. While domestic pigs are a sensitive animal model, miniature pigs are favored in toxicological studies yet their utility as a CARPA model has not yet been explored. Herein, we used liposomal doxorubicin and amphotericin B (Doxil/Caelyx and AmBisome), Cremophor EL and zymosan as CARPA triggers to induce reactions in miniature and domestic pigs, and compared the hemodynamic, hematological, biochemical, and skin alterations. The changes observed after administration of the test agents were very similar in both pig strains, suggesting that miniature pigs are a sensitive, reproducible, and, hence, validatable animal model for CARPA regulatory testing.

***From the Clinical Editor:*** With the advances in nanomedicine research, many new agents are now tested for use in clinical setting. Nonetheless, complement activation-related pseudoallergy (CARPA) is a well known phenomenon which can be caused by nanoparticles. In this study, the authors looked at and compared the use of domestic pigs versus miniature pigs as experimental animals for toxicological studies. Their findings confirmed the possible use of miniature pigs for regulatory testing.


*Key words:* CARPA; Immune toxicity; Miniature pig; Infusion reactions; Regulatory standards

The number of investigational and approved nanomedicines continues to rise, leading to important advances for diagnostic and therapeutic applications.[1,2] With this rapid growth has come increased attention to the regulatory challenges which nanomedicines pose.[3-5] One central issue relates to the i.v. administration of nanomedicines, which can provoke acute hypersensitivity reactions that are classified as complement (C) activation-related pseudoallergy (CARPA).[6-8] Many classes of nanomedicines are known to cause CARPA reactions, including liposomal drugs, micellar drug carriers, contrast agents, and therapeutic antibodies and enzymes.[6-11] Clinical evidence indicates that the frequency of mild to severe CARPA reactions in patients varies between approximately 2% and 30%.[8,12] In rare cases, the reactions are life-threatening and even fatal. The CARPA phenomenon has been recognized as a safety issue for nanomedicines,[13-16] and the European Medicines Agency recommends that generic i.v. liposomal nanomedicines be assessed for CARPA risk in preclinical testing.[15] The latter recommendation underscores the importance of developing and validating standardized predictive assays for CARPA.

*Abbreviations:* C, complement; CARPA, C activation-related pseudoallergy; Cr-EL, Cremophor EL; HR, heart rate; HSR, hypersensitivity reaction; i.v, intravenous; PAP, pulmonary arterial blood pressure; PLT, platelet; SAP, systemic arterial blood pressure; TXB2, thromboxane B2; WBC, white blood cells.

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So far, this goal has remained elusive in the face of numerous *in vitro* and *in vivo* test options for CARPA in a variety of assay systems, animal models, experimental conditions, and endpoints.[11,17-23] Animal experiments help to establish correlations between bolus injections of a test agent and physiological responses, including hemodynamic, hematological, biochemical, and cutaneous changes.[11,17-23] Ultimately, the predictive power of an animal model rests in its sensitivity, reproducibility, and accuracy. The domestic pig has proven to be a unique animal species for predicting the risk of CARPA reactions with high sensitivity and symptoms mimicking severe CARPA reactions in hypersensitive humans.[6,11,24-27] However, the porcine model of CARPA has the limitation that domestic pigs, in general, are not favored as model animals in toxicology studies, particularly long-term toxicology studies, mainly because of their substantial strain variation and the adult animals' large, rapidly growing weight. Miniature pigs, or minipigs, are the regulatory preference when pigs are used for toxicology evaluation.[28,29]

The goal of the present study was a systematic comparison of the activities of known reactogenic agents in the miniature and domestic pig models to answer the question if the minipig represents a mimic of Yorkshire and domestic pigs as a CARPA model. The tested CARPA-triggering agents included liposomal doxorubicin (Doxil, Caelyx), the first FDA-approved nanomedicine,[30] and liposomal amphotericin B (AmBisome), a successful antifungal drug.[31] Cremophor EL (Cr-EL), a micellar solvent of many antincancer drugs including paclitaxel (Taxol), and Zymosan, a widely known C activator yeast membrane polysaccharide[32] were also tested. As demonstrated in past studies, CARPA in pigs consists of a tetrad of physiological changes that include hemodynamic, hematological, biochemical, and skin changes.[11,20] The present study focused mainly on the hemodynamic and hematological endpoints.

## Methods

### *Materials*

Commercial AmBisome (Gilead, Astellas Pharma US, Inc.) and Caelyx (Jansen-Cilag) were stored at 2-8 °C. Purified Zymosan A (Z4250, Sigma-Aldrich) for research use was stored at 2-8 °C. Phosphate-buffered saline (PBS) was obtained from B. Braun Melsungen AG and used to dilute all test samples.

### *Animal studies*

Male mixed breed Yorkshire/Hungarian White Landrace domestic pigs (20-23 kg, 3 months old) and male Ellegaard Göttingen miniature pigs (18-19 kg, 10 months old) were obtained from the Animal Breeding and Nutrition Research Institute (Herceghalom, Hungary) and Ellegaard Göttingen Minipigs ApS (Dalmose, Denmark), respectively. Animals were pre-anesthetized intramuscularly with Calypsol/Xilazine injections (2-4/1.5-2 mL, based on weight), and then transported to the operating room. Anesthesia was maintained using isoflurane inhalation narcosis (2-2.5%) with oxygen. Intubation was performed with 6.5-7 Fr endotracheal tubes in order to maintain free airways with spontaneous breathing.

The pigs were instrumented with a Swan-Ganz catheter which was introduced into the pulmonary artery and used to measure the pulmonary arterial pressure (PAP). Another transducer was connected to the cannula in the femoral artery in order to record the systemic arterial pressure (SAP). The left femoral vein was cannulated for blood sampling. The left external jugular vein was cannulated for administration purposes. Upon completion of the experiments, all pigs were euthanized under anesthesia by the addition of 2 mL Euthasol and 20 mL concentrated KCl through an i.v. injection. All experiments were performed in accordance with the guidelines of the Ethical Committee of Semmelweis University, Budapest.

### *Drug administration*

The test agent was diluted in PBS solution (typically 5 mL) and quickly administered as a bolus i.v. injection into the left external jugular vein. As a negative control, 5 mL PBS without test agent was administered as a bolus i.v. injection and there were no physiological changes.

