Drug Delivery and Translational Research

An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars

--Manuscript Draft--

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Abstract:	Nanoparticles including nanomedicines, are known to be recognised by and interact with the immune system. As these interactions may result in adverse effects, for safety evaluation the presence of such interactions needs to be investigated. Nanomedicines in particular should not unintendedly interact with the immune system, since patient's exposure is not minimised as in the case of "environmental" nanoparticles, and repeated exposure may be required. NLRP3 inflammasome activation and dendritic cell (DC) maturation are two types of immune mechanisms known to be affected by nanoparticles including nanomedicines. NLRP3 inflammasome activation results in production of the pro-inflammatory cytokines IL-1β and IL-18, as well as a specific type of cell death, pyroptosis. Moreover, chronic NLRP3 inflammasome activation has been related to several chronic diseases. Upon maturation, DC activate primary T-cells; interference with this process may result in inappropriate activation and skewing of the adaptive immune response. Here we evaluated the effect of two nanomedicines, representing nanostructured lipid carriers and polymers, on these two assays. Moreover, with a view to possible future standardization and regulatory application, these assays were subject to an inter-laboratory comparison study using common SOPs. One laboratory performed three independent NLRP3 inflammasome activation

	experiments, while the other performed a single experiment. Two laboratories each performed three independent DC maturation experiments. While the nanostructured lipid carrier only showed marginal effects, the polymers showed major cytotoxicity. No evidence for inflammasome activation or DC maturation was demonstrated. Intra- and inter-laboratory comparison showed clearly reproducible results.
Response to Reviewers:	Reviewer #1 The objective of this manuscript is to compare NLRP3 inflammasome activity and dendritic cell maturation between two laboratories for two types of nanoparticles. Because research using immune cells relies strongly on know-how, it is important to make rigorous comparisons between laboratories for the same subjects. However, there are many problems from the stage of experimental design in order to achieve this purpose. Why did the authors target these two nanomedicines? The choice for these two nanomedicines was made for several reasons: (1) they belong to two different classes of nanomedicines, a liposomal nanocarrier and a polymeric nanoparticle. This should enable a comparison between these two classes, potentially revealing differences and similarities between them, (2) the project partners CEA and SINTEF have been producing the liposomal nanocarrier and polymeric nanoparticle, respectively, for many years. They are therefore familiar with the key characterisation measurements and are able themselves to perform these measurements. This also pertains to potential batch differences, (3) the project partners CEA and SINTEF hold the patents for their respective nanomedicines, precluding IP issues. Additionally, these nanomedicines have been applied to all of the experimental work, across the REFINE project. What are they? LipImage™ 815 is a liposomal nanocarrier, PACA is a polymeric nanomedicine. We hope to have addressed sufficient details in the Methods part. LipImage™ 815 is described in: Jacquart A, Kéramidas M, Vollaire J, Boisgard R, Pottier G, Rustique E, Mittler F, Navarro FP, Boutet J, Coll JL, Texier I. LipImage™ 815: novel dye-loaded lipid nanoparticles for long-term and sensitive in vivo near-infrared fluorescence imaging. J Biomed Opt. 2013; 18: 101311. PACA is described in: Øverbye A, Torgersen ML, Sønstevold T, Iversen TG, Mørch Ý, Skotland T, Sandvig K. Cabazitaxel-loaded poly(alkyl cyanoacrylate) nanoparticles: toxicity and changes in the proteome of breast, colon and prostate cancer cells. Nanotoxicology. 2021; 15: 865-84. In addition, there is no description on RIVM laboratory and University of Liverpool laboratory. Since this is a matter of the so-called Good Laboratory Practice, it is important to ensure that the quality of reagents, equipment, SOPs, researchers, and data are properly validated. Therefore, if it is a paper on GLP of immune activity measurement, the reviewer thinks that it is meaningful. Unfortunately, this paper is only a comparison between two laboratories, and the descriptions of samples and experiments are too lenient to draw accurate conclusions. Both RIVM and the University of Liverpool are working under ISO 17025. The originator lab drafted the SOPs based on the protocols running in the lab, the SOP was then reviewed by the project partner laboratory for clarity and by the project's QC partner for regulatory requirements such as adequate reagent and equipment identification, positive and negative controls, data reporting, etc. The SOP was adapted and used for the inter-laboratory comparison. Both labs used a common SOP. For example, it is necessary to clarify differences in cell ID numbers, passage numbers, culture days, etc., differences in the quality of the researchers (education background, research experience etc.), and differences in equipment, instruments, reagents, SOPs, etc., used by two laboratories in this experiment. In particular, in the NLRP3 inflammasome activity, the RIVM laboratory has shown three independent experimental results against Null, whereas the University of Liverpool laboratory uses Null, ASC-deficient cells, and NLRP3-deficient cells, so there is no comparison intra-laboratory variance and inter-laboratory variance for Null. The reviewer is correct, RIVM performed three independent experiments, while the University of Liverpool performed a single experiment. Therefore, intra-laboratory comparison was possible for RIVM, and only limited inter-laboratory comparison between RIVM and the University of Liverpool could be done. In the revised

manuscript we adapted the text accordingly. In the revised manuscript, the data on the deficient cells was omitted as they do not contribute to the intra- or inter-laboratory comparison.

As for dendritic cell maturation, the RIVM laboratory uses stored human buffy coats purchased from the Dutch Blood Bank, but the University of Liverpool laboratory used fresh blood collected from volunteers, so the cells used are different in freshness. And because the immune response varies from person to person, generalization requires blood samples collected from more volunteers (The reviewer does not know how many in this experiment).
RIVM did not use stored human buffy coats, but fresh ones. In the revised manuscript we added: They [the buffy coats] were kept at RT until starting cell isolation the next morning.
We have used monocyte-derived dendritic cells from a single donor for each of the triplicate experiments. We agree with the reviewer that to fully evaluate the response of dendritic cells to a specific agent, the number of donors to be tested should likely be more than 3. However, the aim of the study was an inter-laboratory transferability and comparison study, not a detailed investigation on the nanomedicines themselves, and the observed, similar, trends in the data suggest parity across the results obtained.

Two nanomedicines are used as samples, but no specifics are provided. Which laboratory prepared it? Are the ingredients GMP compliant? Is the lot number the same? Is there any difference between lots? Does the difference in the storage period affect it? Was the preparation done in a clean environment? Have the researchers tested for pyrogens and endotoxins?
The project partner CEA produced LipImage™ 815, while SINTEF produced PACA and PACA-CBZ (see above).
No, not all ingredients were GMP compliant.
Each partner laboratory used the same lot, and the same lot was used for each of the triplicate experiments.
size (nm)PDIζ-potential (mV)
LipImage™ 815 - lot 1490.097-2.5
LipImage™ 815 - lot 2530.150-1.5
PACA – lot 11210.11-3.0
PACA – lot 21340.11-3.2
PACA-CBZ – lot 11350.14-2.5
PACA-CBZ – lot 21400.13-2.9

The integrity of the nanomedicines upon storage was monitored by a third project partner.
Preparation was done in a laminar flow cabinet.
The nanomedicines were tested for endotoxin and the levels were found to be <0.1 IU/ml.

There are many details about the results, but the reviewer would like to ask the above points before going to them.
We thank the reviewer for the constructive comments and hope to have sufficiently addressed the questions by the reviewer.

Reviewer #2
In this manuscript, 'An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars' the authors present the results of a proposed strategy for the efficient hazard assessment of nanomedicines. Their approach is based on the measurement of two endpoints associated with the activation of immune cells after acute exposure in vitro to the nanomedicines. The authors aim to demonstrate the utility of the approach with two case study materials and the reproducibility through an inter-lab comparison of the assay SOPs with the overall goal of promoting the adoption of this approach in the regulatory assessment of new nanomedicines.
In this study the authors are addressing an important unmet need in the hazard assessment and regulation of nanomedicines, in line with the drive to reduce the number of animals used in the research. However in my opinion there are fundamental flaws in some of the underlying concepts and study design that need to be addressed

in order to support the publication of this study. In addition in places the details of methods used and the data presented falls short of the expected standard to allow the reader to independently and critical review the proposed approach and results presented. The major and minor concerns I have with the manuscript are further outlined below.
Major
1. The use of a single endpoint, IL-1b, as an indirect output measurement of a complex biological pathway. The casual use of language, why not measure IL-18. The lack of response in cells with and without the complete pathway is not enough to demonstrate mechanism especially when NO positive controls, either chemical or relevant and comparable material controls have been included in the studies conducted in the knockout cells. At a minimum the authors need more discussion and justification of how a single indirect endpoint, IL-1b secretion, can be considered a sufficient measure of a complex pathway such as NLRP3 activation if the authors seek to further the acceptance of this approach by regulatory agencies. Additional assessment of IL-18 should be conducted or if not the text should be amended to accurately reflect the endpoint measured is IL-1b secretion which is merely an indirect and indicative measure of inflammasome activation.
The reviewer is correct that measuring only IL-1β (and not IL-18) needs some explanation. First, in earlier studies using the same assay we tested three materials known to induce NLRP3 inflammasome activation: SiO2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant. All three materials showed a concentration-dependent increase in IL-1β secretion and a concomitant decrease in viability. Second, in another earlier study we compared IL-1β and IL-18 responses to a concentration range of gold nanoparticles and found only very minor differences in the gold concentration vs. cytokine response profile, except for the absolute amount (pg/ml) of cytokine secreted.
We decided to in the revised manuscript omit the work on the deficient cell lines as they do not contribute to the intra- and inter-laboratory comparison (see also our response to Reviewer 1).
Still, we agree with the reviewer that it is of added value to measure additional parameters. In the revised manuscript we added: Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3 activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death. Please, see also the response to Reviewer 3.

2. Lack of appropriate controls, both positive and negative materials and chemical controls (nigericin missing from UoL data). The ISO standard (ISO 10993-5:2009. Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity') for the assessment of requires the inclusion of positive control materials in a study and also recommends the study includes a negative control materials 'The purpose of the positive control is to demonstrate an appropriate test system response. For example, an organotin-stabilized polyurethane1) has been used as positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts. In addition to a material, pure chemicals can also be used to demonstrate the performance of the test system.'
The reviewer is correct that in the present manuscript we did not include that in a separate inter-laboratory comparison study within the project, the cytotoxicity of the three materials LipImage™815, PACA, and PACA-CBZ were tested. In the revised manuscript we added: In a separate inter-laboratory comparison study within the REFINE project the cytotoxicity of the same batches of the three materials LipImage™815, PACA, and PACA-CBZ was tested in four different cell lines using both the WST-8 and the LDH release assay. LipImage™ 815 was non-cytotoxic up to a concentration of 128 µg/ml, whereas PACA caused dose-dependent cytotoxic effects starting from 8 µg/ml. PACA-CBZ showed a less pronounced dose-dependent effect with the lowest concentration of 2 µg/mL causing cytotoxic effects (Eder et al. submitted for publication).
We used SiO2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant as positive control materials for NLRP3 inflammasome activation. In addition, we used nigericin as low-molecular weight “pure chemical” positive control.

The inclusion of both these controls would not only provide data to allow the reliability

and reproducibility of the assays to be demonstrated, which appears to be the point of conducting the inter-lab comparison but also aid in the interpretation of results and justification of the authors that a certain effect level (e.g. 30% cytotoxicity) has some biological relevance. Currently these justifications are simply speculative and the interpretation of results greatly weakened as the lack of response from cells to exposure to the case study materials for the endpoints selected. A negative response could be reflective of a weakness or limitation of the test system rather than a lack of hazard of the materials. The authors do not provide any rationale for the selection of exposure concentrations selected, time-course of exposure selected or deposition or interaction between the materials and cells. The inclusion of positive control materials would at least allow the authors to demonstrate there are operating within relevant ball-park of exposure dose and time.
When setting up the NLRP3 inflammasome activation assay, we have gone through great lengths to optimise PMA stimulation (concentration/time chessboard) and exposure time. The exposure concentration is similar to what we routinely use for other nanomaterials. As mentioned above, we used SiO2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant as positive control materials. In addition, we used nigericin as the low-molecular weight positive control. The ISO standard 10993 states 70% viability as threshold of cytotoxicity.

3. Number of replicates for IL-1b secretion conducted by UoL, the authors report in numerous places in the manuscript e.g. abstract and discussion, that ', two laboratories performed each of the assays in three independent experiments'. This is not accurate and misrepresents the amount of data presented.
The reviewer is correct. In the Abstract and Discussion of the revised manuscript we changed the text to: One laboratory performed the assay in three independent experiments, while the other laboratory performed the experiment once. One laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. Two laboratories each performed three independent DC maturation assays in three independent experiments.
4. Authors rely too heavily on descriptive terms to report quantitative results....e.g. 'sharp increase'...'gradual decrease' but these terms are defined inconsistently throughout the manuscript and appear to more reflect the authors conclusions/intuitive interpretations of the results than a clear reporting of the results themselves. For example in Figure 5, LipImage exposure shows a consistent exposure concentration trend of increasing DC-SIGN....with the % difference much greater than the 30% reduction reported for the positive controls...why have the authors only reported 'slightly increased DC-SIGN expression at the highest conc tested'? While PACA and PACA-CBZ induced what looks like a more dramatic decrease in DC-SIGN than the positive control treatments but this is not clearly reported in the results text. Does LipImage cause a conc dependent increase in PD-L1 expression? The trend on the graphs presented looks slight but an effect could be masked but the dramatic increase caused by positive control represented on the same graph. Given the differences in mode of action and bioavailability of a soluble receptor agonists and a particle exposure this trend in response to LipImage should not be ignored. For clarity and transparency the results section should include tables reporting quantitative results across the exposure concentrations rather than rely on selective reporting and vague terms as the manuscript is presented here. Furthermore switching between % response in comparison to untreated control, % increase/decrease in endpoint measured, fold-change increase/decrease when reporting results making the text unclear and difficult to read in places.
We attempted to find a balance between being precise (mentioning percentages and fold increase/decrease) and having a text that is still readable. In the revised manuscript the wording is more precise, and we added the exact values in the Supplementary Information. There, WST-1 values are given as absorbance values, not as normalised values. The choice to use percentages vs. fold increase/decrease is caused by the effect size. Generally, effects >150% increase are given as a fold increase as a percentage would implicate an accuracy that is unrealistic.

5. I question the interpretation of results reporting the functional activation of immune cells in response to stimulus from populations with high levels of cytotoxicity e.g. Exposure to PACA resulted in a sharp decrease in viability, from 70% viability at 8 µg/ml to 9% viability at 16 µg/ml. Such a dramatic loss in viability will not be reflective of cells stimulated to mount an immune response, the inclusion of intermediate

exposure concentrations would add confidence that the immune responses i.e. IL-1b secretion and DC activation, are not being missed or masked by excessive cell death in the populations and also likely improve the accuracy of the benchmark dose modelling.
The aim of the study was an inter-laboratory comparison study, not an in-depth evaluation of the potential effects in a rather narrow exposure window. Still, an in-depth study on the specific nanomedicine would indeed require investigating what is happening in that exposure window.

6. Lack of characterisation of the materials. In the methods the authors allude to the measurement of hydrodynamic diameter and zeta potential of the NP dispersions but do not provide the data. Was this conducted by both partners? Where NP dispersions consistent across both sites? Do the authors have any indication that the NP deposit on the cells within the timeframe of exposure? Ideally the authors would report the rate of deposition for each material within the test system.
The reviewer is correct that this information is missing. We apologise for this omission. In the revised manuscript we included the following Table:
Size (nm)PDIζ-pot (mV)Drug loading (wt %)Dye loading (wt %)
LipImage53 ± 10.15-1.5 ± 1-0.35%
PACA136.20.11-4.8--
PACA-CBZ121.80.14-5.510.8%-
Table 1. Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF.
Given that for sample preparation the nanomedicines only needed vortexing and not (probe or bath) sonication we did not feel the need to perform a comparison of the dispersions.
Based on the density of the nanomedicines (close to 1), given the dimensions of the cell culture well, the deposition of a 100 µg/ml dispersion amounted 3.76 µg (LipImage), 2.34 µg (PACA), and 2.48 µg (PACA-CBZ) within a 24-hr period (calculated by the ISDD model).

