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Using the air pouch model for assessing in vivo inflammatory activity of nanoparticles

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Dear editor

I would like to make some comments and clarifications regarding the article “Intradermal air pouch leukocytosis as an in vivo test for nanoparticles” recently published in the *International Journal of Nanomedicine*. In this study, Vandooren et al¹ used the air pouch model for evaluating potential pro-inflammatory effects of nanoparticles (NPs). This model is certainly suitable for investigating the inflammatory process which occurs during sterile inflammation and is suitable for determining if an agent, including a given NP, possesses pro-inflammatory activity or not.

We have been using the murine air pouch model in our laboratory for more than 12 years. In 2001, we were the first to successfully use this model in an immunotoxicological study to demonstrate that a chemical of environmental concern, the insecticide dieldrin, was found to induce an inflammatory response in vivo, as evidenced by a neutrophilic infiltration into the air pouches.² Since then, we have used this model for determining the role of several molecules in inflammation, including a plant lectin (*Visum album* agglutinin-I)³ and a malleable protein matrix,⁴ both possessing anti-inflammatory activity, and cytokines (interleukin [IL]-4, IL-15 and IL-21), possessing pro-inflammatory activity as evidenced by leukocyte infiltration into air pouches,^{5–7} and a local increased production of pro-inflammatory mediators, including cytokines and chemokines. In addition, we have used this model to evaluate the role of specific proteins in inflammation using knockout mice.^{8,9}

More recently, in 2011, we used the murine air pouch model to demonstrate for the first time that a given NP, namely titanium dioxide (TiO₂), was pro-inflammatory, as evidenced by a rapid recruitment of leukocytes into the air pouch, where >80% of cells were neutrophils.¹⁰ Although this study was not cited/discussed by Vandooren et al,¹ it is important to clarify that this air pouch model is a model of acute inflammation in which the three phases of inflammation are observed: initiation; amplification; and resolution. Initiation occurred in the first few minutes to hours following administration of a given agent. When an agent is pro-inflammatory, the maximum number of attracted leukocytes is normally observed after 6–9 hours. Under normal circumstances, resolution of inflammation rapidly occurs after 12–24 hours, when the number of leukocytes drastically decreases to reach similar levels to those of control mice treated with the vehicle only (negative control). Of note, we not only determined that TiO₂ NPs are pro-inflammatory in this in vivo model, but we also demonstrated that TiO₂ NPs act very rapidly, since leukocyte infiltration was already observed after 3 hours,

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a time point where no significant cell infiltration was observed following treatment with different pro-inflammatory agents, including the very potent pro-inflammatory lipopolysaccharides (LPS). In the study of Vandooren et al,¹ all the NPs were tested after 24 hours of administration, ie, during the resolution phase of inflammation. Although they were able to observe a weak-to-moderate pro-inflammatory activity for the tested NPs, the experiments should have also been done a few hours after the administration of the NPs to better appreciate the pro-inflammatory activity. In this respect, and knowing that a given NP, TiO_2 , can rapidly attract leukocytes after 3 hours,¹⁰ it is highly recommended to test the effect of a given NP in the air pouch model at several periods of time following administration, as we have done in the past.^{2,3,5–11} Typically, a suitable kinetic is to test the potential pro-inflammatory effect at 3, 6, 9, 12 and 24 hours following NP administration. It is clear that, in our experiment conditions, if TiO_2 NPs had only been tested after 24 hours, our conclusion would be different, since the pro-inflammatory activity would have been completely missed. Therefore, short periods of time must be included for evaluating the pro-inflammatory effect of a given NP using the air pouch model. In addition, concentration experiments should also be performed. Therefore, although the air pouch model is a very good model for investigating the effect of a given NP on inflammation (pro- or anti-inflammatory activity), one has to consider that the model is not that simple and could be time consuming if used for screening purposes.