### *In vivo monitoring of cardiopulmonary responses*

Through continuous recording of the pulmonary and systemic pressure signals, online averaging was performed and recorded, and the heart rate (HR) was also derived (from the SAP signal) and averaged. From the mean PAP, SAP curves at determined time points about 20 s intervals were averaged and evaluated by the ADInstruments LabChart Pro v8 software modules.

### *Hematological analysis*

For determination of plasma levels of vasoactive substances, blood was collected and stored in K3-EDTA vials, and an additional 3 mL of blood was stored in spray-dried hirudin blood collecting tubes. The samples were taken just before administration of the test agent at 0 min, and 1, 3, 5, 10, and 15 min after administration. If the reaction was long, additional samples were collected at later time points (20, 30, 45 min). After sampling, the blood was kept on ice until centrifugation. Blood cell counting was performed using an Abacus Hematology Analyzer (Diatron Messtechnik GmbH). After centrifugation, the supernatant plasma was stored at −20 °C, and then transferred within 5 hr to −80 °C until analysis.

### *Measurement of plasma thromboxane B2*

Plasma thromboxane B2 (TXB2), the stable metabolite of TXBA2, was measured with an ELISA kit (Cayman Chemicals, Ann Arbor, MI).

### *ELISA assays of blood C3 levels*

Complement activation in pig blood was measured by combined use of MicroVue's Pan-Specific C3 (PS-C3) Reagent kit and the human SC5b-9 Plus EIA kit (TECO*medical* AG, Sissach, Switzerland). The former kit converts the porcine C3 level into human SC5b-9, whose level is measured by the second kit.

Table 1
Description of test agents for CARPA prediction in domestic and miniature pigs.

| Test agent | Clinical/Experimental use | Composition | Particle diameter | References |
|---|---|---|---|---|
| Caelyx | Liposomal formulation to deliver the chemotherapeutic agent Doxorubicin for cancer therapy | HSPC (56.3 mol%)<br>Cholesterol (38.4 mol%)<br>2 K-PEG-DSPE (5.3 mol%) | 80-85 nm | 10, 12, 16, 34 |
| AmBisome | Liposomal formulation to deliver the drug Amphotericin B for treatment of systemic fungal infections | HSPC (49 mol%)<br>Cholesterol (23 mol%)<br>DSPG (18 mol%)<br>Vitamin E (0.3 mol%<br>Amphotericin B (9 mol%) | 78 nm | 26, 35 |
| Cremophor EL | Nonionic emulsifier widely used to solubilize water insoluble drugs, such as the anticancer drug, paclitaxel (Taxol) | Complex mixture of unmodified castor oil and a large variety of polyethylene glycols, polyethoxylated glycerols, fatty acids, and mono-, di-, and tri-esters of glycerol that are polyethoxylated to different degrees. | 8-22 nm micelles (in saline)<br>50-300 nm droplets (in serum) | 36-38 |
| Zymosan | Positive control that is used to stimulate the innate immune response | Component of the yeast (*Saccharomyces cerevisiae*) cell wall that consists of protein-carbohydrate complexes, the latter glucan structures have repeating glucose units connected by β-1,3-glycosidic linkages. | 3 μm | 32, 39, 40 |

Two liposomal nanomedicines (one agent with and one agent without surface-grafted polyethylene glycol (PEG)) were tested along with a micellar carrier and a yeast cell extract.

### *Statistical analysis*

Data are expressed as the mean ± standard deviation of the mean where appropriate, and statistical analysis of continuous variables was performed using the unpaired *t* test in the GraphPad QuickCalcs computer program (GraphPad Software, Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant (*).

## Results

### *Features of CARPA triggering drugs*

Table 1 details the use, composition, and size of CARPA-genic agents tested, along with references to their CARPA reactogenicity.

### *Hemodynamic and blood cell changes caused by Caelyx*

Figure 1 presents the hemodynamic and blood cell changes that arise immediately after i.v. bolus administration of Caelyx into miniature and domestic pigs. Panels A-D present data from 2 animals (pig A and B), treated, as first bolus, with low (0.1 mg/kg) and 10-fold higher doses (high: 1.0 mg/kg phospholipid) of Caelyx, respectively. After these injections both animals were re-challenged with a high-dose (1 mg/kg) Caelyx to establish the presence of tachyphylaxis. In panel A, the first low-dose bolus led to a minor reaction involving an 11% increase in PAP within 2 min paralleled by a minimal 3% change in SAP and persistent 20% leukopenia (Figure 1, *A-C*, blue circles). The PLT count did not change relative to baseline values (Figure 1, *D*, blue circles). Upon the next sequential bolus at 1 mg/kg (not shown), the PAP signal exhibited a biphasic signature with an initial 17% increase followed by a 9% decrease relative to the baseline before final stabilization at the baseline level. In this case, leukopenia was minor (10%), while there was again a minimal 3% increase in the SAP and no change in the PLT level (not shown). This low reactogenecity at high dose is suggestive of partial tachyphylaxis, leading to further experiments at high dose with naïve miniature pigs.

Figure 1, *A* also shows that in a subsequent experiment using another naïve minipig (Pig B) the first bolus administration of Caelyx at 1 mg/kg led to a rapid and sharp, up to 100% increase in PAP, followed by gradual stabilization over 10 min (red squares). This response was paralleled by a 15% increase in SAP (Figure 1, *B*, red squares). There was also moderate 20% to 30% leukopenia and minor 5% to 10% thrombocytopenia (Figure 1, *C* and *D*, red squares). After stabilization of the physiological parameters, a second sequential bolus at 1 mg/kg was then administered (not shown). There was a delayed 10% to 25% increase in PAP within 6 min, while the SAP increased by 4% to 10% within 2 min. There were no changes in hematological parameters. Collectively, the evidence supports that the first bolus of 0.1 mg/kg or 1 mg/kg Caelyx provides similar levels of desensitization, as indicated by significant tachyphylaxis.