7. Potential for NP interference in the assays was not fully assessed. The potential adsorbance of the WST-1 reagent and secreted IL-1b should be addressed. In figure 3 a consistent decrease in IL-1b measured between 0 and 2-4 ug/ml exposure conc when cells exposed to PACA is seen...could this be due to particle interference? The authors need to also provide more detail in the methods section explaining how the assays were conducted, e.g. were cells washed and particles removed before exposure to WST-1 reagent? Were NP removed by centrifugation from the supernatant for the ELISA? These details are important for the standardisation of an SOP for NP exposure.
WST-1 was added without prior washing of the cells. As a control, wells were included that contain a dilution series of the nanomedicine but without cells. This corrects for possible light absorbance of the nanomedicines but not for possible interference of colour (due to substrate conversion) by the nanomedicines.
Since the ELISA protocol contains multiple washing steps, we did not centrifuge the supernatants.

8. Do the authors consider an n=3 to have sufficient statistical power to conduct benchmark dose modelling? The majority of endpoints reported could not be included in modelling. The authors report this is 'When an EC30 could not be calculated, or the ratio between the upper (95%) and lower (5%) limit around the EC30 was > 5, the data were not considered.' But do not provide detail on which caveat applied to each situation where the modelling could not be carried out. The authors should more clearly report whether the inability to use this exposure-response modelling approach is due to a lack of response generated or due to too great level of variation between the replicates. In the currently format it is difficult to assess the utility of the modelling approach and reason for including it in the manuscript.
In the revised manuscript we added which of the two (could not be calculated, or ratio between BMDU and BMDL was >5) applied.

9. The authors acknowledge the subjective nature of flow cytometry population gating. Given the aim of this study is to conduct an interlab comparison both the 2D scatter plots and subjective gating strategies conducted at each site need to be included, a

least in Supplementary Information.
The gating strategy is included in the SOP. In the revised manuscript the SOP (in fact, both SOPs) is included in the Supplementary Information.

10. Alluding to results from other studies without the presentation of data or citing a source the reader can access to support their conclusion as the authors have done in the Discussion paragraph 3 is inappropriate. If the authors cannot provide the data or source this paragraph should be removed.
In the revised manuscript we included the data on the Avanti liposome as Supplementary Information.

Minor
1. Justification for the use of both a cell-line and primary DC cells. The rationale for the use of primary DC cells is clear but the benefits for the use of THP-1 cells over primary monocyte-derived macrophages is speculative. Would there not be some added value to assessing the NRLP3 activation in the matched MDM from the same donor blood as the DC's? The user will have to go through the collection and preparation of samples but not use the MDM component, while in parallel maintain the THP-1 cells in culture. The authors are proposing this is a more efficient approach to hazard assessment?
We greatly appreciate the suggestion by the reviewer. On the one hand, it is indeed efficient to use the same monocyte population for both DC maturation and NLRP3 inflammasome activation assays. On the other hand, the two assays have been regarded as two individual (not inter-related) assays in a larger series of in vitro assays for effects on the immune system. The fact that the DC maturation assay requires monocytes from blood donors refrains its use as a regulatory test, albeit an important output parameter. Therefore, when both options are open (cell line or primary cells), as is the case for the NLRP3 inflammasome activation assay, we chose for the cell line-based assay.

2. Page 4, line 12-13: 'The assays were performed by two laboratories using common SOPs'. Provide references to these 'common SOPs'.
In the revised manuscript both SOPs are attached as Supplementary Information.

3. Very short exposure to PMA for differentiation, has this protocol been validated by more extensive characterisation of macrophage differentiation than simple adherence of cells? e.g. demonstration of macrophage markers CD11b etc?
We forgot to mention that the cells were also visually inspected for macrophage-like appearance. In the revised manuscript we added: The cells were visually inspected for macrophage-like appearance.

4. Inflammasome activation leads to the cleavage of pro-IL-1b and secretion of the mature form. Secretion is a more accurate term for the endpoint i.e. level of IL-1b released into the supernatant. The term IL-1b 'production' is more indicative of de novo protein synthesis and should be amended through the text to better reflect the pathway if it is being used as a marker of inflammasome activation. However I would continue to argue the level of IL-1b secretion being measured could be due to multiple mechanisms including an increase in protein translation and therefore not specific to inflammasome activation. Is the ELISA kit used by the authors specific for the mature form of IL-1b?
The ELISA is not specific for mature IL-1β.

5. Legend of Figure 4 appears to be incorrect, are the green dots reporting cell viability or IL-1b secretion?
In the revised manuscript the dot colour has been corrected.

6. In Figure 5a there is a consistent and complete loss of viability for cells exposed to PACA-CBZ at a dose of 16ug/ml which appears to be an anomaly when considering the other doses and responses measured. Can the authors confirm the independence of each of the experimental replicates? Were cells from different donors? Different preparations of particles? Conducted on different days?
Yes, we can indeed confirm that the experiments were done on cells from different donors, using different preparations of the dilution series, and on different days. So, indeed they are independent experiments.

7. In Fig 5d the authors report 'For PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml and 64 µg/ml, respectively. Possibly, CD14+ cells are less sensitive to PACA and PACA-CBZ, resulting in an increased MFI.' The bases of this speculative interpretation of this result is not clear.
We agree with the reviewer that this is indeed speculative. In the revised manuscript, this sentence has been removed.

Reviewer #3
The manuscript by Vandebriel and colleagues describes an evaluation of the effects of two different nanocarriers, one of which is loaded with a chemotherapeutic agent towards immune cells. The strength of the study is both in the use of very different nano-carriers to assess the effect of these relevant nano-medicines but also in the comparative approach employed by the two institutions to look at reproducibility. In particular, the finding that the Live/ Dead assay shows lower reproducibility as well as wider issues around flow cytometry as a tool is interesting, useful and addressable during future standardisation.
A criticism of the study is that the evaluation of NALP3 inflammasome activation is somewhat basic as it solely rests on the expression of IL-1B, rather than a more comprehensive measure such as caspase-1 activation. However, this is mitigated by the use of knock out (KO) THP-1 cells using siRNA to target ACS and NALP3 by one of the partners. Considering the regulatory use of such an approach, thought should be given to a) if IL-1B expression is sufficient as a marker of activation b) if repeat experiments with KO cells is efficient or c) if there are other markers that can be used in addition to IL-1B expression, preferably in the same experiment.
We thank the reviewer for this suggestion. In another study we compared IL-1β and IL-18 responses to a concentration range of gold nanoparticles and found only a very minor difference in the gold concentration vs. cytokine response profile, except for the absolute amount (pg/ml) of cytokine secreted. In addition, cleavage of pro-IL-1β and pro-IL-18 to IL-1β and IL-18, respectively, both depend on caspase-1 activation. Therefore, addition of IL-18 does not seem useful. Adding measurement of caspase-1 activity may seem useful as this points to inflammasome activation (not necessarily NLRP3, e.g. also AIM2 has caspase-1 activation as downstream effect). Another possible addition could be to measure caspase-3, with the aim to evaluate cell death not caused by pyroptosis but by apoptosis. These caspases should be measured in lysed (or fresh) cells, not in supernatants, somewhat complicating the assay. More generally, the purpose of the work was not to delineate/define specific mechanistic responses but, to produce a reliable and straightforward SOP to indicate inflammasome activation; hence the focus on IL-1β as a prototypical marker.
In the revised manuscript we added: Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3 activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death. Please, see also the response to Reviewer 2.

Overall, the study is both robust, interesting and well written with the materials and Methods section being particularly detailed and well described. However, from an editorial perspective the Results section could do with some more thought. This is because currently, the sequential reporting of the RIVM results then the University of Liverpool results for each endpoint makes the results section very long, repetitive and a little hard to follow. I'm not sure how best to address this due to the comparative nature of the study but perhaps greater use of supplementary data to reduce the number of figures and a side-by-side comparative table of results; either providing a qualitative summary (e.g. ++/-) or quantitative values.
We have discussed how to improve the flow of the text but could not come up with a better solution. The heat map as shown for the DC data is a direct comparison between the data of both partners.

Major Points:

1. Whilst size and surface charge characterisation of the nano-carriers was conducted as reported in the methods, this data seems to be missing from the paper. As parameters like surface charge may offer an explanation for the differential cytotoxicity of these two substances, it is important to report and discuss these.

	The reviewer is correct that this information is missing. We apologise for this omission. In the revised manuscript we included the following Table: Size (nm)PDIζ-pot (mV)Drug loading (wt %)Dye loading (wt %) LipImage™ 81553 ± 10.15-1.5 ± 1-0.35% PACA136.20.11-4.8-- PACA-CBZ121.80.14-5.510.8%- Table 1. Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF. Minor Points: 1. From a readability perspective, the authors should re-examine their rather excessive use of commas in the text as this makes many of the sentences disjointed and hard to read. For example: "In a recent analysis, we listed, among others, immune system endpoints for which, however required by regulatory authorities, no generally accepted assays exist". In the revised manuscript we will as much as possible reduce the use of commas. 2. It would be useful for the reader if the nature of the chemotherapeutic agent Cabazitaxel is explained. This could be at either its first mention at the end of the introduction or with the Materials and Methods. The reviewer is correct that this data is missing from the manuscript and we apologise for this omission. In the revised manuscript we added: Cabazitaxel was obtained from Shanghai Biochempartner Co., Ltd. (Shanghai, China).

Dear Dr. Alonso,

Please find attached the revised manuscript “An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars” which we submit for publication in the “REFINE” special issue of Drug Delivery and Translational Research (DDTR-D-22-00024).

In this manuscript we present the results of an inter-laboratory comparison study of two *in vitro* assays on two close-to-market nanomedicines, a nanostructured lipid carrier and a polymer. Both assays fall within the regulatory information requirements for the evaluation of possible immunotoxic effects of nanomedicines, but they are not yet standardised. The present study should be a relevant step towards standardization of these assays. All work has been done within the EU REFINE project.

The information presented in the manuscript is original and has not been submitted for publication elsewhere. All authors have disclosed any actual or potential competing interests, have read the manuscript, agree that the work is ready for submission, accept responsibility for the contents of the manuscript and agree for its submission to Drug Delivery and Translational Research.

We have provided a point-by-point reply to the reviewers’ queries, indicated the changes made to the original manuscript and provided a revised manuscript with Track Changes (including 5 Supplementary files that were not included in the original manuscript). We also include a clean version of the revised manuscript.

We hope to have addressed the comments of the reviewers sufficiently so that the manuscript is now acceptable for publication.

kind regards

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Reviewer #1

The objective of this manuscript is to compare NLRP3 inflammasome activity and dendritic cell maturation between two laboratories for two types of nanoparticles. Because research using immune cells relies strongly on know-how, it is important to make rigorous comparisons between laboratories for the same subjects. However, there are many problems from the stage of experimental design in order to achieve this purpose.

Why did the authors target these two nanomedicines?

The choice for these two nanomedicines was made for several reasons: (1) they belong to two different classes of nanomedicines, a liposomal nanocarrier and a polymeric nanoparticle. This should enable a comparison between these two classes, potentially revealing differences and similarities between them, (2) the project partners CEA and SINTEF have been producing the liposomal nanocarrier and polymeric nanoparticle, respectively, for many years. They are therefore familiar with the key characterisation measurements and are able themselves to perform these measurements. This also pertains to potential batch differences, (3) the project partners CEA and SINTEF hold the patents for their respective nanomedicines, precluding IP issues. Additionally, these nanomedicines have been applied to all of the experimental work, across the REFINE project.

What are they?

LipImage™ 815 is a liposomal nanocarrier, PACA is a polymeric nanomedicine. We hope to have addressed sufficient details in the Methods part.

LipImage™ 815 is described in:

Jacquart A, Kéramidas M, Vollaire J, Boisgard R, Pottier G, Rustique E, Mittler F, Navarro FP, Boutet J, Coll JL, Texier I. LipImage™ 815: novel dye-loaded lipid nanoparticles for long-term and sensitive in vivo near-infrared fluorescence imaging. J Biomed Opt. 2013; 18: 101311.

PACA is described in:

Øverbye A, Torgersen ML, Sønstevold T, Iversen TG, Mørch Ý, Skotland T, Sandvig K. Cabazitaxel-loaded poly(alkyl cyanoacrylate) nanoparticles: toxicity and changes in the proteome of breast, colon and prostate cancer cells. Nanotoxicology. 2021; 15: 865-84.

In addition, there is no description on RIVM laboratory and University of Liverpool laboratory. Since this is a matter of the so-called Good Laboratory Practice, it is important to ensure that the quality of reagents, equipment, SOPs, researchers, and data are properly validated. Therefore, if it is a paper on GLP of immune activity measurement, the reviewer thinks that it is meaningful. Unfortunately, this paper is only a comparison between two laboratories, and the descriptions of samples and experiments are too lenient to draw accurate conclusions.

Both RIVM and the University of Liverpool are working under ISO 17025.

The originator lab drafted the SOPs based on the protocols running in the lab, the SOP was then reviewed by the project partner laboratory for clarity and by the project's QC partner for regulatory

requirements such as adequate reagent and equipment identification, positive and negative controls, data reporting, etc. The SOP was adapted and used for the inter-laboratory comparison. Both labs used a common SOP.

For example, it is necessary to clarify differences in cell ID numbers, passage numbers, culture days, etc., differences in the quality of the researchers (education background, research experience etc.), and differences in equipment, instruments, reagents, SOPs, etc., used by two laboratories in this experiment. In particular, in the NLRP3 inflammasome activity, the RIVM laboratory has shown three independent experimental results against Null, whereas the University of Liverpool laboratory uses Null, ASC-deficient cells, and NLRP3-deficient cells, so there is no comparison intra-laboratory variance and inter-laboratory variance for Null.

The reviewer is correct, RIVM performed three independent experiments, while the University of Liverpool performed a single experiment. Therefore, intra-laboratory comparison was possible for RIVM, and only limited inter-laboratory comparison between RIVM and the University of Liverpool could be done. In the revised manuscript we adapted the text accordingly. In the revised manuscript, the data on the deficient cells was omitted as they do not contribute to the intra- or inter-laboratory comparison.

As for dendritic cell maturation, the RIVM laboratory uses stored human buffy coats purchased from the Dutch Blood Bank, but the University of Liverpool laboratory used fresh blood collected from volunteers, so the cells used are different in freshness. And because the immune response varies from person to person, generalization requires blood samples collected from more volunteers (The reviewer does not know how many in this experiment).

RIVM did not use stored human buffy coats, but fresh ones. In the revised manuscript we added: They [the buffy coats] were kept at RT until starting cell isolation the next morning.

We have used monocyte-derived dendritic cells from a single donor for each of the triplicate experiments. We agree with the reviewer that to fully evaluate the response of dendritic cells to a specific agent, the number of donors to be tested should likely be more than 3. However, the aim of the study was an inter-laboratory transferability and comparison study, not a detailed investigation on the nanomedicines themselves, and the observed, similar, trends in the data suggest parity across the results obtained.

Two nanomedicines are used as samples, but no specifics are provided. Which laboratory prepared it? Are the ingredients GMP compliant? Is the lot number the same? Is there any difference between lots? Does the difference in the storage period affect it? Was the preparation done in a clean environment? Have the researchers tested for pyrogens and endotoxins?

The project partner CEA produced LipImage™ 815, while SINTEF produced PACA and PACA-CBZ (see above).

No, not all ingredients were GMP compliant.