The other important point that has to be discussed is the characterization of NPs. Whether this is performed using dynamic light scattering analysis or other methods, it has to be carried out as closely as possible to the same experimental conditions that the NPs would be administered in *in vivo* (and *in vitro*) experiments, and not in pure water, as is frequently observed in the literature. One can easily understand that an NP will display completely different characteristics under water conditions than dispersed in culture medium with the presence or absence of serum, or in other biological fluids, for example, in regards to the phenomenon known as the corona of the NPs.¹² Knowing that NP preparations can contain some contaminants, including endotoxins that are extremely pro-inflammatory when present at certain levels, it remains important to measure the level of endotoxins in the NP preparations that will be administered *in vivo*, and not simply in pure water or directly from the stock solution, including for commercially available NPs. Sterilization of NPs could also be carried out to destroy endotoxins by simply autoclaving the NPs. However, the characterization must be

done after this procedure. Although the characterization of the tested NPs was performed by Vandooren et al,¹ it was not specified if this has been done in the same buffer that had been used for the air pouch administration of NPs, or in pure water, or other experimental conditions.

Temperature is another condition that must be taken into account for this characterization, since when the NPs are administered *in vivo*, body temperature is $\sim 37^\circ\text{C}$, which differs from so-called “room temperature” ($\sim 23^\circ\text{C}$). Recently, we performed the characterization at 37°C (the temperature used in the *in vitro* study) and at room temperature, and we observed important differences using dynamic light scattering (Goncalves and Girard, unpublished data, 2014).

Although Vandooren et al¹ have determined the endotoxin levels of the tested NP preparations using the classical Limulus amoebocyte lysate (LAL) assay, it is not specified if this was performed in the same solutions that were administered in air pouches, or in pure water. In addition, although we are also using this assay for determining endotoxin levels in the tested NP solutions, it is important to mention that NPs or engineered particles could interfere with the assay, leading to either an enhancement or an inhibition of the amount of endotoxins, and causing an overestimation or underestimation, respectively.¹³ Therefore, careful technical controls have to be performed to eliminate possible interference of a given NP with the assay. To circumvent that, Neun and Dobrovolskaia¹³ have proposed two assays. In our studies, we are currently testing the NPs solution in parallel in Lysogeny broth agar plates incubated for a period of 24–72 hours, and are verifying the presence (positive control) or absence of colonies.¹¹

More recently, using the murine air pouch model, we have demonstrated that even if a given NP is not pro-inflammatory by itself, it can act by accelerating/amplifying the response of another agent,¹¹ a situation that is likely to occur *in vivo*. This has been observed for fullereneol and LPS, where both agents did not significantly increase the number of leukocytes into air pouches after 3 hours when used alone, but did when they were both administered together.¹¹ Therefore, this further testifies how complex it is to determine potential effects of a given NP in inflammation, and it becomes evident that even if an NP appears to be safe, since it did not demonstrate any apparent pro-inflammatory effect by itself, it can act by amplifying the effect of other agents. This is particularly true in humans, frequently fighting against pathogens: a situation that differs using mice in animal facilities under pathogen-free conditions.

In summary, I agree with the conclusion of Vandooren et al¹ that the air pouch model is suitable for evaluating

inflammatory activity of a given NP, and that this model could be used as a future standard assay in nanotoxicology studies, as long as it was performed adequately. However, due to the above observations, I cannot agree with the recommendation to use only NPs yielding air pouch leukocytosis equivalent to the negative control. Several criteria must be considered, including: 1) kinetic experiments including time points in the first few hours following administration of NPs into the air pouch; 2) more complete dose–response experiments; 3) careful characterization of the NPs in the same experimental conditions used for air pouch administration; 4) careful determination of endotoxins in the NP solutions tested by adding interference experiments with the LAL assay or by trying other methods, eg, agar plates, as proposed by Neun and Dobrovolskaia¹³ and previously published by us.¹¹ In addition, one has to consider that even if a given NP appears to have no effect by itself when compared to the control, it can act with other agents in vivo, causing inflammation. This testifies as to the complexity of evaluating the effect of a given NP on inflammation. Future experiments will help to better refine a scientific procedure that could be standardized for determining the pro-inflammatory effect of a given NP.

Disclosure

The author reports no conflicts of interest in this communication.