For comparison, repetitive bolus administrations at 1 mg/kg were also performed in naïve domestic pigs. The first bolus led to a rapid 34% to 47% increase in PAP and a 13% to 16% increase in SAP. Initially moderate 20% to 40% leukopenia was observed along with 10% to 30% thrombocytopenia, both of which began to recover within 3 min. By contrast, the second bolus provided evidence for nearly complete tachyphylaxis in the domestic pig, with a minor 8% increase in PAP within 4 min and no changes in the other physiological parameters. In general, the PAP changes recorded in miniature pigs (170% ± 22%) were comparable to those recorded in domestic pigs (141% ± 9%), as presented in Figure 1, *E*. On the other hand, the SAP changes were nearly identical in both miniature pigs (114% ± 2%) and domestic pigs (115% ± 2%) (Figure 1, *F*).

### *Hemodynamic and blood cell changes caused by AmBisome*

Figure 2 presents the hemodynamic and blood cell responses upon the i.v. administration of AmBisome into miniature pigs. Boluses at 0.1 mg/kg were first administrated, yielding rapid

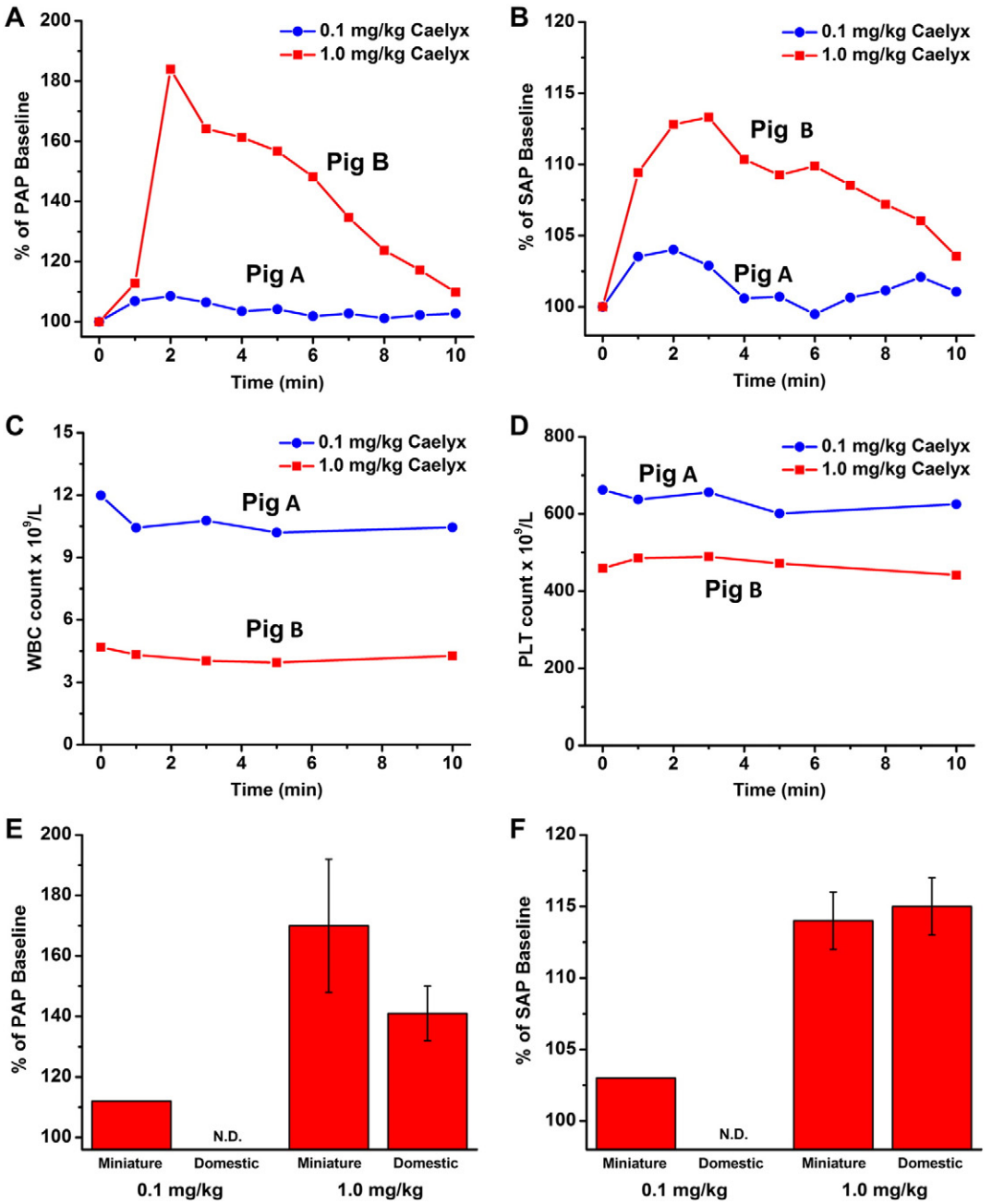


Figure 1. Physiological changes in miniature pigs upon Caelyx administration. Representative changes in **(A)** PAP, **(B)** SAP, **(C)** WBC, and **(D)** PLT in miniature pigs as a function of time in response to Caelyx bolus administrations at low dose (blue circles) and high dose (red squares) test concentrations. The injection time was normalized to $t = 0$ min. Comparison of **(E)** PAP and **(F)** SAP changes in miniature and domestic pigs. Note that only the high dose concentration was administered to domestic pigs. N.D. means not determined.

46% to 52% increases in PAP that gradually decreased over 10 min (Figure 2, *A*, blue circles). A parallel change in SAP (12% to 14% increase) with minor leukopenia were observed, and there were no changes in PLT counts (Figure 2, *B-D*, blue circles). In one exceptional case (not shown), the 0.1 mg/kg bolus triggered a 167% increase in PAP, a biphasic SAP response (36% increase followed by a 9% decrease relative to baseline), and significant 37% leukopenia and 20% thrombocytopenia. Bolus administrations at 0.1 mg/kg in domestic pigs caused more tempered cardiopulmonary stress. There was an approximately 30% increase in PAP with gradual decline, along with an 11% to 15% increase in SAP, moderate 18% to 25% leukopenia, and negligible thrombocytopenia.