Each partner laboratory used the same lot, and the same lot was used for each of the triplicate experiments.

	size (nm)	PDI	ζ-potential (mV)
LipImage™ 815 - lot 1	49	0.097	-2.5
LipImage™ 815 - lot 2	53	0.150	-1.5
PACA – lot 1	121	0.11	-3.0
PACA – lot 2	134	0.11	-3.2
PACA-CBZ – lot 1	135	0.14	-2.5
PACA-CBZ – lot 2	140	0.13	-2.9

The integrity of the nanomedicines upon storage was monitored by a third project partner.

Preparation was done in a laminar flow cabinet.

The nanomedicines were tested for endotoxin and the levels were found to be <0.1 IU/ml.

There are many details about the results, but the reviewer would like to ask the above points before going to them.

We thank the reviewer for the constructive comments and hope to have sufficiently addressed the questions by the reviewer.

Reviewer #2

In this manuscript, 'An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars' the authors present the results of a proposed strategy for the efficient hazard assessment of nanomedicines. Their approach is based on the measurement of two endpoints associated with the activation of immune cells after acute exposure in vitro to the nanomedicines. The authors aim to demonstrate the utility of the approach with two case study materials and the reproducibility through an inter-lab comparison of the assay SOPs with the overall goal of promoting the adoption of this approach in the regulatory assessment of new nanomedicines.

In this study the authors are addressing an important unmet need in the hazard assessment and regulation of nanomedicines, in line with the drive to reduce the number of animals used in the research. However in my opinion there are fundamental flaws in some of the underlying concepts and study design that need to be addressed in order to support the publication of this study. In addition in places the details of methods used and the data presented falls short of the expected standard to allow the reader to independently and critical review the proposed approach and results presented. The major and minor concerns I have with the manuscript are further outlined below.

Major

1. The use of a single endpoint, IL-1b, as an indirect output measurement of a complex biological pathway. The casual use of language, why not measure IL-18. The lack of response in cells with and without the complete pathway is not enough to demonstrate mechanism especially when NO positive controls, either chemical or relevant and comparable material controls have been included in the studies conducted in the knockout cells. At a minimum the authors need more discussion and justification of how a single indirect endpoint, IL-1b secretion, can be considered a sufficient measure of a complex pathway such as NLRP3 activation if the authors seek to further the acceptance of this approach by regulatory agencies. Additional assessment of IL-18 should be conducted or if not the text should be amended to accurately reflect the endpoint measured is IL-1b secretion which is merely an indirect and indicative measure of inflammasome activation.

The reviewer is correct that measuring only IL-1β (and not IL-18) needs some explanation. First, in earlier studies using the same assay we tested three materials known to induce NLRP3 inflammasome activation: SiO_2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant. All three materials showed a concentration-dependent increase in IL-1β secretion and a concomitant decrease in viability. Second, in another earlier study we compared IL-1β and IL-18 responses to a concentration range of gold nanoparticles and found only very minor differences in the gold concentration vs. cytokine response profile, except for the absolute amount (pg/ml) of cytokine secreted.

We decided to in the revised manuscript omit the work on the deficient cell lines as they do not contribute to the intra- and inter-laboratory comparison (see also our response to Reviewer 1).

Still, we agree with the reviewer that it is of added value to measure additional parameters. In the revised manuscript we added: Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3

activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death. Please, see also the response to Reviewer 3.

2. Lack of appropriate controls, both positive and negative materials and chemical controls (nigericin missing from UoL data). The ISO standard (ISO 10993-5:2009. Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity') for the assessment of requires the inclusion of positive control materials in a study and also recommends the study includes a negative control materials 'The purpose of the positive control is to demonstrate an appropriate test system response. For example, an organotin-stabilized polyurethane1) has been used as positive control for solid materials and extracts. Dilutions of phenol, for example, have been used as a positive control for extracts. In addition to a material, pure chemicals can also be used to demonstrate the performance of the test system.'

The reviewer is correct that in the present manuscript we did not include that in a separate inter-laboratory comparison study within the project, the cytotoxicity of the three materials LipImage™815, PACA, and PACA-CBZ were tested. In the revised manuscript we added: In a separate inter-laboratory comparison study within the REFINE project the cytotoxicity of the same batches of the three materials LipImage™815, PACA, and PACA-CBZ was tested in four different cell lines using both the WST-8 and the LDH release assay. LipImage™ 815 was non-cytotoxic up to a concentration of 128 µg/ml, whereas PACA caused dose-dependent cytotoxic effects starting from 8 µg/ml. PACA-CBZ showed a less pronounced dose-dependent effect with the lowest concentration of 2 µg/mL causing cytotoxic effects (Eder et al. submitted for publication).

We used SiO_2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant as positive control materials for NLRP3 inflammasome activation. In addition, we used nigericin as low-molecular weight “pure chemical” positive control.

The inclusion of both these controls would not only provide data to allow the reliability and reproducibility of the assays to be demonstrated, which appears to be the point of conducting the inter-lab comparison but also aid in the interpretation of results and justification of the authors that a certain effect level (e.g. 30% cytotoxicity) has some biological relevance. Currently these justifications are simply speculative and the interpretation of results greatly weakened as the lack of response from cells to exposure to the case study materials for the endpoints selected. A negative response could be reflective of a weakness or limitation of the test system rather than a lack of hazard of the materials. The authors do not provide any rationale for the selection of exposure concentrations selected, time-course of exposure selected or deposition or interaction between the materials and cells. The inclusion of positive control materials would at least allow the authors to demonstrate there are operating within relevant ball-park of exposure dose and time.

When setting up the NLRP3 inflammasome activation assay, we have gone through great lengths to optimise PMA stimulation (concentration/time chessboard) and exposure time. The exposure concentration is similar to what we routinely use for other nanomaterials. As mentioned above, we used SiO_2 nanoparticles, multi-walled carbon nanotubes, and aluminium hydroxide adjuvant as positive control materials. In addition, we used nigericin as the low-molecular weight positive control. The ISO standard 10993 states 70% viability as threshold of cytotoxicity.

3. Number of replicates for IL-1b secretion conducted by UoL, the authors report in numerous places in the manuscript e.g. abstract and discussion, that ', two laboratories performed each of the assays in three independent experiments'. This is not accurate and misrepresents the amount of data presented.

The reviewer is correct. In the Abstract and Discussion of the revised manuscript we changed the text to: One laboratory performed the assay in three independent experiments, while the other laboratory performed the experiment once. One laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. Two laboratories each performed three independent DC maturation assays in three independent experiments.

4. Authors rely too heavily on descriptive terms to report quantitative results....e.g. 'sharp increase'...'gradual decrease' but these terms are defined inconsistently throughout the manuscript and appear to more reflect the authors conclusions/intuitive interpretations of the results than a clear reporting of the results themselves. For example in Figure 5, LipImage exposure shows a consistent exposure concentration trend of increasing DC-SIGN....with the % difference much greater than the 30% reduction reported for the positive controls...why have the authors only reported 'slightly increased DC-SIGN expression at the highest conc tested'? While PACA and PACA-CBZ induced what looks like a more dramatic decrease in DC-SIGN than the positive control treatments but this is not clearly reported in the results text. Does LipImage cause a conc dependent increase in PD-L1 expression? The trend on the graphs presented looks slight but an effect could be masked but the dramatic increase caused by positive control represented on the same graph. Given the differences in mode of action and bioavailability of a soluble receptor agonists and a particle exposure this trend in response to LipImage should not be ignored. For clarity and transparency the results section should include tables reporting quantitative results across the exposure concentrations rather than rely on selective reporting and vague terms as the manuscript is presented here. Furthermore switching between % response in comparison to untreated control, % increase/decrease in endpoint measured, fold-change increase/decrease when reporting results making the text unclear and difficult to read in places.

We attempted to find a balance between being precise (mentioning percentages and fold increase/decrease) and having a text that is still readable. In the revised manuscript the wording is more precise, and we added the exact values in the Supplementary Information. There, WST-1 values are given as absorbance values, not as normalised values. The choice to use percentages vs. fold increase/decrease is caused by the effect size. Generally, effects >150% increase are given as a fold increase as a percentage would implicate an accuracy that is unrealistic.

5. I question the interpretation of results reporting the functional activation of immune cells in response to stimulus from populations with high levels of cytotoxicity e.g. Exposure to PACA resulted in a sharp decrease in viability, from 70% viability at 8 µg/ml to 9% viability at 16 µg/ml. Such a dramatic loss in viability will not be reflective of cells stimulated to mount an immune response, the inclusion of intermediate exposure concentrations would add confidence that the immune responses i.e. IL-1b secretion and DC activation, are not being missed or masked by excessive cell death in the populations and also likely improve the accuracy of the benchmark dose modelling.

The aim of the study was an inter-laboratory comparison study, not an in-depth evaluation of the potential effects in a rather narrow exposure window. Still, an in-depth study on the specific nanomedicine would indeed require investigating what is happening in that exposure window.

6. Lack of characterisation of the materials. In the methods the authors allude to the measurement of hydrodynamic diameter and zeta potential of the NP dispersions but do not provide the data. Was this conducted by both partners? Where NP dispersions consistent across both sites? Do the authors have any indication that the NP deposit on the cells within the timeframe of exposure? Ideally the authors would report the rate of deposition for each material within the test system.

The reviewer is correct that this information is missing. We apologise for this omission. In the revised manuscript we included the following Table:

	Size (nm)	PDI	ζ-pot (mV)	Drug loading (wt %)	Dye loading (wt %)
LipImage	53 ± 1	0.15	-1.5 ± 1	-	0.35%
PACA	136.2	0.11	-4.8	-	-
PACA-CBZ	121.8	0.14	-5.5	10.8%	-

Table 1. Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF.

Given that for sample preparation the nanomedicines only needed vortexing and not (probe or bath) sonication we did not feel the need to perform a comparison of the dispersions.

Based on the density of the nanomedicines (close to 1), given the dimensions of the cell culture well, the deposition of a 100 µg/ml dispersion amounted 3.76 µg (LipImage), 2.34 µg (PACA), and 2.48 µg (PACA-CBZ) within a 24-hr period (calculated by the ISDD model).

7. Potential for NP interference in the assays was not fully assessed. The potential adsorbance of the WST-1 reagent and secreted IL-1b should be addressed. In figure 3 a consistent decrease in IL-1b measured between 0 and 2-4 ug/ml exposure conc when cells exposed to PACA is seen...could this be due to particle interference? The authors need to also provide more detail in the methods section explaining how the assays were conducted, e.g. were cells washed and particles removed before exposure to WST-1 reagent? Were NP removed by centrifugation from the supernatant for the ELISA? These details are important for the standardisation of an SOP for NP exposure.

WST-1 was added without prior washing of the cells. As a control, wells were included that contain a dilution series of the nanomedicine but without cells. This corrects for possible light absorbance of the nanomedicines but not for possible interference of colour (due to substrate conversion) by the nanomedicines.

Since the ELISA protocol contains multiple washing steps, we did not centrifuge the supernatants.

8. Do the authors consider an n=3 to have sufficient statistical power to conduct benchmark dose modelling? The majority of endpoints reported could not be included in modelling. The authors report

this is 'When an EC30 could not be calculated, or the ratio between the upper (95%) and lower (5%) limit around the EC30 was > 5, the data were not considered.' But do not provide detail on which caveat applied to each situation where the modelling could not be carried out. The authors should more clearly report whether the inability to use this exposure-response modelling approach is due to a lack of response generated or due to too great level of variation between the replicates. In the currently format it is difficult to assess the utility of the modelling approach and reason for including it in the manuscript.

In the revised manuscript we added which of the two (could not be calculated, or ratio between BMDU and BMDL was >5) applied.

9. The authors acknowledge the subjective nature of flow cytometry population gating. Given the aim of this study is to conduct an interlab comparison both the 2D scatter plots and subjective gating strategies conducted at each site need to be included, a least in Supplementary Information.

The gating strategy is included in the SOP. In the revised manuscript the SOP (in fact, both SOPs) is included in the Supplementary Information.

10. Alluding to results from other studies without the presentation of data or citing a source the reader can access to support their conclusion as the authors have done in the Discussion paragraph 3 is inappropriate. If the authors cannot provide the data or source this paragraph should be removed.

In the revised manuscript we included the data on the Avanti liposome as Supplementary Information.

Minor

1. Justification for the use of both a cell-line and primary DC cells. The rationale for the use of primary DC cells is clear but the benefits for the use of THP-1 cells over primary monocyte-derived macrophages is speculative. Would there not be some added value to assessing the NRLP3 activation in the matched MDM from the same donor blood as the DC's? The user will have to go through the collection and preparation of samples but not use the MDM component, while in parallel maintain the THP-1 cells in culture. The authors are proposing this is a more efficient approach to hazard assessment?

We greatly appreciate the suggestion by the reviewer. On the one hand, it is indeed efficient to use the same monocyte population for both DC maturation and NLRP3 inflammasome activation assays. On the other hand, the two assays have been regarded as two individual (not inter-related) assays in a larger series of in vitro assays for effects on the immune system. The fact that the DC maturation assay requires monocytes from blood donors refrains its use as a regulatory test, albeit an important output parameter. Therefore, when both options are open (cell line or primary cells), as is the case for the NLRP3 inflammasome activation assay, we chose for the cell line-based assay.

2. Page 4, line 12-13: 'The assays were performed by two laboratories using common SOPs'. Provide references to these 'common SOPs'.

In the revised manuscript both SOPs are attached as Supplementary Information.

3. Very short exposure to PMA for differentiation, has this protocol been validated by more extensive characterisation of macrophage differentiation than simple adherence of cells? e.g. demonstration of macrophage markers CD11b etc?

We forgot to mention that the cells were also visually inspected for macrophage-like appearance. In the revised manuscript we added: The cells were visually inspected for macrophage-like appearance.

4. Inflammasome activation leads to the cleavage of pro-IL-1b and secretion of the mature form. Secretion is a more accurate term for the endpoint i.e. level of IL-1b released into the supernatant. The term IL-1b 'production' is more indicative of de novo protein synthesis and should be amended through the text to better reflect the pathway if it is being used as a marker of inflammasome activation. However I would continue to argue the level of IL-1b secretion being measured could be due to multiple mechanisms including an increase in protein translation and therefore not specific to inflammasome activation. Is the ELISA kit used by the authors specific for the mature form of IL-1b?

The ELISA is not specific for mature IL-1β.

5. Legend of Figure 4 appears to be incorrect, are the green dots reporting cell viability or IL-1b secretion?

In the revised manuscript the dot colour has been corrected.

6. In Figure 5a there is a consistent and complete loss of viability for cells exposed to PACA-CBZ at a dose of 16ug/ml which appears to be an anomaly when considering the other doses and responses measured. Can the authors confirm the independence of each of the experimental replicates? Were cells from different donors? Different preparations of particles? Conducted on different days?

Yes, we can indeed confirm that the experiments were done on cells from different donors, using different preparations of the dilution series, and on different days. So, indeed they are independent experiments.

7. In Fig 5d the authors report 'For PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml and 64 µg/ml, respectively. Possibly, CD14+ cells are less sensitive to PACA and PACA-CBZ, resulting in an increased MFI.' The bases of this speculative interpretation of this result is not clear.

We agree with the reviewer that this is indeed speculative. In the revised manuscript, this sentence has been removed.

Reviewer #3

The manuscript by Vandebriel and colleagues describes an evaluation of the effects of two different nanocarriers, one of which is loaded with a chemotherapeutic agent towards immune cells. The strength of the study is both in the use of very different nano-carriers to assess the effect of these relevant nano-medicines but also in the comparative approach employed by the two institutions to look at reproducibility. In particular, the finding that the Live/ Dead assay shows lower reproducibility as well as wider issues around flow cytometry as a tool is interesting, useful and addressable during future standardisation.

A criticism of the study is that the evaluation of NALP3 inflammasome activation is somewhat basic as it solely rests on the expression of IL-1B, rather than a more comprehensive measure such as caspase-1 activation. However, this is mitigated by the use of knock out (KO) THP-1 cells using siRNA to target ACS and NALP3 by one of the partners. Considering the regulatory use of such an approach, thought should be given to a) if IL-1B expression is sufficient as a marker of activation b) if repeat experiments with KO cells is efficient or c) if there are other markers that can be used in addition to IL-1B expression, preferably in the same experiment.