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Authors' reply

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Dear editor

Thank you for forwarding the Letter to the Editor about our recent publication¹ in which we advocated the use of air pouch leukocytosis as a second line in vivo test for nanoparticles, destined for parenteral administration. We are grateful to the group of Dr Girard for mentioning additional work on this topic. The intentions of our work were multifold. First, we wanted to advocate the use of the air pouch test as an accessible and straightforward in vivo analytical tool in an anticipatory way, ie, before doing more laborious in vivo tests in animals, of course, with the view to using the minimal number of animals. Second, within a consortium of nanoparticle specialists of the European Community Save-Me project (No 263307), biosafety issues were raised and it was concluded that we should test beyond the level of cellular toxicity and endotoxin and other microbial contaminations. Third, for the study of functionalized nanoparticles, often only limited quantities are available, with the effect that the manufacturers do not necessarily have the opportunity to do broad testing. Finally, in such testing, positive and negative control samples are essential and we suggested using chlorite oxidized oxyamylose (COAM) as a positive control and phosphate buffered saline (PBS) as a negative control.

We were aware of the kinetic aspects of leukocyte influxes and that the maximal level of specific leukocyte subsets depends on the cell type (eg, neutrophil migration is faster than monocyte and lymphocyte migration) and on the biological context of the trigger (eg, acute versus chronic inflammation and cancer). For this aspect we cited specific manuscripts,^{2,3} but recent work^{4,5} reinforces this idea very well. Cell influxes to air pouches and other compartments are often based on the production of specific chemokines. The kinetics of direct fluxes of neutrophils and other leukocyte types in mice have been well established in many studies.^{6,7} Nanoparticles may also induce cytokine and chemokine production⁴ and thus indirectly contribute to air

pouch leukocytosis at a later stage than 3 hours, certainly for monocytes and lymphocytes that tend to infiltrate at a slower pace than neutrophils. In other words, depending on the immune effector cells being activated, the ideal analytical time point may differ considerably and, for that reason, analysis at different time points results in a more complete picture. This is the case for all screening tests. For adaptive immune responses and indirect effects, one may have to look beyond 24 hours; for acute and direct effects of nanoparticles, shorter time intervals than 24 hours are relevant. For an inexpensive and general in vivo screening, 24 hours is a reasonable compromise.

Another aspect that we address is the differential cell analysis. The specific counting of macrophages/monocytes, lymphocytes and granulocytes is an easy routine test that enhances the information content of the air pouch test. As outlined by our colleagues⁵ and as indicated in our work, the readout (of the positive control COAM) has a biological dose-response relationship.¹ Here, it is essential to also draw attention to the fact that most chemokines have bell-shaped dose-response effects⁸ and that this may also be the case for some nanoparticles.

We used pyrogen-free PBS as an isotonic buffer system in order to avoid any cytolysis. An alternative could be pyrogen-free 0.9% NaCl solution. We advise using isotonic pyrogen-free solutions, rather than water, because of the hemolytic effects of pure water. We agree that for all comparisons, the same buffer system needs to be used. Prior to the analysis of endotoxin contents and the evaluation of air pouch leukocytosis, the positive control compound (COAM) and all nanoparticles were dissolved and diluted in PBS.

The comment on possible corona effects is justified. Indeed, we mentioned in our manuscript that corona properties may contribute to immunological effects. The biophysical conditions of the nanoparticles (temperature-dependence, aggregation, corona-effect, stability) will co-determine the in vivo effects and need to be considered by any manufacturer of nanoparticles.

We were aware of the different specificities of various commercial endotoxin tests and that Limulus-based tests may vary for different nanoparticle preparations.^{9,10} On the basis of this information, we used the chromogenic LAL assay with a dilution series as a compromise. In sharp contrast with endogenous pyrogen or interleukin-1, endotoxin is heat-stable.¹¹ As a consequence, simple autoclaving is not sufficient to eliminate lipopolysaccharides. In conclusion, differential leukocytosis analysis in air pouches is a cost-effective and simple second line in vivo safety test for nanoparticle screening,

and we thank our colleagues for their advice to test more conditions, beyond our minimal recommendations.

Disclosure

The authors report no conflicts of interest in this communication.

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