At 1 mg/kg, boluses led to more pronounced reactions, with a 190% to 206% increase in PAP and a 26% to 50% increase in SAP (Figure 2, *A* and *B*, red squares). These hemodynamic

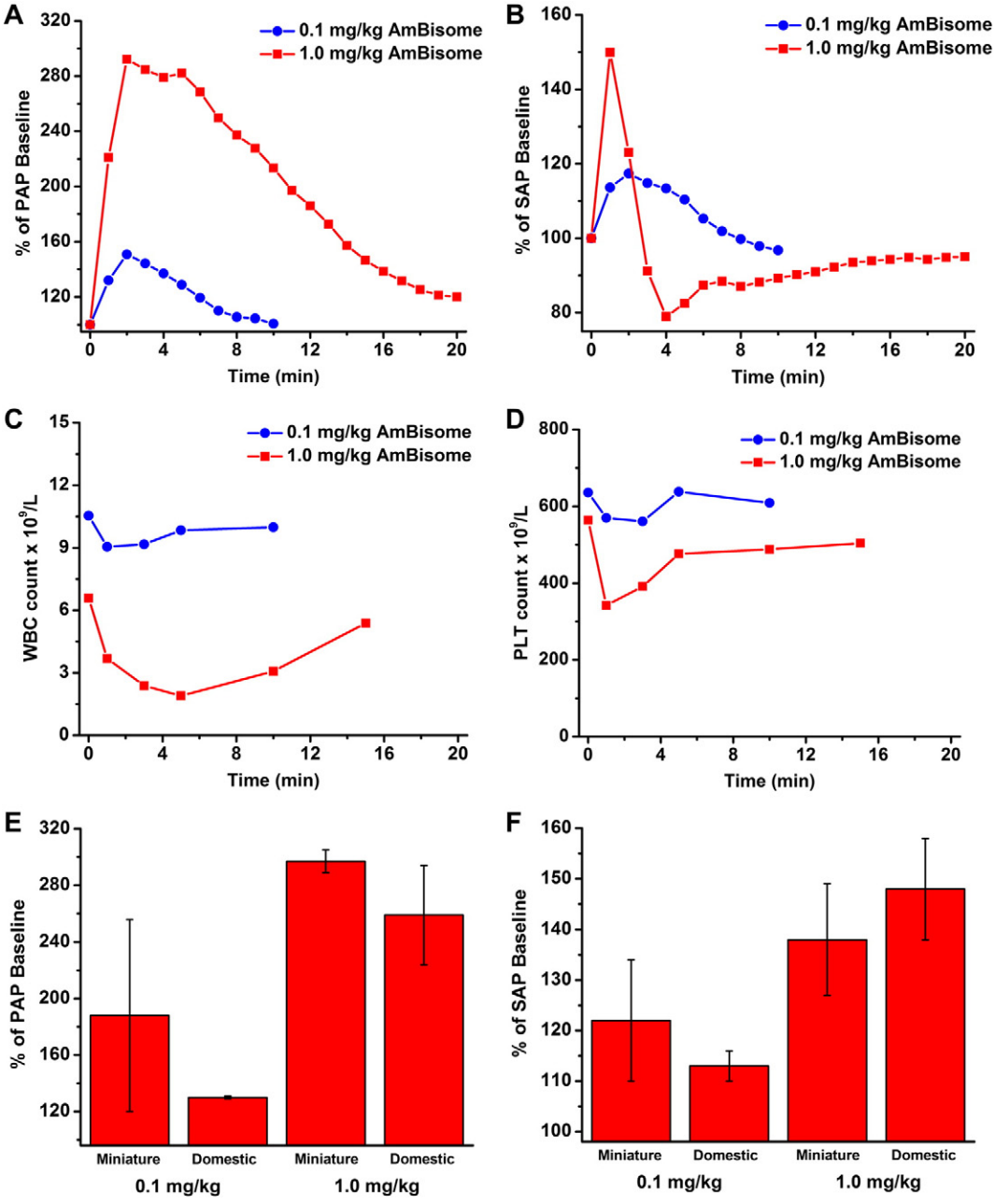


Figure 2. Physiological changes in miniature pigs upon AmBisome administration. Similar experiments were performed as in Figure 1 except that AmBisome boluses were administered.

changes were accompanied by significant hematological changes, including severe 40% to 72% leukopenia and moderate 20% to 30% thrombocytopenia that began to recover after 5 min (Figure 2, *C* and *D*, red squares). Likewise, strong reactions were observed in domestic pigs following 1 mg/kg boluses. There was a 134% to 184% increase in PAP with sustained 10-min peak intervals, as well as a 41% to 50% increase in SAP. As with miniature pigs, significant transient hematological changes were recorded in domestic pigs, particularly 40% to 47% leukopenia as well as moderate 14% to 25% thrombocytopenia. Overall, the hemodynamic and hematological responses are quantitatively comparable between the two species. The PAP changes recorded in miniature pigs at 0.1 mg/kg and 1.0 mg/kg boluses were 188% ± 66% and 297% ± 8%, respectively, and these values are statistically comparable to the equivalent values recorded in domestic pigs which were 130% ± 1% and 259% ± 35%, respectively (Figure 2, *E*). In addition, the SAP changes recorded in miniature pigs at 0.1 mg/kg and 1.0 mg/kg boluses were 122% ± 12% and 138% ± 11%, respectively, and these values are also statistically comparable to the equivalent values recorded in domestic pigs which were 113% ± 3% and 148% ± 10%, respectively (Figure 2, *F*).