We thank the reviewer for this suggestion. In another study we compared IL-1β and IL-18 responses to a concentration range of gold nanoparticles and found only a very minor difference in the gold concentration vs. cytokine response profile, except for the absolute amount (pg/ml) of cytokine secreted. In addition, cleavage of pro-IL-1β and pro-IL-18 to IL-1β and IL-18, respectively, both depend on caspase-1 activation. Therefore, addition of IL-18 does not seem useful. Adding measurement of caspase-1 activity may seem useful as this points to inflammasome activation (not necessarily NLRP3, e.g. also AIM2 has caspase-1 activation as downstream effect). Another possible addition could be to measure caspase-3, with the aim to evaluate cell death not caused by pyroptosis but by apoptosis. These caspases should be measured in lysed (or fresh) cells, not in supernatants, somewhat complicating the assay. More generally, the purpose of the work was not to delineate/define specific mechanistic responses but, to produce a reliable and straightforward SOP to indicate inflammasome activation; hence the focus on IL-1β as a prototypical marker.

In the revised manuscript we added: Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3 activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death. Please, see also the response to Reviewer 2.

Overall, the study is both robust, interesting and well written with the materials and Methods section being particularly detailed and well described. However, from an editorial perspective the Results section could do with some more thought. This is because currently, the sequential reporting of the RIVM results then the University of Liverpool results for each endpoint makes the results section very long, repetitive and a little hard to follow. I'm not sure how best to address this due to the comparative nature of the study but perhaps greater use of supplementary data to reduce the number of figures and a side-by-side comparative table of results; either providing a qualitative summary (e.g. ++/-) or quantitative values.

We have discussed how to improve the flow of the text but could not come up with a better solution. The heat map as shown for the DC data is a direct comparison between the data of both partners.

Major Points:

1. Whilst size and surface charge characterisation of the nano-carriers was conducted as reported in the methods, this data seems to be missing from the paper. As parameters like surface charge may offer an explanation for the differential cytotoxicity of these two substances, it is important to report and discuss these.

The reviewer is correct that this information is missing. We apologise for this omission. In the revised manuscript we included the following Table:

	Size (nm)	PDI	ζ-pot (mV)	Drug loading (wt %)	Dye loading (wt %)
LipImage™ 815	53 ± 1	0.15	-1.5 ± 1	-	0.35%
PACA	136.2	0.11	-4.8	-	-
PACA-CBZ	121.8	0.14	-5.5	10.8%	-

Table 1. Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF.

Minor Points:

1. From a readability perspective, the authors should re-examine their rather excessive use of commas in the text as this makes many of the sentences disjointed and hard to read. For example: "In a recent analysis, we listed, among others, immune system endpoints for which, however required by regulatory authorities, no generally accepted assays exist".

In the revised manuscript we will as much as possible reduce the use of commas.

2. It would be useful for the reader if the nature of the chemotherapeutic agent Cabazitaxel is explained. This could be at either its first mention at the end of the introduction or with the Materials and Methods.

The reviewer is correct that this data is missing from the manuscript and we apologise for this omission. In the revised manuscript we added: Cabazitaxel was obtained from Shanghai Biochempartner Co., Ltd. (Shanghai, China).

An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars

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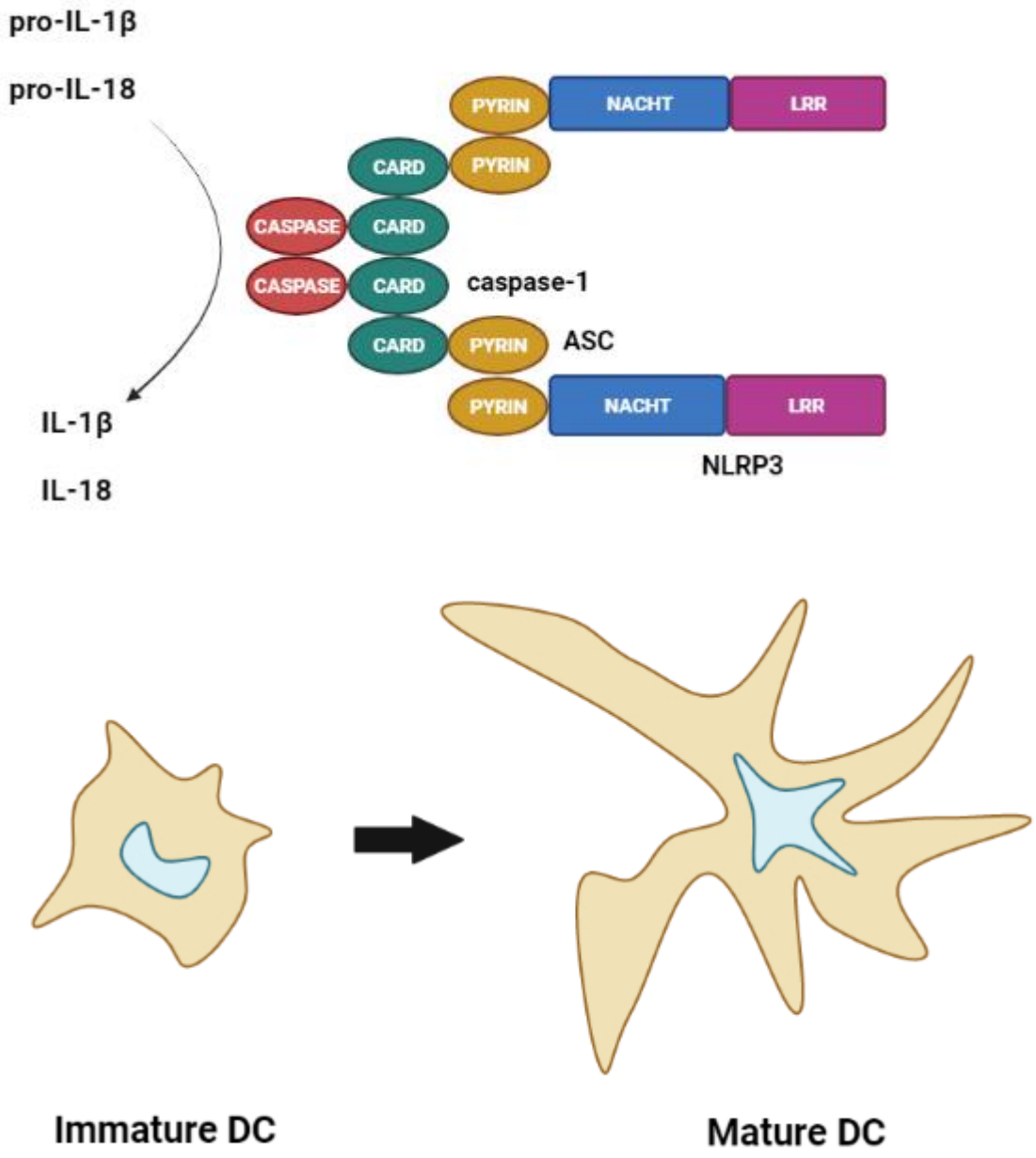
Abstract

Nanoparticles~~,~~ including nanomedicines, are known to be recognised by~~,~~ and interact with~~,~~ the immune system. As these interactions may result in adverse effects, for safety evaluation the presence of such interactions needs to be investigated. Nanomedicines in particular should not~~,~~ unintendedly~~,~~ interact with the immune system, since patient's exposure is not minimised~~,~~ as in the case of "environmental" nanoparticles, and repeated exposure may be required. NLRP3 inflammasome activation and dendritic cell (DC) maturation are two types of immune mechanisms known to be affected by nanoparticles including nanomedicines. NLRP3 inflammasome activation results in production of the pro-inflammatory cytokines IL-1β and IL-18, as well as a specific type of cell death, pyroptosis. Moreover, chronic NLRP3 inflammasome activation has been related to several chronic diseases. Upon maturation, DC activate primary T-cells; interference with this process may result in inappropriate activation and skewing of the adaptive immune response. Here we evaluated the effect of two nanomedicines, representing nanostructured lipid carriers and polymers, on these two assays. Moreover, with a view to possible future standardization and regulatory application, these assays were subject to an inter-laboratory comparison study~~,~~ using common SOPs. One laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. ~~Initially, t~~Two laboratories each performed three independent DC maturation~~each of the assays in three independent~~ experiments. While the nanostructured lipid carrier~~s~~ only showed marginal effects, the polymers showed major cytotoxicity. No evidence for inflammasome activation or DC maturation was demonstrated. Intra- and inter-laboratory comparison showed clearly reproducible results.



Graphical Abstract

Keywords

NLRP3 inflammasome activation; monocyte-derived dendritic cell; dendritic cell maturation; nanostructured lipid carrier; polymeric nanomedicine; inter-laboratory comparison

Introduction

Nanoparticles (NP) are known to interact with the immune system [1]. This also holds for nanomedicines [2]. Generally, effects on the immune system can be regarded as detrimental as it disturbs the intricate homeostasis of the system. Especially nanomedicines should not interact with the immune system, since patients are intentionally exposed and often so for a prolonged period. The degree and nature of NP interaction with the immune system depends on the NP's characteristics [3-4]. However, the relationship between these is still not~~,~~ completely~~,~~ understood meaning that prediction of effects on the immune system from these characteristics is limited. A series of (preferably *in vitro* and high throughput) assays~~,~~ is therefore required, to establish possible effects on the immune system. In a recent analysis~~,~~ we ~~listed~~, among others, listed immune system endpoints for which, however required by regulatory authorities, no generally accepted assays exist [5]. From this list of endpoints two assays, measuring different immune mechanisms, are the subject of the current study. Both mechanisms are known to be affected by nanomaterials and are linked to adverse immune effects [6-8].

The NLR family, pyrin domain-containing 3 (NLRP3) inflammasome consists of a NLRP3 scaffold, an apoptosis-associated speck-like protein containing a CARD (ASC) adaptor, and pro-caspase-1. Upon activation, NLRP3 recruits ASC. ASC then binds to pro-caspase-1, resulting in auto-cleavage of this pro-enzyme to become the active enzyme~~,~~ caspase-1. Caspase-1 processes pro-IL-1β and pro-IL-18 to bioactive IL-1β and IL-18, respectively [9]. Please refer to this publication also for a clear representation of the pathways involved. These cytokines are potent mediators of inflammation. Next to host-derived molecules and a multitude of infectious agents [9], the NLRP3 inflammasome can be induced by a wide range of xenobiotics including NP [6]. Its activation is associated with various inflammatory diseases, including lung fibrosis, obesity and type-2 diabetes [7].

Dendritic cells (DCs) are sentinel cells that are pivotal in the initiation of adaptive immune responses [10]. Moreover, they integrate various stimuli, such as from different pathogen-associated molecular patterns (PAMPs) and the cytokine milieu. PAMPs are detected by pattern recognition receptors (PRRs) that are highly expressed by DCs. Important classes of PRR form the Toll-like receptors and Nod-like receptors. Importantly, the nature of the immune response following DC maturation is significantly influenced by the PRR (or combination of different PRR). In this way, DCs form an important link between the innate and adaptive immune response. DCs appear as immature DCs that are well capable of ingesting protein antigens and as mature DCs that are especially capable of presenting peptides to naive T cells. This process of DC maturation is central to the functioning of DC. Various types of NP can influence the process of DC maturation and by that immune function [8]. DC maturation can be readily measured by cell surface marker expression and cytokine production. The panel of cell surface markers used to measure DC maturation generally comprises HLA-DR (MHC class II) and the T-cell co-receptors CD40, CD80, and CD86, although additions to this panel such as~~are, for example,~~ CD83, PD-L1~~,~~ and DC-SIGN, are sometimes included, while in other studies~~,~~ the panel used is more limited. The cytokines measured to evaluate DC maturation are mostly IL-12p40 or IL-12p70, but also IL-10 and TNF-α.

Although DC harbour a fully functional NLRP3 inflammasome, for the NLRP3 inflammasome activation assay we chose to use macrophages derived from THP-1 human monocytes, since (1) the NLRP3 inflammasome is more strongly expressed in macrophages compared to DC, (2) a monocyte cell line

is likely to provide more reproducible data than primary monocytes, and (3) there is no possible interference by DC maturation on NLRP3 inflammasome activation. ~~As far as we know, NLRP3 inflammasome activation by NP has only be tested in murine bone marrow-derived DC, not in human monocyte-derived DC. On the other hand,~~ DC obtained from cell lines have limited functionality compared to those obtained from primary monocytes, justifying primary monocytes as a source for the DC maturation assay.

Here we present the results of an inter-laboratory comparison study of two assays, NLRP3 inflammasome activation (using macrophages derived from THP-1 monocytes), and DC maturation (using DC derived from primary monocytes). The assays were performed by two laboratories using common SOPs. While RIVM performed three independent replicate experiments~~studies~~ of the NLRP3 inflammasome activation assay~~studies~~, the University of Liverpool performed one experiment~~study using wild-type cells, one study using ASC-deficient cells and one study using NLRP3-deficient cells~~. RIVM and the University of Liverpool each performed the DC maturation assay in three independent replicate experiments~~studies~~. Two types of nanomedicines were tested: the nanostructured lipid carrier LipImage™ 815, and a nanocarrier composed of poly (alkyl cyanoacrylate) polymer. The latter was tested both empty and loaded with Cabazitaxel.

Materials and methods

Nanomedicines

Two types of nanomedicines were tested: (i) the nanostructured lipid carrier LipImage™ 815 [11], (ii) the nanocarrier composed of the poly (alkyl cyanoacrylate) (PACA) polymer: poly (2-ethylbutyl cyanoacrylate) (PEBCA). PEBCA was tested both empty and loaded with Cabazitaxel (CBZ) [12]. In the present paper, these polymer nanocarriers are designated PACA and PACA-CBZ, respectively.

LipImage™ 815 synthesis and characterisation

Batches of LipImage™ 815 were prepared by high-pressure homogenization (HPH). The lipid phase comprised 19.125 g of soybean oil, 6.375 g of Suppocire™ NB, 4.875 g of lecithin, and 150 mg of IR-870 oleyl (molar mass: 986.29 g/mol), which was synthetized as previously described [11]. The aqueous phase comprised 25.875 g of Myrj™ S40 and 110 ml NaCl 154 mM. Mixtures of lipid and aqueous phases were pre-emulsified using a mechanical disperser (Ultra-T25 Digital Turrax, IKA) operated at 15,000 rpm for 5 minutes. The emulsion was then processed with a High-Pressure Homogenizer (Panda Plus 2000, GEA Niro Soavi, Italy) operated for 16 cycles with a total pressure of 1250 bars, the pressure of the second stage chamber being set at 50 bars and the cooling system at 30°C. Batches of 200 g of particles were then purified by 5 µm filtration followed by tangential flow filtration (Labscale TFF system, Millipore) against NaCl 154 mM through a Pellicon XL Biomax™ cassette (Merck) operated at a trans-membrane pressure of 1 bar at a flow rate of 2 ml/min. The nanoparticle dispersion was adjusted to a concentration of 100 mg/ml and filtered through a 0.22 µm Millipore membrane for sterilization before storage and use.

Dynamic light scattering (DLS) was used to determine the particle hydrodynamic diameter and zeta potential (Zeta Sizer Nano ZS, Malvern Instrument, Orsay, France). Particle dispersions were diluted to 2 mg/ml of lipids in 0.22 µm filtered 0.1 X PBS and transferred in Zeta Sizer Nano cells (Malvern

Instrument) before each measurement, performed in triplicate. Results (Z-average diameter, dispersity index, ζ-potential) were expressed as mean and standard deviation of three independent measurements performed at 25 °C. The encapsulation efficiency and payload of IR780-oleyl dye in the LipImage™ 815 were determined by high-performance liquid chromatography (HPLC WATERS Alliance 2695 / Fluorescence 2475 detector) and compared with a calibration curve established from the reference fluorophore IR780-Oleyl alone, as previously described [13]. The theoretical amount of IR780-Oleyl encapsulated in a batch of LipImage™ 815 at 100 mg/ml lipid nanoparticles is 266 µM. The size, polydispersity index, ζ-potential, and dye loading of LipImage™ 815 is shown in Table 1.