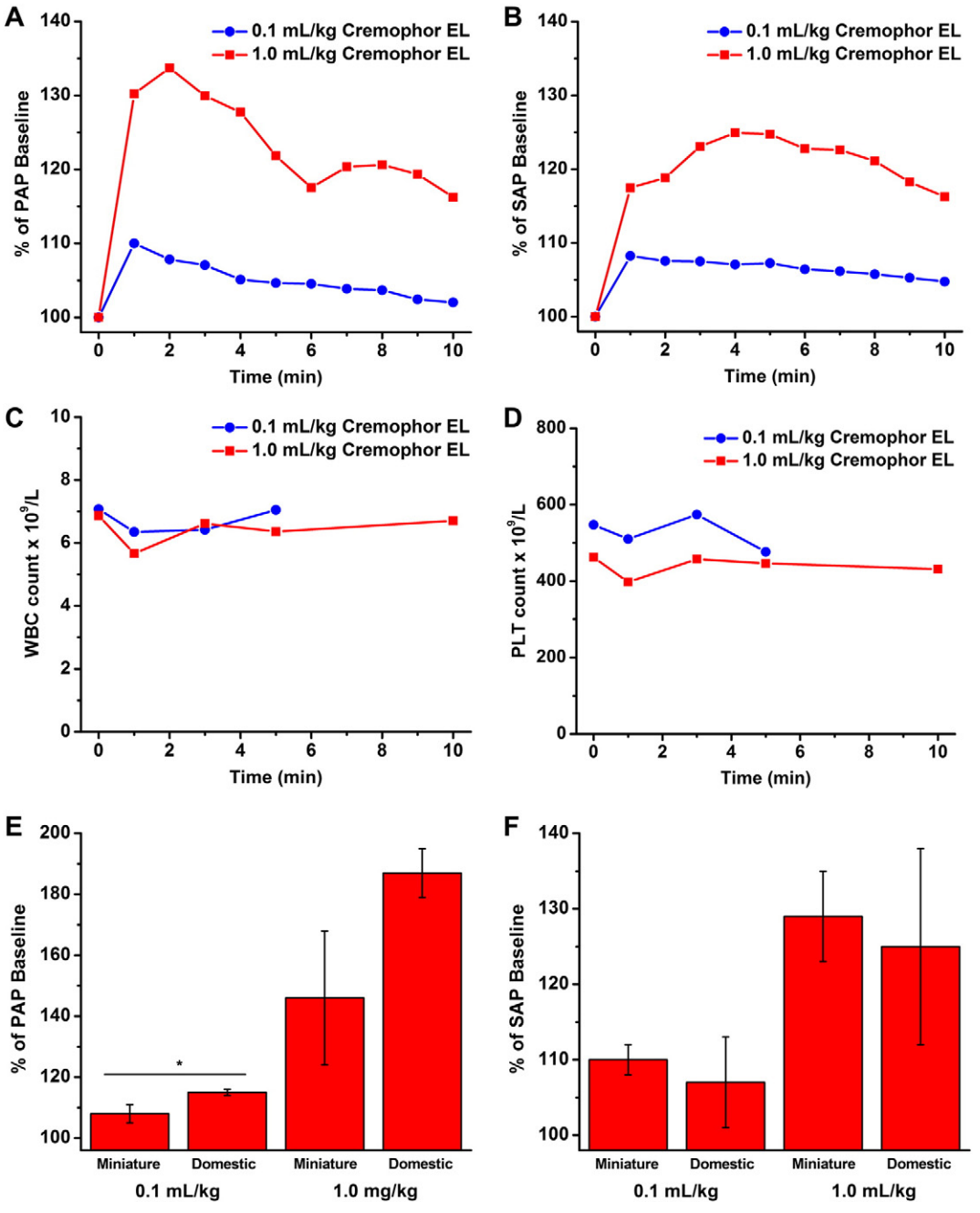


Figure 3. Physiological changes in miniature pigs upon Cremophor EL administration. Similar experiments were performed as in Figure 1 except that Cremophor EL boluses were administered.

*Hemodynamic and blood cell changes caused by Cremophor EL*

Figure 3 presents the hemodynamic and blood cell changes upon the i.v. administration of Cr-EL in miniature and domestic pigs. Bolus administration at 0.1 mL/kg caused minor hemodynamic effects, with a 5% to 10% increase in PAP and an 8% to 11% increase in SAP (Figure 3, *A* and *B*, blue circles). There were no hematological changes (Figure 3, *C* and *D*, blue circles). In domestic pigs, bolus administration at 0.1 mL/kg also led to only minor hemodynamic changes, as demonstrated by a 14% to 15% increase in PAP. There were also variable changes in the SAP but no hematological changes.

Sequential bolus administrations at 1.0 mL/kg provoked stronger reactions, with a 30% to 71% increase in PAP in miniature pigs along with a 26% to 35% increase in SAP (Figure 3, *A* and *B*, red squares). There was also minor 7% to 17% leukopenia and negligible changes in PLT (Figure 3, *C* and *D*, red squares). As Cr-EL was prepared in an 80/20 v/v% water–ethanol mixture, another bolus of 20% ethanol without test agent was also administered in a control experiment. In this