PACA synthesis

PACA nanoparticles were synthesized under aseptic conditions at SINTEF (Trondheim, Norway) by mini-emulsion polymerization. Prior to synthesis, all solutions were sterile filtered, and all equipment was autoclaved. An oil phase consisting of poly(ethyl butyl cyanoacrylate) (PEBCA) (Cuantum Medical Cosmetics) containing 2 wt % Miglyol 812 (Cremer) and 10 wt % vanillin was prepared. For drug-loaded particles, 12 wt % CBZ (~~BioChemPartner~~Shanghai Biochempartner Co., Ltd. (Shanghai, China).) was added to the oil phase and only 2 wt % vanillin was used. For dye-loaded particles, either 0.4 wt % IR-780-Oleyl (custom synthesis at CEA LETI) or NR668 (modified Nile Red, custom synthesis at SINTEF [14]) was added to the oil phase.

An aqueous phase consisting of 0.1 M HCl containing the two PEG stabilisers (Brij®L23 and Kolliphor®HS15, both Sigma-Aldrich, 5 wt % of each) was added to the oil phase. The water and oil phases were mixed and immediately sonicated for 3 min on ice (6 × 30 s intervals, 60% amplitude, Branson Ultrasonics digital sonifier). The solution was rotated (15 rpm) at room temperature (RT) overnight. The pH was then adjusted to 5.0 to allow further polymerisation at RT for 5 hours. The dispersions were dialyzed (Spectra/Por dialysis membrane MWCO 100.000 Da) against 1 mM HCl to remove unreacted PEG. The size (z-average), polydispersity index (PDI) and the ζ-potential of the NPs in phosphate buffer (10 mM, pH 7.0) were measured by DLS and laser Doppler Micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments).

To calculate the amount of encapsulated drug, the drug was extracted from the particles by dissolving them in acetone (1:10), and quantified by liquid chromatography coupled to mass spectrometry (LC-MS/MS) using an Agilent 1290 HPLC system coupled to an Agilent 6490 triple quadrupole mass spectrometer. The size, polydispersity index, ζ-potential, and drug loading of PACA and PACA-CBZ is shown in Table 1.

	Size (nm)	PDI	ζ-pot (mV)	Drug loading (wt %)	Dye loading (wt %)
LipImage™815	53 ± 1	0.15	-1.5 ± 1	-	0.35%
PACA	136.2	0.11	-4.8	-	-
PACA-CBZ	121.8	0.14	-5.5	10.8%	-

Table 1: Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF.

NLRP3 inflammasome activation

Cell line maintenance

THP-1 cells (ATCC TIB-202)~~, THP1-defASC cells and THP1-defNLRP3 cells (InvivoGen) were used in this study. The cells~~ were cultured in complete cell culture medium (CCM), that is: RPMI 1640 (Gibco) supplemented with foetal calf serum (10% v/v, Greiner-Bio), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco). ~~Additionally, the ASC- and NLRP3-deficient THP-1 cells were cultured in CCM supplemented with HygroGold (200 µg/ml, InvivoGen) to maintain the siRNA responsible for the suppression of ASC or NLRP3.~~ The cells~~All cell lines~~ were sub-cultured twice per week, seeded to a cell density of $2x10^5$ cells/ml, and not allowed to grow to a density beyond $1x10^6$ cells/ml. Cells were not cultured for more than twenty passages to prevent genetic divergence.

Differentiation of THP-1 cells

The ~~wild-type, ASC- and NLRP3-deficient~~ THP-1 cells were differentiated into macrophage-like cells by culturing for 3 hours in the presence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) in 96-well format at a cell density of $5x10^5$ cells/ml, 100 µl/well. After this incubation the cells were adherent. The cells were visually inspected for macrophage-like appearance. The medium was replaced with fresh culture medium without PMA and the plates were incubated for 24 hours at standard conditions (humidified incubator at 37°C, 5% CO2). After this incubation period, the cells were exposed to a two-fold dilution series of LipImage™ 815, PACA, or PACA-CBZ (2, 4, 8, 16, 32, 64, and 128 µg/ml), for 48 hours at standard conditions. As positive control for NLRP3 inflammasome activation, nigericin (InvivoGen) was used (0.625 and 1.25 µg/ml). CCM was used as negative control. Cells were used for viability testing; culture supernatants were frozen at -80°C until further use (ELISA).

Viability of THP-1 cells

The viability of the cells after exposure was assessed using the cell proliferation reagent WST-1 (Sigma-Aldrich). Exposed cells (and controls) were incubated for 2.5 hours under standard conditions in the presence of 10% (v/v) WST-1 reagent. After incubation, the absorbance (A) was measured in each well at 440 nm (A_{440}) and corrected for background absorbance at 620 nm (A_{620}). Exposures for viability assessment were performed in triplicate and the viability was calculated as follows: (A (cells in medium, X) – A (medium only, X))/A (cells in medium, C) – A (medium only, C), where X is a specific concentration nanomedicine or positive control and C the CCM control. The viability was expressed as percentage of the control. As a control, for each nanomedicine at the highest exposure concentration (in CCM), the A_{440}-A_{620} signal was measured and found not to interfere with the read-out signal of the WST-1 assay.

IL-1β ELISA

The IL-1β concentrations in the culture supernatant were determined using ELISA (eBioscience) according to the manufacturer's instructions. An 8-point, 2-fold dilution series of a cytokine standard was prepared, diluent was used as blank. A calibration curve was calculated using 5-parameter curve fitting. Exposures for the assessment of IL-1β secretion were performed in four wells per condition. The supernatants were tested in a 2-fold dilution, except nigericin (0.625 µg/ml in a 5-fold dilution and 1.25 µg/ml in a 30-fold dilution) to stay within the standard curve concentration range.

Concentration-response modelling

Concentration-response modelling for viability and IL-1β production was performed with the statistical software package PROAST [15] (version 70.3) within the software environment 'R' [16] (version 4.1.0).

In this approach, a concentration-response dataset is evaluated as a whole by fitting a concentration-response model over the entire concentration range studied. Having fitted a concentration-response model to the data, this curve is used to assess the benchmark concentration (BMC) associated with the benchmark response (BMR) of 50%. The choice of the model for deriving the BMC follows from a procedure of applying likelihood ratio tests to the five members of the following two nested families of models:

Exponential family	Hill family
E1: $y = a$	H1: $y = a$
E2: $y = a \exp(b\,x)$	H2: $y = a\,(1 - x / (b + x))$
E3: $y = a \exp(b\,x^d)$	H3: $y = a\,(1 - x^d / (b^d + x^d))$
E4: $y = a\,(c - (c - 1)) \exp(b\,x)$	H4: $y = a\,(1 + (c - 1)\,x / (b + x))$
E5: $y = a\,(c - (c - 1)) \exp(b\,x^d)$	H5: $y = a\,(1 + (c - 1)\,x^d / (b^d + x^d))$

where y is any continuous endpoint and x denotes the concentration. In these models, the parameter "a" represents the background response and the parameter "b" can be considered as the parameter reflecting the efficacy of the chemical (or the sensitivity of the subject). First, the likelihood ratio test was used to establish whether extension of a model by increasing the number of parameters resulted in a statistically significant improvement of the fit. The model that could not be significantly improved was considered as the most appropriate member (which adequately fits but does not overfit the data) within each family. In addition, a goodness of fit test ($P > 0.05$) was applied by comparing the log-likelihood of the fitted model to that associated with the so-called "full model." The full model simply consists of the observed (mean) responses at each applied concentration. The model is accepted when the log-likelihood value of the fitted model is not significantly worse than that of the full model. Subsequently, the BMCs are derived from the different models and the 90% confidence intervals (CIs) surrounding the BMCs are calculated using the profile-likelihood method. The BMC used in the analysis was the geometric average of the BMCs derived for the different models. The 90% CI surrounding this BMC comprised the BMCL and the BMCU found for the BMC estimates derived from the different models.

The Standard Operating Procedure is included (Supplementary Information S1).

Dendritic cell maturation

Isolation of $CD14^+$ cells

Human buffy coats were purchased from the Dutch blood bank (Sanquin, Amsterdam) and obtained the night before isolation of the cells. They were kept at RT until starting cell isolation the next

morning. At the University of Liverpool, blood was obtained from healthy volunteers on the day of the experiment. The buffy coat was diluted 1:1 with PBS. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat by centrifugation (1000g, 30 min, 20°C) over Ficoll (Lymphoprep; Axis Shield, Oslo, Norway). After washing with PBS, red blood cells were lysed by resuspending the cell pellet in ACK buffer (156 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) and a subsequent wash with PBS. $CD14^+$ cells were positively selected from the cell suspension using a magnetic-activated cell sorting (MACS) kit with CD14-specific antibodies (MACS, Miltenyi Biotec, Leiden, the Netherlands) according to the manufacturer's instructions. To achieve a high purity, a lower amount of antibody was used than recommended (5 µl per 10^7 cells). The positive and negative fractions from the MACS were analysed by flow cytometry to determine $CD14^+$ purity (see Table 2 for antibody panel).

Marker	Label	Dilution	Manufacturer
CD14	PE	1:50	Becton Dickinson
Live/dead	Aqua	1:400	Invitrogen

Table 2: Antibody panel for the assessment of $CD14^+$ purity of cell samples by flow cytometry.

Staining was done in two consecutive steps. First, the cells were stained with Live/dead (in PBS, 0.2 mM EDTA) at 4 °C for 30 min. Second, the cells were washed with FACS buffer (PBS pH 7.2, 0.2 mM EDTA, 0.5% BSA), and stained with anti-CD14 antibody in FACS buffer at 4 °C for 30 min.

The $CD14^+$ cell fraction was resuspended in complete culture medium (CCM): RPMI 1640 GlutaMAX (Gibco), 10% Foetal Calf Serum (FCS; Hyclone; GE Healthcare), 1% pen/strep (Gibco), 450 U/ml GM-CSF (PeproTech) and 350 U/ml IL-4 (Active Bioscience). For a flow diagram see Fig. 1.

Differentiation of $CD14^+$ cells to immature DC, and exposure to nanomedicines

The $CD14^+$ cells were seeded in 12-well plates, 1 ml/well, $3x10^5$ cells/ml and incubated at 37°C and 5% CO_2 in a humidified incubator. After 3 days, 100 µl RPMI 1640 GlutaMAX containing 10% FCS, 4500 U/ml GM-CSF and 3500 U/ml IL-4 was added to each well to a final concentration of approximately 450 U/ml GM-CSF and 350 U/ml IL-4. After 6 days, 750 µl culture medium was removed and spun down. The pellet was resuspended in CCM and seeded back into the wells with a dilution series of LipImage™ 815, PACA, or PACA-CBZ. LPS (100 ng/ml) and R848 (5 µg/ml) were used as positive controls, 10% PBS as negative control. The plates were placed back in the incubator for 44-48 hours until harvest for analysis. For harvesting, from each well the culture medium was collected and spun down. Each supernatant was individually transferred to a fresh tube and stored at -80°C for ELISA. In the meantime, cold PBS was put on the cells that were attached to the wells. After detaching the cells by gentle scraping and pipetting, they were collected and added to the tube in which already part of the cells was collected. These cells were divided over two wells for staining with the two separate antibody panels.

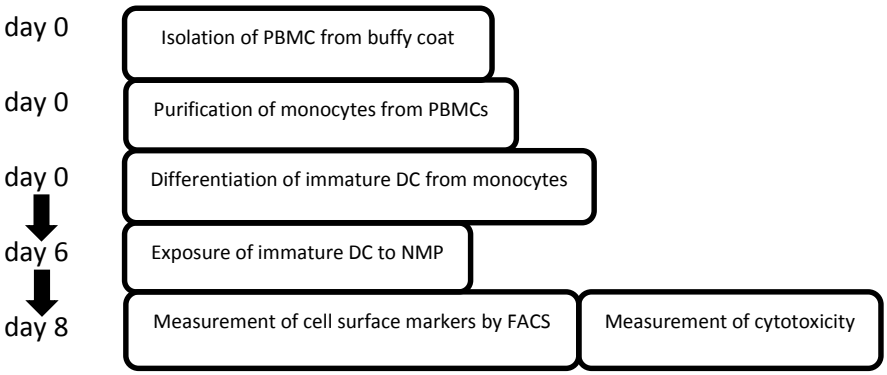


Fig. 1: Flow diagram of DC maturation assay.

Flow cytometric analysis of cultured cells

Maturation of the DCs was assessed by flow cytometry (FACS) using two antibody panels (Table 3). In addition, Forward Scatter (FSC; a measure of cell size) and Side Scatter (SSC; a measure of internal complexity (i.e. granularity)) were measured.

Panel 1

Marker	**Label**	**Dilution**	**Manufacturer**
CD80	FITC	1:40	Becton Dickinson
CD14	PE	1:50	Becton Dickinson
PD-L1	APC	1:400	eBioscience
HLA-DR	Pacific Blue	1:1000	Biolegend
Live/dead	Aqua	1:1000	Invitrogen

Panel 2

Marker	**Label**	**Dilution**	**Manufacturer**
CD83	FITC	1:20	Becton Dickinson
CD40	PE	1:10	Becton Dickinson
DC-SIGN	APC	1:200	Becton Dickinson
CD86	Pacific Blue	1:800	Biolegend
Live/dead	Aqua	1:1000	Invitrogen

Table 3: Antibody panels used for the assessment of DC maturation by flow cytometry.

First, the cells were washed twice with PBS. Second, the cells were stained with Live/dead in PBS, 0.2 mM EDTA at 4 °C for 30 min. Third, the cells were washed once FACS buffer (PBS pH 7.2, 0.2 mM EDTA, 0.5% BSA). To 100 µl of these cells, 100 µl of panel 1 or panel mix 2 (see Table 3) was added. After incubation at 4 °C for 30 min, the cells were washed twice, spun down, and included in FACS buffer. Data was acquired using the FACS Canto II (Becton Dickinson Biosciences) using the settings: (1) FSC: 150; SSC: 350; PE: 488 nm laser (blue), 585/42 filter; Aqua: 405 nm laser (violet), 510/50 filter. (2) Sample flow rate 3 µl/sec; sample volume 170 µl; mixing volume 70 µl; mixing speed 180 µl; number of mixes 3; washing volume 800 µl. (3) Compensations were set using beads and DC, on a population of 50% living and 50% dead cells. To obtain dead cells, the living cells were heat-shocked.

Data were analysed using FlowJo software (Becton Dickinson). Gating was done according to Fig. 2.

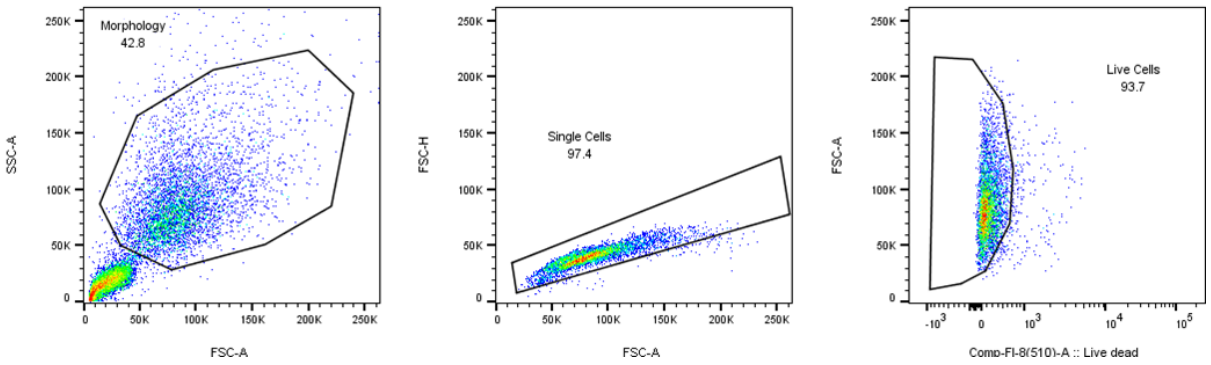


Fig. 2: Gating procedure. (1) Gating was done based on the morphology of the cells (left graph). The lower left corner (<50K FSC-A (X-axis) and <50 K-SSC-A (Y-axis)) is excluded. (2) Within the cell population gated under (1), the single cells were gated (middle graph). In the FSC-A (X-axis) vs. FSC-H (Y-axis) plot, doublet cells form a population below the diagonal. (3) Within the cell population gated under (2), the live cells were gated (right graph). In the Live/dead staining (X-axis) vs. FSC-A (right axis), the dead cells scatter to the right.

Determination of cultured dendritic cell viability

The $CD14^+$ cells were seeded in 96-well plates, 200 µl/well, $3x10^5$ cells/ml and incubated at 37°C and 5% CO_2 in a humidified incubator. The protocol as described above "Differentiation of CD14+ cells to immature DC, and exposure to nanomedicines" was used (and done concurrently). For viability assessment, the protocol as described above "Viability of THP-1 cells" was used.

Concentration-response modelling

The method as described above "Concentration-response modelling" was used.

The Standard Operating Procedure is included (Supplementary Information S2).

Results

Inflammasome activation

RIVM

PMA-activated THP-1 cells were incubated for 48 hr with the positive control nigericin at two concentrations (0.625 and 1.25 µg/ml) and a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, 4, and 2 µg/ml, plus a medium control (0)). After thisthat, viability was evaluated using the WST-1 assay and the IL-1β concentration using an ELISA. The results shown are from three independent experiments (Fig. 3).

The positive control for NLRP3 inflammasome activation, nigericin, showed a strong reduction in viability (25% and 8% of the medium control for the low and high nigericin concentration, respectively) and a strong increase in IL-1β production (720 and 3200 pg/ml for the low and high nigericin concentration, respectively, where the medium control amounted 110 pg/ml). Thisese data of concentration-dependent reduction in viability and concomitant concentration-dependent increase in IL-1β production suggest an appropriate function of the positive control nigericin and thus suggests a proper functioning of the NLRP3 inflammasome activation assay.

Exposure to LipImage™ 815 resulted in a 30% ~~slight concentration-dependent~~ decrease in viability ~~, with a 30% decrease~~ at the highest concentration tested (128 µg/ml). A ~~slight concentration-dependent increase in IL-1β production was seen, with a~~ 50% increase in IL-1β production (from 110 pg/ml to 165 pg/ml) was seen at the highest concentration tested ~~(from 110 pg/ml to 165 pg/ml)~~. Although a ~~A concentration-dependent~~ decrease in viability and a concomitant ~~concentration-dependent~~ increase in IL-1β production is seen, a hallmark of NLRP3 inflammasome activation~~. However~~, the effects observed are too small to suggest that LipImage™ 815 activates the NLRP3 inflammasome.

Exposure to PACA resulted in a clear~~sharp~~ decrease in viability within a 2-fold concentration range, from 70% viability at 8 µg/ml to 9% viability at 16 µg/ml. At these same concentrations, only a small~~slight~~ increase in IL-1β production was seen, from 50 pg/ml to 70 pg/ml. Exposure to PACA-CBZ also resulted in a clear~~sharp~~ decrease in viability, albeit within a 4-fold concentration range~~less pronounced compared to PACA alone~~, from~~being~~ 85% viability at 8 µg/ml, 37% viability at 16 µg/ml, to~~and~~ 8% viability at 32 µg/ml. At these same concentrations, only a small~~slight~~ increase in IL-1β production was seen, being 50 pg/ml at 8 µg/ml, and 60 pg/ml at 16 µg/ml and 32 µg/ml. This data suggests a strong cytotoxic effect of PACA, both with and without CBZ, and no evidence for NLRP3 inflammasome activation.

Comparison of the results between the individual experiments shows a high reproducibility, with some quantitative differences in IL-1β production throughout individual concentration-response curves, but a highly similar shape of both the viability and the IL-1β production concentration-response curves.

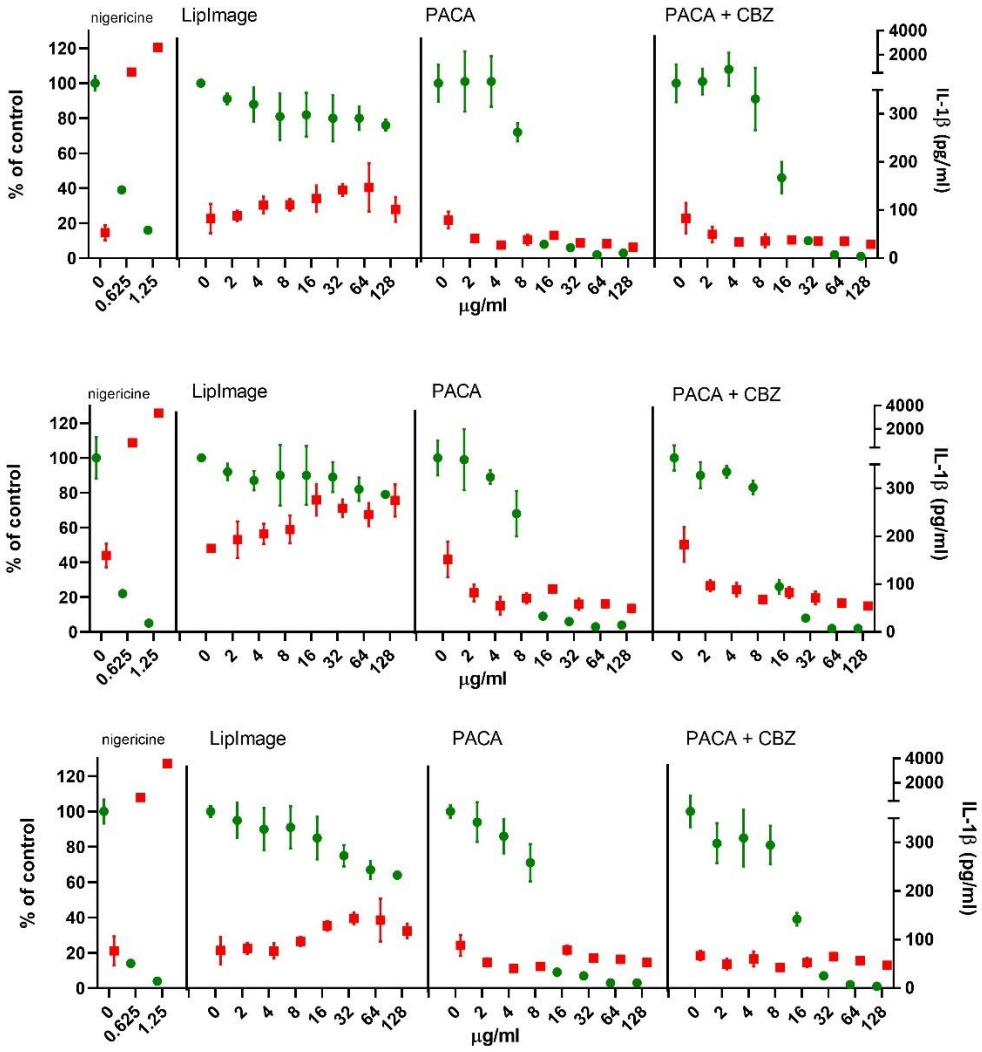


Fig. 3 NLRP3 inflammasome activation by LipImage™ 815, PACA, and PACA-CBZ. Nigericin: positive control. Green (plotted to the left Y-axis): viability (percentage of untreated control). Red (plotted to the right Y-axis): IL-1β production (pg/ml). Three independent experiments are shown. Mean ± SD, with N = 4 replicates per experiment.

Inflammasome activation was also assessed using concentration-response modelling. The three experiments presented in Fig. 3 were analysed (together). Since ISO [17] takes 70% viability as a threshold for cytotoxicity, 30% reduction was chosen as effect size. The concentration at which a 30% effect is obtained is designated here as the Effective Concentration $(EC)_{30}$. Since no guidance exists on an effect size for markers of NLRP3 inflammasome activation, by default we chose a 30% effect (in this

case an increase), similar in size to viability. Next to establishing the EC, the software tool PROAST provides a 90% confidence interval (CI) around a specific EC (here EC_{30}). In Table 4, the EC_{30} values and corresponding 90% CIs are shown.

When an EC_{30} could not be calculated (LipImage™ 815: viability; PACA-CBZ: IL-1β production), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: IL-1β production; PACA: IL-1β production), the data were not considered. This was the case for viability for LipImage™ 815, and for IL-1β production for all three nanomedicines. The data in Table 43 show that the effects of PACA and PACA-CBZ on viability are highly similar, suggesting that in this assay the reduced viability is only due to PACA and not to CBZ.

	viability	
	EC_{30}	**90% CI**
PACA	8.15	6.31-10.1
PACA-CBZ	8.00	6.41-9.83

Table 4: EC_{30} and 90% CI values of viability (µg/ml).

University of Liverpool

Exposure to LipImage™ 815 resulted in a slight concentration-dependent increase in viability, with a 26% increase at the highest concentration tested (128 µg/ml). A slight concentration-dependent decrease in IL-1β production was seen, with a 10% decrease in IL-1β production was seen at the highest concentration tested (from 50 pg/ml to 45 pg/ml). The effects observed do not suggest that LipImage™ 815 activates the NLRP3 inflammasome. Similar observations were made for THP-1 cells deficient in the NLRP3 inflammasome, induced by gene knockdown of ASC or of NLRP3. These cells were similarly pre-treated with PMA and similarly exposed compared to the wild-type THP-1 cells. Exposure to LipImage™ 815 resulted in a slight concentration-dependent increase in viability, with a 7% increase at the highest concentration tested (128 µg/ml) for the ASC-deficient cells and an 18% increase at the highest concentration tested for the NLRP3-deficient cells. Together, these data suggest that LipImage™ 815 does not induce NLRP3 inflammasome activation.

Exposure to PACA resulted in a clearsharp decrease in viability within a 2-fold concentration range, from 79% viability at 8 µg/ml to 19% viability at 16 µg/ml, so at the same concentrations as seen in the experiments performed at RIVM. At these same concentrations, no effect on IL-1β production (48 pg/ml) was seen. Exposure to PACA-CBZ also resulted in a decrease in viability, albeit within a 4-fold concentration range less pronounced compared to PACA alone, frombeing 97% viability at 8 µg/ml, 78% viability at 16 µg/ml, toand 44% viability at 32 µg/ml. Thus, similar to the findings at RIVM, PACA-CBZ showed a more gradual decrease in viabilitytoxicity compared to PACA alone. At these same concentrations, no effect on IL-1β production (48 pg/ml for PACA and 45 pg/ml for PACA-CBZ) was seen. This data suggests a strong cytotoxic effect of PACA, both with and without CBZ, and no evidence for NLRP3 inflammasome activation.

Data from RIVM (Supplementary Information S3A) and the University of Liverpool (Supplementary Information S3B) is included.

Regarding THP-1 cells deficient in the NLRP3 inflammasome, for PACA, similar to the wild-type cells a sharp although less profound decrease in viability was seen: for ASC deficient cells 32% viability at 8 µg/ml and 14% at 16 µg/ml, and for NLRP3 deficent cells 61% at 8 µg/ml and 37% at 16 µg/ml. For PACA-CBZ, however, an almost 50% reduction in viability was seen already at the lowest concentration

~~tested (2 µg/ml). At these same concentrations, no effect on IL-1β production was seen: 44 pg/ml for PACA and 45 pg/ml for PACA-CBZ, both for ASC-deficient and NLRP3-deficient cells. This data suggests a strong cytotoxic effect of PACA, both with and without CBZ, and no evidence for NLRP3 inflammasome activation.~~

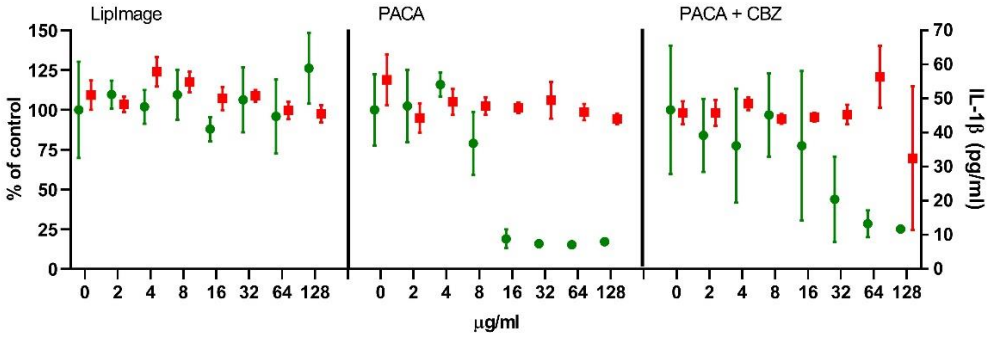


Fig. 4 NLRP3 inflammasome activation by LipImage™ 815, PACA, and PACA-CBZ. Green (plotted to the left Y-axis): viability (percentage of untreated control). Red (plotted to the right Y-axis): IL-1β production (pg/ml). ~~Top: wild-type THP-1. Middle: ASC-deficient THP-1. Bottom: NLRP3-deficient THP-1.~~ Mean ± SD, with N = 4 replicates per experiment.

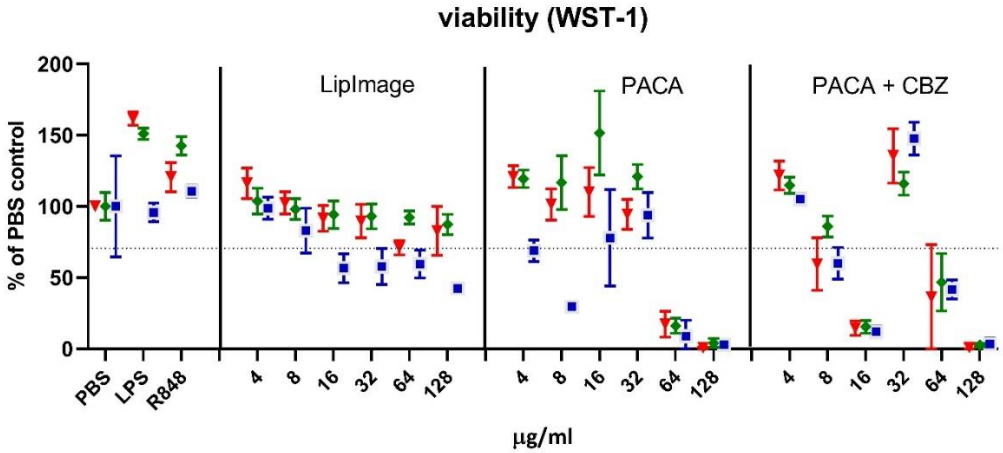
Dendritic cell maturation

RIVM

Monocytes were isolated from buffy coats and differentiated to immature DC. These were incubated for 48 hr with a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, and 4 µg/ml, plus a medium control (0)). After thisthat, viability was evaluated using the WST-1 assay and the surface marker expression using a FACS. The results shown are from three independent experiments (Fig. 5).