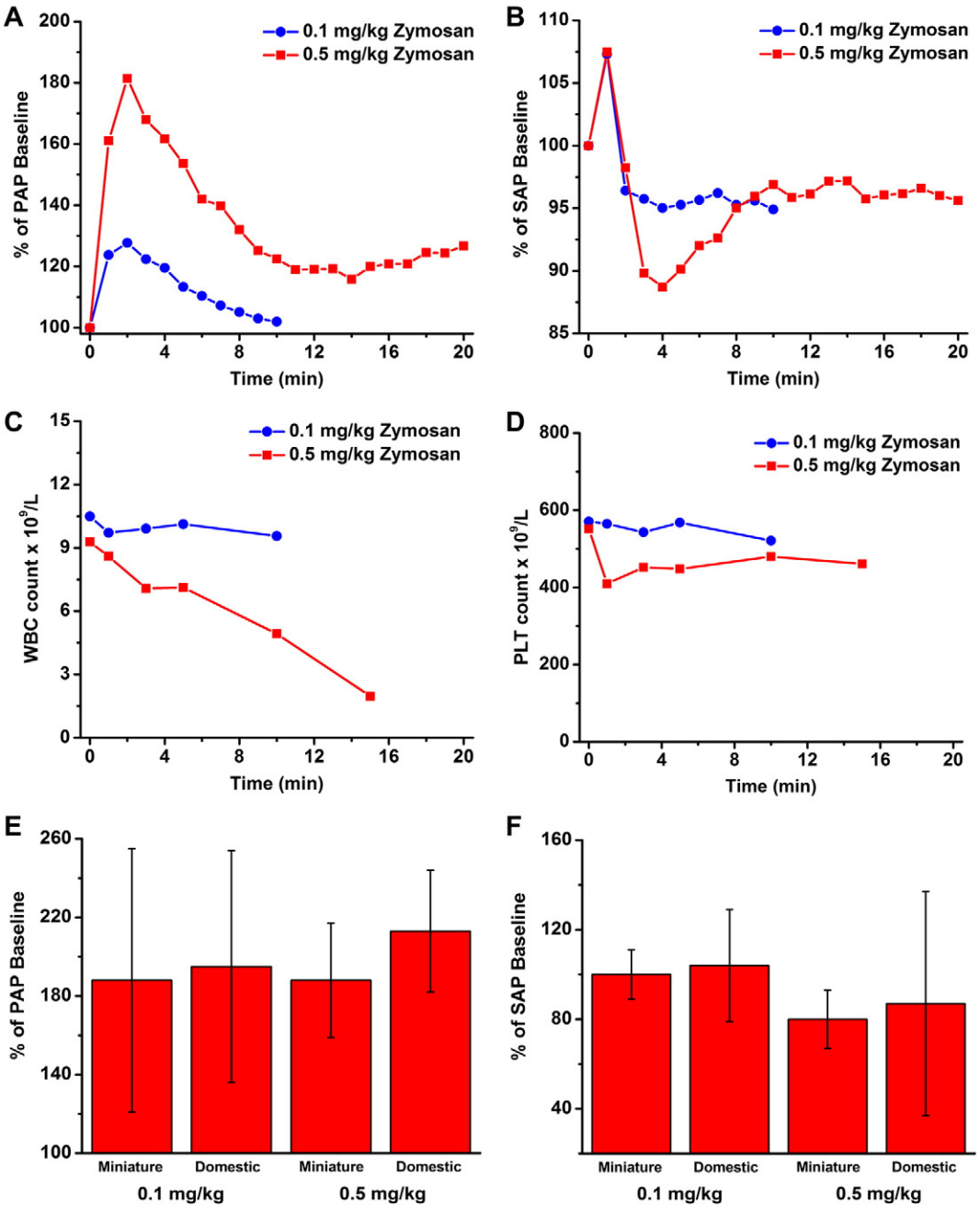


Figure 4. Physiological changes in miniature pigs upon Zymosan administration. Similar experiments were performed as in Figure 1 except that Zymosan boluses were administered.

case, there were no hemodynamic and hematological responses, confirming that the reaction was due to the nanomedicine, not the solvent. In domestic pigs, 1.0 mL/kg boluses were also administered, leading to 81% to 92% increases in PAP. The SAP changes were more moderate, and exhibited variable behavior, including a sharp increase or biphasic trends. The hematological changes were again nearly negligible, with only minor 0% to 16% leukopenia. Taken together, the findings support that the PAP change serves as a reliable signature for detecting reactions caused by i.v. administration of Cr-EL. Surprisingly, in contrast to the liposomal nanomedicines, the domestic pig appeared to have more sensitive PAP responses to Cr-EL than miniature pigs (Figure 3, *E* and *F*). The PAP changes recorded in miniature pigs at 0.1 mL/kg and 1.0 mL/kg boluses were 108% ± 3% and 146% ± 22%, respectively, as compared to the equivalent values recorded in domestic pigs which were 115% ± 1% and 187% ± 8%, respectively (Figure 3, *E*). In particular, the difference in PAP changes recorded in miniature versus domestic pigs at 0.1 mL/kg bolus injection was statistically significant ($P < 0.05$). By contrast, the SAP changes recorded in miniature pigs at 0.1 mL/kg and 1.0 mL/kg boluses were 110% ± 2% and 107% ± 6%, respectively, and these

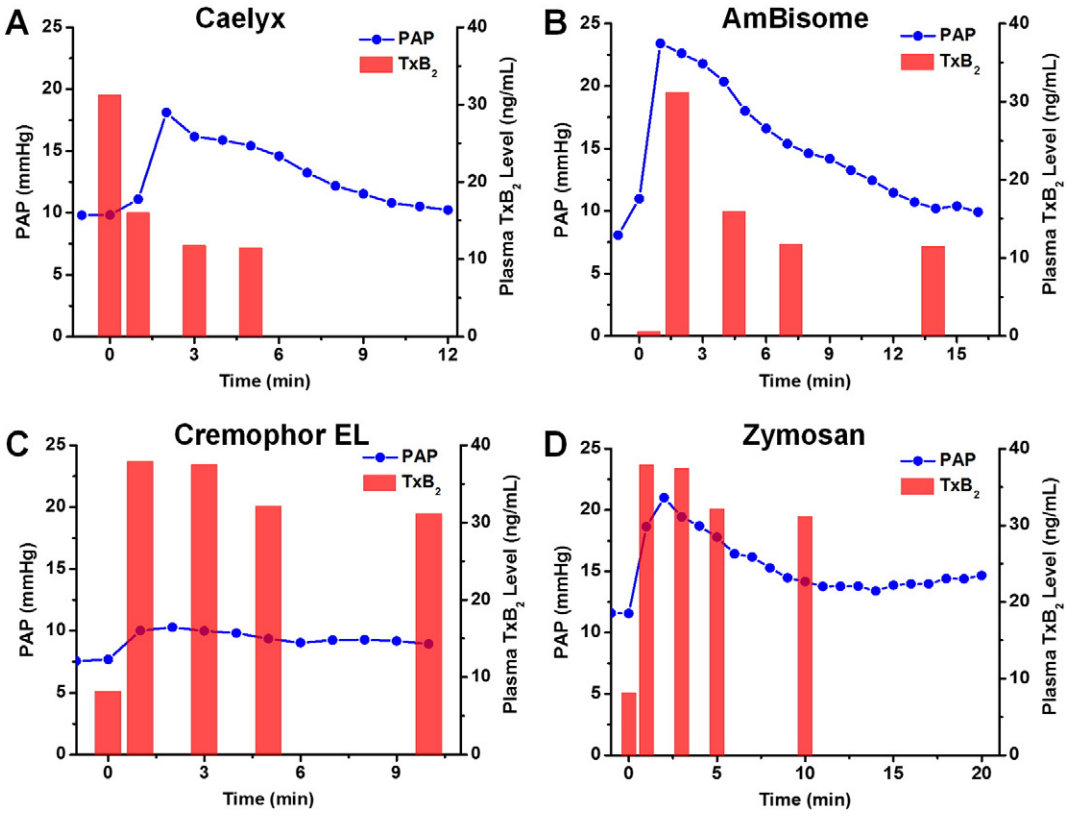


Figure 5. Plasma TXB2 levels in miniature pigs and their relationship with the changes of PAP. The time course of the PAP change (blue circles) and plasma TXB2 concentration is presented in response to bolus administration of **(A)** Caelyx at 1 mg/kg, **(B)** AmBisome at 1 mg/kg, **(C)** Cr-EL at 1 mL/kg, and **(D)** Zymosan at 0.5 mg/kg. The injection time was normalized to $t = 0$ min.

values are statistically comparable to the equivalent values recorded in domestic pigs which were 129% ± 6% and 125% ± 13%, respectively (Figure 3, *F*).

*Hemodynamic and blood cell changes caused by Zymosan*

Figure 4 presents the hemodynamic and blood cell changes upon the i.v. administration of Zymosan in miniature pigs. Bolus administration at 0.1 mg/kg led to variable PAP increases ranging from 28% to 158% (Figure 4, *A*, blue circles). The SAP change was either stable or had a minor change (±10%) (Figure 4, *B*, blue circles). There was 7% to 20% leukopenia and stable PLT levels (Figure 4, *C* and *D*, blue circles). Similar responses were also observed in domestic pigs, with a 53% to 136% increase in PAP, and a variable but minor SAP change. The hematological changes were also stable.

A subsequent bolus administration at 0.5 mg/kg yielded strong physiological responses in miniature pigs, with 63% to 120% increases in PAP as well as pronounced decreases in SAP leading to cardiac arrest in some cases (Figure 4, *A* and *B*, red squares). There was also significant 73% and 79% leukopenia, and 26% to 33% thrombocytopenia (Figure 4, *C* and *D*, red squares). In domestic pigs, the responses were comparable and there was a 91% to 135% change in PAP as well as a variable change in SAP, which in some cases led to cardiac arrest. The coincident leukopenia was less intense (up to 29%) and there were similar levels of thrombocytopenia between 24% and 33%. From this evidence, it can be concluded that similar reactions were observed in both miniature and domestic pigs in response to Zymosan (Figure 4, *E* and *F*). The PAP changes recorded in miniature pigs at 0.1 mg/kg and 0.5 mg/kg boluses were 188% ± 67% and 188% ± 29%, respectively, and these values are statistically comparable to the equivalent values recorded in domestic pigs which were 195% ± 59% and 213% ± 31%, respectively (Figure 4, *E*). In addition, the SAP changes recorded in miniature pigs at 0.1 mg/kg and 0.5 mg/kg boluses were 100% ± 11% and 80% ± 13%, respectively, and these values are also statistically comparable to the equivalent values recorded in domestic pigs which were 104% ± 25% and 87% ± 50%, respectively (Figure 4, *F*). Taken together, depending on the particular nanomedicine or control agent, the miniature and domestic pigs demonstrated variable sensitivity in the corresponding PAP change which support that prototypical CARPA reactions in fact represent a class of reactions with a milieu of factors involved.

*Plasma thromboxane B2 changes in miniature pigs caused by the different CARPA inducers: relationship with PAP changes*

Figure 5 shows the effects of the four CARPA triggers on plasma TXB2 in miniature pigs, along with the temporal correlation between TXB2 and PAP changes.

Just as described for domestic pigs, Caelyx, AmBisome and Zymosan caused immediate, major rises in plasma TXB2, the

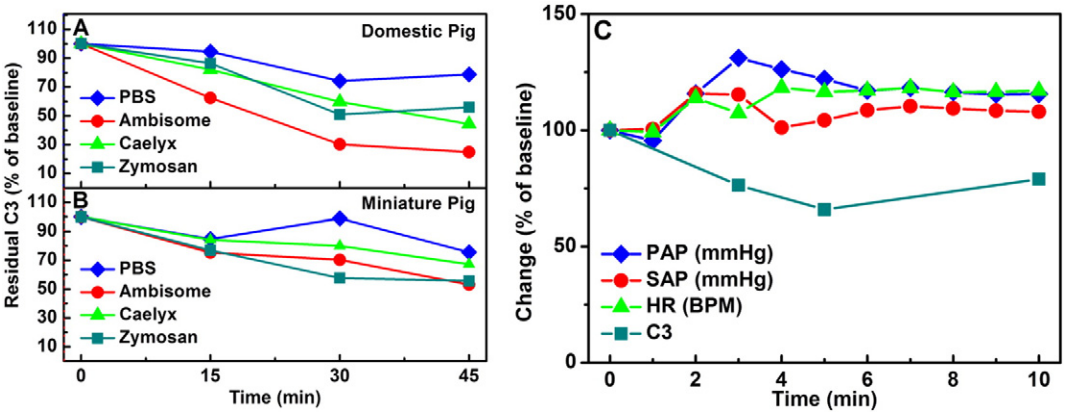


Figure 6. C3 changes *in vitro* and *in vivo*. Time course of C3 decline in the hirudinized plasma of **(A)** domestic and **(B)** miniature pigs following incubation with the indicated C activators. Ambisome and Caelyx were applied at 16 mg phospholipid/mL and zymosan at 1.2 mg/mL. C activation was measured by the PAN-C3 assay. Further details are described in the methods section. **(C)** Time course of hemodynamic and plasma C3 changes in a domestic pig injected with 0.1 mg/kg Zymosan. All other conditions are the same as those described for Figures 1-4.

rises of which showed close temporal correlation with the changes in PAP (Figure 5, *A, B, D*). However, surprisingly, Cr-EL induced large and sustained increases in TXB2 were not associated with major changes in PAP (Figure 5, *C*), a fact which was also established for domestic pigs in the present study (data not shown). This finding raises the possibility that TXB2 does not play a causal role in CrEL-induced pulmonary hypertension.

### *Effects of complement activators on plasma C3 changes in vitro and in vivo*

Figure 6, *A* and *B* show the C3 levels in plasma from domestic and miniature pigs, respectively, which were incubated with Ambisome, Caelyx or Zymosan for 45 min at 37 °C. There was significant decline of the C3 levels with all C activators, and the differences between domestic and miniature pigs were minor. Likewise, the *in vivo* experiment presented in Figure 6, *C* shows significant decline and then recovery of plasma C3, and these changes coincide with the rise and normalization of PAP, SAP and heart rate. Taken together, the data are consistent with the causal involvement of C activation in the hemodynamic changes observed in the present study.