Viability

The positive controls for DC maturation, LPS and R848, did not affect viability. Incubation with LipImage™ 815 marginally affected cell viability, averaging 70% at the highest concentration tested (128 µg/ml). PACA showed a clearsharp decrease in viability within a 4-fold concentration range, fromaveraging 103% at 32 µg/ml, 14% at 64 µg/ml, and 3% at 128 µg/ml. PACA-CBZ, curiously, showed a biphasic viability curve, averaging 114%, 69%, 14%, 133%, 42%, and 2% for the entire concentration range. It should be noted that the results are obtained from three independent experiments using DCs cultured from monocytes of different donors, performed on different weeks. A complete loss of viability at 16 µg/ml does not fit the viability seen for PACA, for PACA and PACA-CBZ as evaluated by Live/dead staining (see below), or for PACA and PACA-CBZ in the experiments performed by the University of Liverpool (see below). However, in a study dedicated to evaluate the cytotoxicity of LipImage™ 815, PACA, and PACA-CBZ, in four different cell lines using two different viability assays, both PACA and PACA-CBZ showed a clear reduction in viability from 2 µg/ml, depending on the cell line and the assay ~~and in other viability assays using PACA and PACA-CBZ~~ (Eder et al. submitted~~this issue~~).



]

Fig. 5a Viability of DC after incubation with LipImage™ 815, PACA, and PACA-CBZ. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation.

Live/dead, FSC, and SSC

The positive controls for DC maturation, LPS and R848, had a minor effect on Live/dead staining, and did not affect FSC (a measure of cell size) and SSC (a measure of internal complexity (i.e. granularity)). No exposure effects of LipImage™ 815 on Live/dead staining, FSC and SSC were seen. PACA and PACA-CBZ induced a clear~~sharp~~ increase in Live/dead staining from 32 µg/ml and from 64 µg/ml, respectively. PACA and PACA-CBZ decreased FSC from 32 and 64 µg/ml, respectively. PACA and PACA-CBZ did not affect SSC. ~~So, WST-1, Live/dead-staining and FSC seems to be similarly sensitive effect parameters for PACA and PACA-CBZ.~~

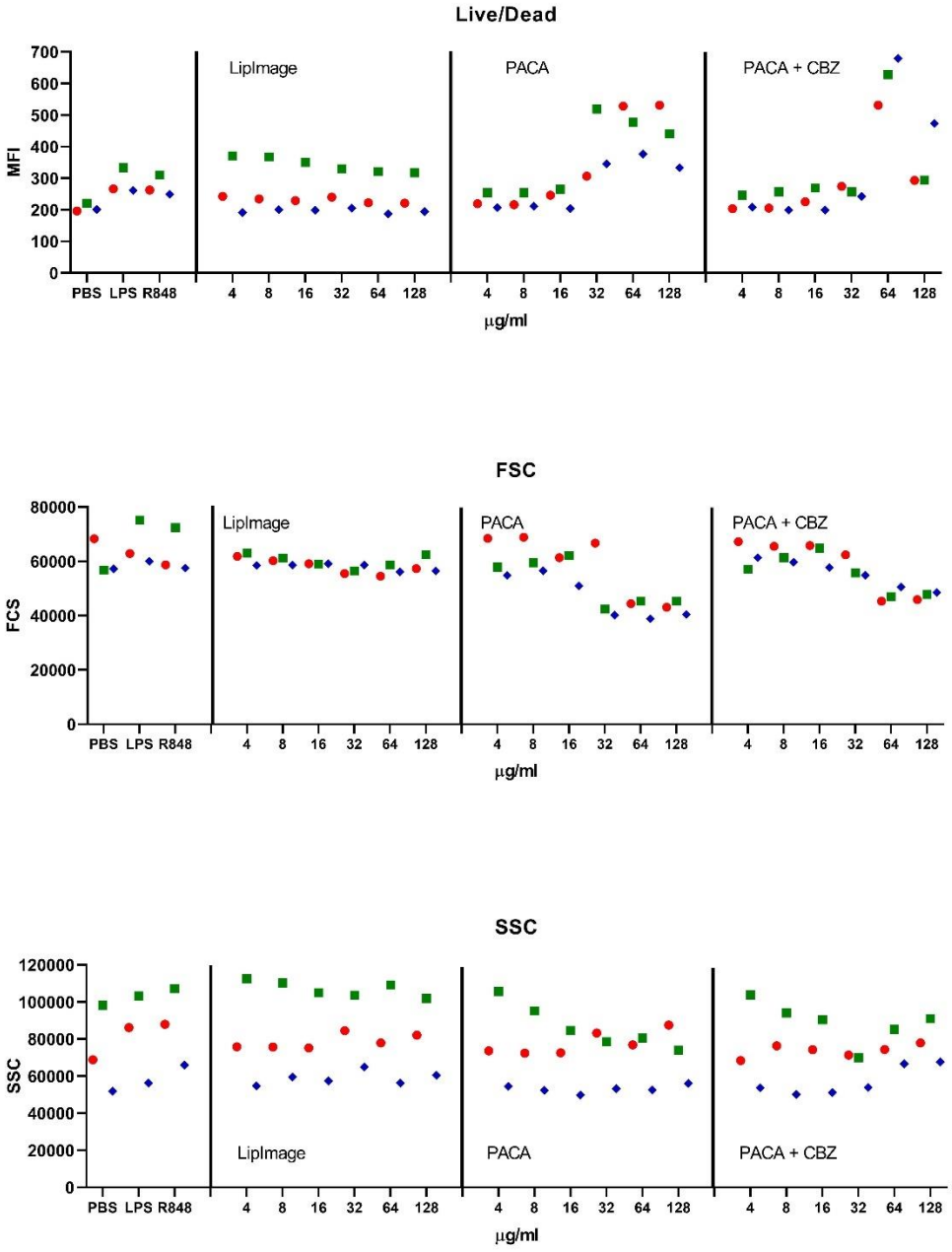


Fig. 5b Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on Live/dead staining, FSC, and SSC. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD80, CD83, and CD86

The positive controls LPS and R848 clearly induced CD80, CD83, and CD86 expression (5- and 3.5-fold for CD80; 7.5- and 6-fold for CD83; 30- and 23-fold for CD86, for LPS and R848, respectively), strongly suggestive of DC maturation. LipImage™ 815, PACA, and PACA-CBZ failed to do so, suggesting that none of the three nanomedicines induced DC maturation.

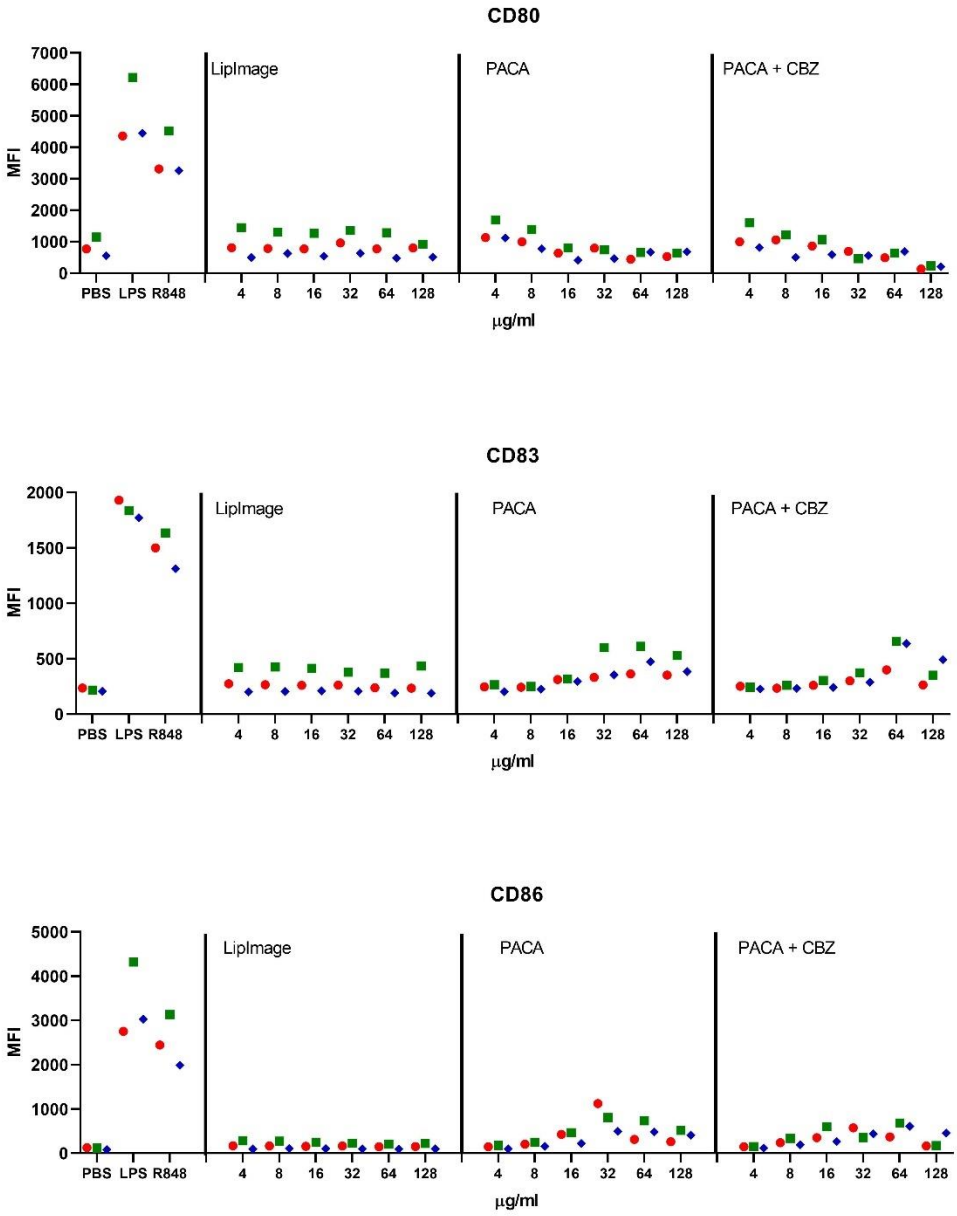


Fig. 5c Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD80, CD83, and CD86 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD14, DC-SIGN, and PD-L1

LPS and R848 rather similarly downregulated CD14 expression, by 40%. CD14 downregulation by LPS is in line with previous data showing combined endocytosis of LPS, TLR4, and CD14 [18]. DC-SIGN expression was reduced by 30% and 20% by LPS and R848, respectively. PD-L1 expression was induced 11-fold and 7-fold by LPS and R848, respectively. Decreased DC-SIGN expression and increased PD-L1 expression both suggest DC maturation. DC-SIGN is typically downregulated in DC upon maturation [19]. PD-L1 is upregulated in DC upon maturation [20].

LipImage™ 815 did not affect CD14 expression. For PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml and 64 µg/ml, respectively. ~~Possibly, CD14$^+$ cells are less sensitive to PACA and PACA-~~

~~CBZ, resulting in an increased MFI.~~ LipImage™ 815 slightly increased DC-SIGN expression at the highest concentration tested (128 µg/ml), while PACA and PACA-CBZ slightly decreased DC-SIGN expression at this concentration. LipImage™ 815, PACA, and PACA-CBZ did not affect PD-L1 expression, in line with a lack of effect on the maturation markers CD80, CD83, and CD86.

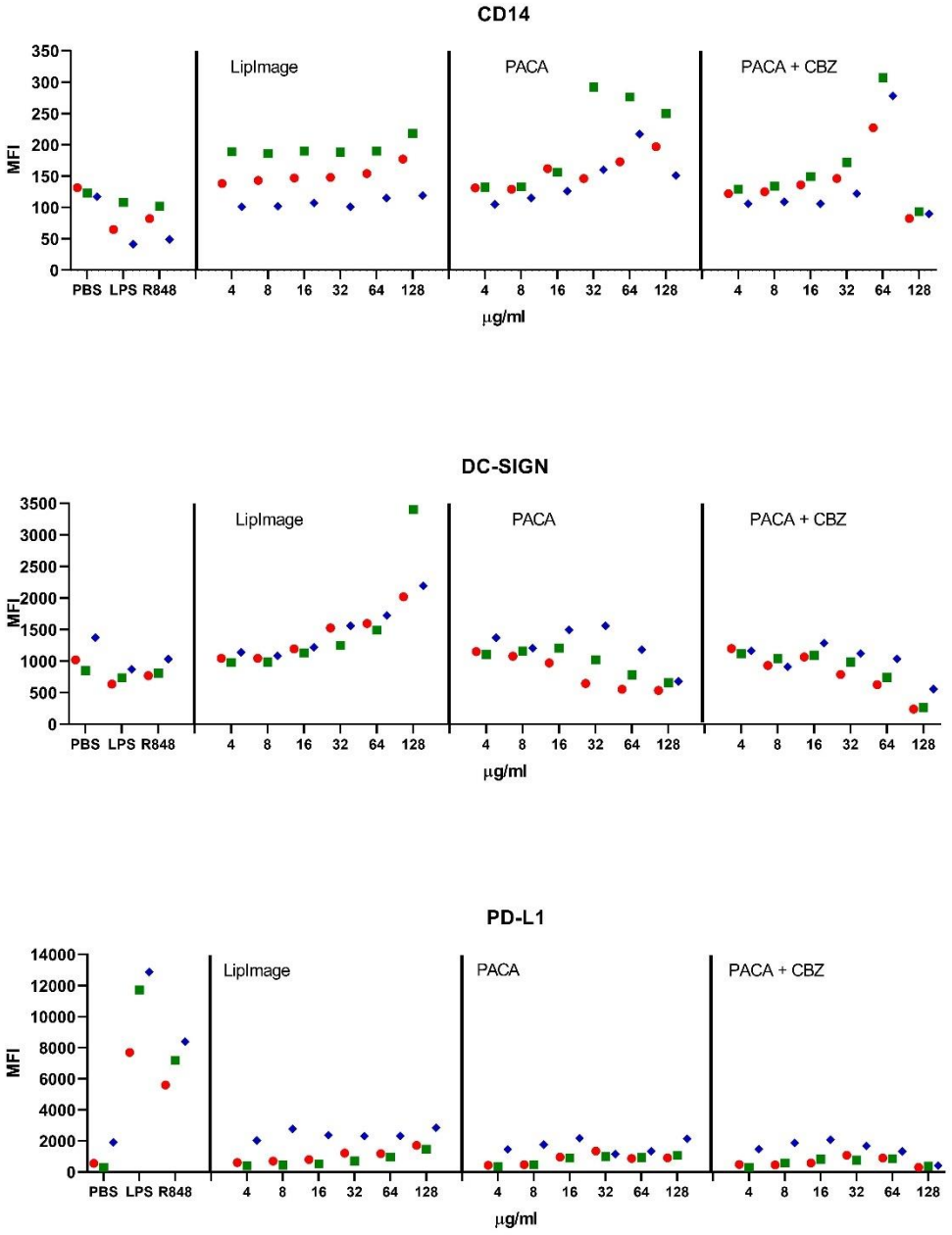


Fig. 5d Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD14, DC-SIGN, and PD-L1 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD40 and HLA-DR

The positive controls LPS and R848 clearly induced CD40 expression (3.5- and 3-fold, respectively) and to a lesser extent HLA-DR expression (1.6- and 2-fold, respectively), suggestive of DC maturation.

LipImage™ 815 failed to induce expression of either CD40 or HLA-DR. PACA and PACA-CBZ induced a 2-fold CD40 expression from 32 µg/ml and 64 µg/ml, respectively, and a 2-fold HLA-DR expression from 16 µg/ml and 32 µg/ml, respectively.