## Discussion

### *Animal models of CARPA: features of the porcine model*

The consequences of C activation have been studied in many animal models, including rats, dogs, rabbits, and pigs (see Ref.[18] and references therein), as well as non-human primates (see, e.g., Ref.[33]). Among these models, the porcine model appears to most closely mimic human CARPA in terms of reaction kinetics, spectrum of symptoms, and the conditions of reaction induction.[11,20] For Doxil, for example, it was calculated that the drug dose that triggers CARPA in pigs corresponds to the dose that triggers infusion reactions in *hypersensitive* man.[34]

Pigs closely resemble man in many features of its anatomy, physiology and biochemistry, in particular the cardiovascular system, the skin and digestive tract are considered to be very good models for man.[29,35] Importantly, the immune system of the pig is functionally similar to that of man.[36] Furthermore, pigs are more acceptable experimental animals than dogs or non-human primates. These facts taken together rationalize the use of pigs as CARPA model.

### *Miniature pig vs domestic pig comparison in toxicology studies*

Minipigs represent a breed of the species *Sus scrofa,* bred for the purpose of animal research. Their characteristic feature is that they are markedly smaller than other farmyard varieties of the species; moreover, unlike domestic pigs, minipigs do not grow after reaching adulthood. For this reason, beside beagle dogs, minipigs have become a favored non-rodent model in regulatory toxicology.[28,37-39]

Of particular importance, minipigs were suggested to be appropriate models for immunogenicity testing of biopharmaceuticals, such as the recombinant human interleukin-1 receptor antagonist, anakinra[40] and the TNF-alpha blocker monoclonal antibodies, adalimumab and infliximab.[41] The minipig model was found equally predictive to the antibody response to the aforementioned protein therapeutics as nonhuman primate models. In another recent study, Huang et al used minipigs for the evaluation of the allergenicity of orally administered, genetically modified food (soybean beta-conglycinin) and found the model appropriate to predict type 1, IgE-mediated allergy.[42] These remarkable data taken together suggest that minipigs are appropriate models for the testing of acquired, specific immunity. However, until to date, we are not aware of studies using minipigs to test the nonspecific, innate immune responses to drugs, in particular CARPAgenic nanomedicines and other agents.

### *Use of minipigs as a CARPA model*

The findings in the present study indicate that minipigs respond to CARPAgenic drugs in similar fashion to domestic pigs, which was shown for four different kinds of reactions triggers: two different liposomes, an emulsifier, and Zymosan. The liposomes studied are widely used, successful

nanomedicines, representing two fundamental features of reactogenic liposomes: Caelyx is PEGylated and weakly negative, while AmBisome is a non-PEGylated and highly negatively charged liposome. Cr-EL represents micelles, while Zymosan is a complex lipo-polysaccharide; all these agents activate C via different pathways and different mechanisms, so the fact that minipigs and pigs reacted comparably to all four reaction triggers suggests that their immune systems do not differentiate among different types of C activators. In other words, the immune responses of domestic and minipigs to various C triggers appear to be similar.

### *Cr-EL reactions in pigs*

In addition to establishing the identity of CARPA in miniature pigs, this study led to the novel finding that Cr-EL caused reactions in pigs. Previous attempts to show the reactogenicity of Cr-EL in pigs failed, leaving us previously to believe that Cr-EL is not reactive in pigs.[18] This emulsifier was shown to cause C activation via the alternative pathway,[18,43,44] and it is extremely reactogenic in dogs.[45] The failure to show reactogenicity in pigs previously is most likely due to different dilution of the emulsifier in ethanol/water, which determines the particle size in the emulsion.[46] It should also be noted that the Cr-EL reaction in both domestic and minipigs was not usual, inasmuch as the pulmonary hypertension it caused did not correlate with plasma TXB2. This observation represents the first case that these CARPA biomarkers are not linked, which raises the possibility that TXB2 is not the only direct trigger of PAP rise, and/or CrEL has additional biochemical effects in animals that interfere with PAP and TXB2 rising hand-in-hand.

### *Pathway of C activation in pigs*

An interesting question raised by our study is which pathway of C activation in pigs is induced by the activators used. In general, the molecular mechanism of C activation depends both on the activator and the species. Thus, liposomes can activate C via the classical pathway, amplified by the alternative pathway feedback loop, or directly via the alternative pathway. Zymosan, on the other hand, is known to activate C via all three pathways, primarily via the alternative pathway. As for the validity of these statements for pigs, we are not aware of information in the literature, and we are not aware, either, of specific inhibitors or other tools to distinguish the activation pathways in this species. Further studies and new tools will hopefully clarify these questions in the future.

### *Causality between C activation and hypersensitivity reactions in pigs*

The close time correlation between hemodynamic changes and the decline of plasma C3 (Figure 6, *C*) represents key proof for the relationship between these two processes. However, the reported observations alone do not reveal whether C activation plays a causal role in the hemodynamic changes, or the two processes are independent consequences of another, yet unclarified basic immune derangement. While this question has not been entirely settled, the following facts support the former conclusion; 1) the physiological effects of anaphylatoxins explain the symptoms of CARPA but not those of IgE-mediated classical allergy[24-27]; 2) the hemodynamic changes underlying CARPA in pigs could be inhibited by soluble complement receptor type I (sCR1) and an anti-porcine C5a antibody, GS-1, which are specific C inhibitors[6]; 3) the hemodynamic changes in liposome-induced CARPA in pigs could be mimicked by human C5a[25]; and, finally, 4) we have shown significant correlation of C activation by liposomes *in vitro* and their reactogenicity in pigs *in vivo*.[24-27]

### *Outlook*

In summary, our findings establish a foundation for minipigs as a validatable immune toxicity model of CARPA towards continued standardization and validation in order to match regulatory standards. Ultimately, such a regulatory model should be able to predict whether a drug candidate has the capability to cause CARPA in general. As discussed and emphasized in all previous publications on the issue, the pig is an oversensitive model for CARPA, the presence of a reaction in pigs (and minipigs) does not reflect the human response in general; it reflects the response of hypersensitive individuals, whose percentage in the human population may vary between 0.001-10%, depending on the drug. Consequently, the pig/minipig model highlights the risk that HSRs can occur in a small percentage of people. Importantly, the model allows the development of safe administration protocols for reactogenic drugs, as exemplified by the development of tolerance induction against Doxil reactions by Doxebo.[27] Further development and understanding of the pig/minipig model will hopefully contribute to increasing the safety, and, hence, human use of nanomedicines with immune reactivity.

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