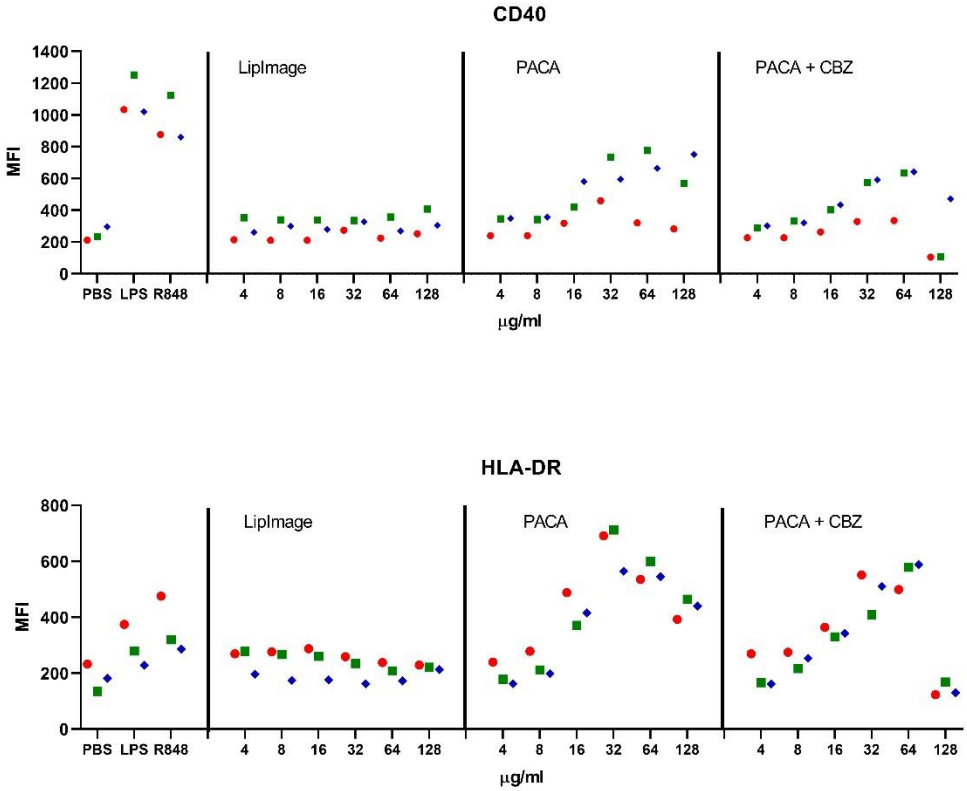


Fig. 5e Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD40 and HLA-DR surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

We analysed DC maturation also by concentration-response modelling, using the PROAST software tool. The three experiments presented in Fig. 5 were analysed (together). Since ISO [17] takes 70% viability as a threshold for cytotoxicity, 30% was chosen as effect size. The concentration at which a 30% effect is obtained is designated here as the Effective Concentration $(EC)_{30}$. Since no guidance exists on an effect size for markers of DC maturation, by default we chose a 30% effect, similar to viability. Next to establishing the ED, the software tool PROAST allows for generation of a 90% confidence interval (CI) around a specific ED (here EC_{30}). In Table 5, the EC_{30} values and corresponding 90% CIs are shown.

When an EC_{30} could not be calculated (LipImage™ 815: all parameters except DC-SIGN and PD-L1; PACA: FSC; PACA-CBZ: CD14, CD40), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: DC-SIGN and PD-L1; PACA: all parameters except FSC, WST-1 and Live/dead; PACA-CBZ: all parameters except WST-1, Live/Dead, CD14, and CD40), the data were not considered. ~~This was the case for all measurements for LipImage™ 815, and for FSC, SSC, CD80, CD83, CD86, CD14, DC-SIGN, PD-L1, CD40, and HLA-DR measurements for PACA and PACA-CBZ.~~ For WST-1 and Live/dead staining (Table 5~~4~~), the EC_{30} values for PACA-CBZ were 1.5 times higher than for PACA, possibly suggesting that in these assays PACA-CBZ may be slightly less cytotoxic compared to PACA.

	PACA		PACA-CBZ	
	EC_{30}	90% CI	EC_{30}	90% CI
WST-1	43.1	28.7-46.8	63.7	34.1-84.6
Live/dead	24.8	15.3-29.7	36.2	25.7-46.3

Table 5: EC_{30} and 90% CI values (µg/ml).

University of Liverpool

Monocytes were isolated from buffy coats and differentiated to immature DC. They were incubated for 48 hr with a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, 4, and 2 µg/ml, plus a medium control (0)). After that, viability was evaluated using the WST-1 assay and ~~the~~ surface marker expression using a FACS. The results shown are from three independent experiments (Fig. 6).

Viability

The positive controls for DC maturation, LPS and R848, did not affect viability. Incubation with LipImage™ 815 marginally affected cell viability, averaging 80% at the highest concentration tested (128 µg/ml). PACA showed a clear concentration-dependent decrease in viability within a 4-fold concentration range, from~~averaging~~ 91% at 32 µg/ml, 58% at 64 µg/ml, to~~and~~ 27% at 128 µg/ml. PACA-CBZ showed a similar~~, albeit less pronounced~~ decrease in viability, from~~averaging~~ 91% at 32 µg/ml, 74% at 64 µg/ml, and 37% at 128 µg/ml.

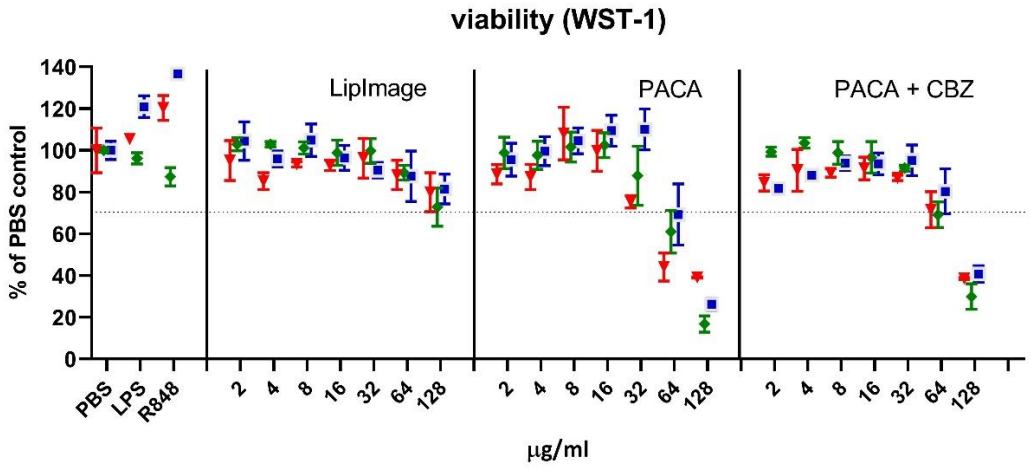


Fig. 6a Viability of DC after incubation with LipImage™ 815, PACA, and PACA-CBZ. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation.

Live/dead, FSC, and SSC

The positive controls for DC maturation, LPS and R848, did not affect Live/dead staining, FSC, and SSC. LipImage™ 815 induced a ~~gradual~~ concentration-dependent increase in Live/dead staining, while no exposure effects on FSC and SSC were seen. PACA ~~and PACA-CBZ~~ induced a clear~~sharp~~ increase in Live/dead staining from 16 ~~to 32~~ µg/ml in two out of three independent experiments.~~and at 32 µg/ml, respectively.~~ PACA-CBZ induced a clear increase in Live/dead staining from 32 µg/ml. PACA decreased FSC and SSC from ~~8 to~~ 16 µg/ml, while PACA-CBZ affected FSC and SSC ~~in a more gradual~~

~~way~~ from ~~8 to~~ 32 µg/ml. ~~So, FSC and SSC seem to be the most sensitive effect parameters for PACA and PACA-CBZ, followed by Live/dead staining. WST-1 seemed to be less sensitive than these three FACS-based parameters.~~

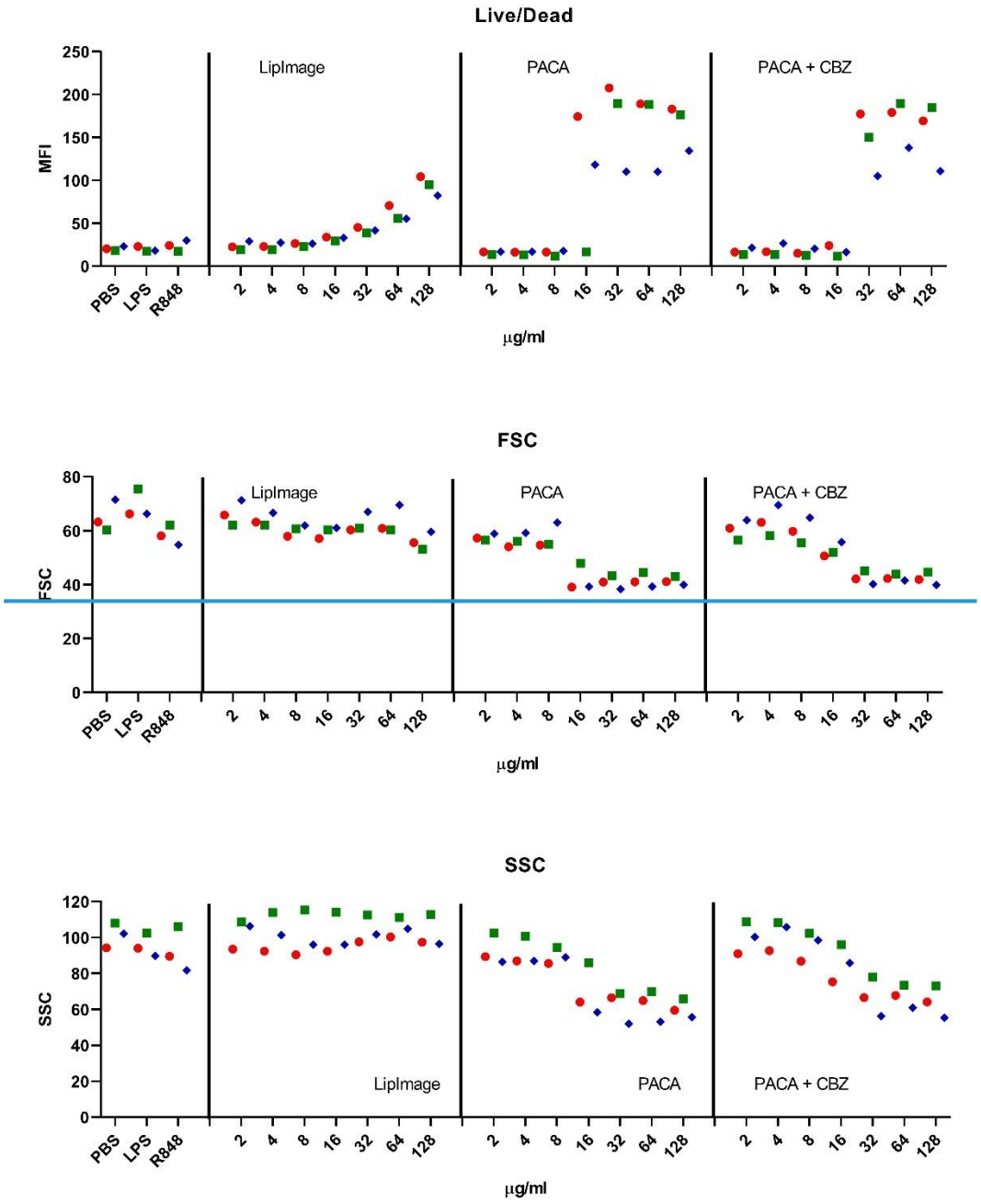


Fig. 6b Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on Live/dead staining, FSC, and SSC. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD80, CD83, and CD86

While LPS and R848 clearly induced CD80, CD83, and CD86 expression showing DC maturation, LipImage™ 815, PACA, and PACA-CBZ failed to do so. This suggests that none of the three nanomedicines induceds DC maturation.

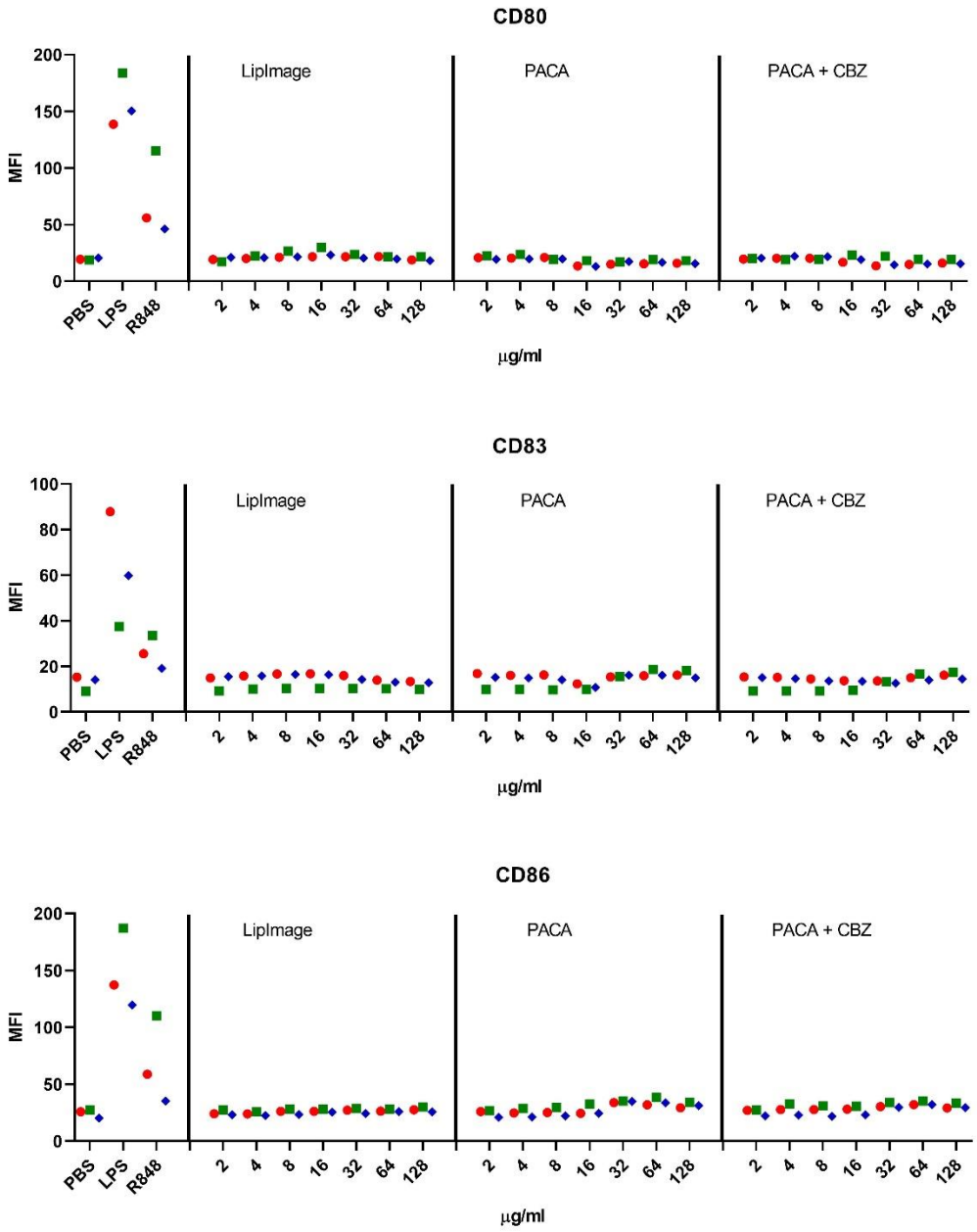


Fig. 6c Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD80, CD83, and CD86 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD14, DC-SIGN, and PD-L1

~~CD14 expression was quite variable between the different experiments, most likely due to inter-donor variability.~~ LPS clearly downregulated CD14 expression, in line with previous data showing combined

endocytosis of LPS, TLR4, and CD14 [18]. R848 decreased CD14 to a lesser extent than LPS did. DC-SIGN expression was not affected by LPS or R848. PD-L1 expression was induced by LPS and R848.

LipImage™ 815 did not affect CD14 expression. For one of the three independent experiments, for both PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml. ~~Possibly, CD14$^+$ DC are less sensitive to PACA and PACA-CBZ.~~ LipImage™ 815 induced the expression of DC-SIGN and PD-L1 from 64 µg/ml. DC-SIGN is typically downregulated in DC upon maturation [19]. No effects on DC-SIGN and PD-L1 expression by PACA and PACA-CBZ were~~are~~ seen.

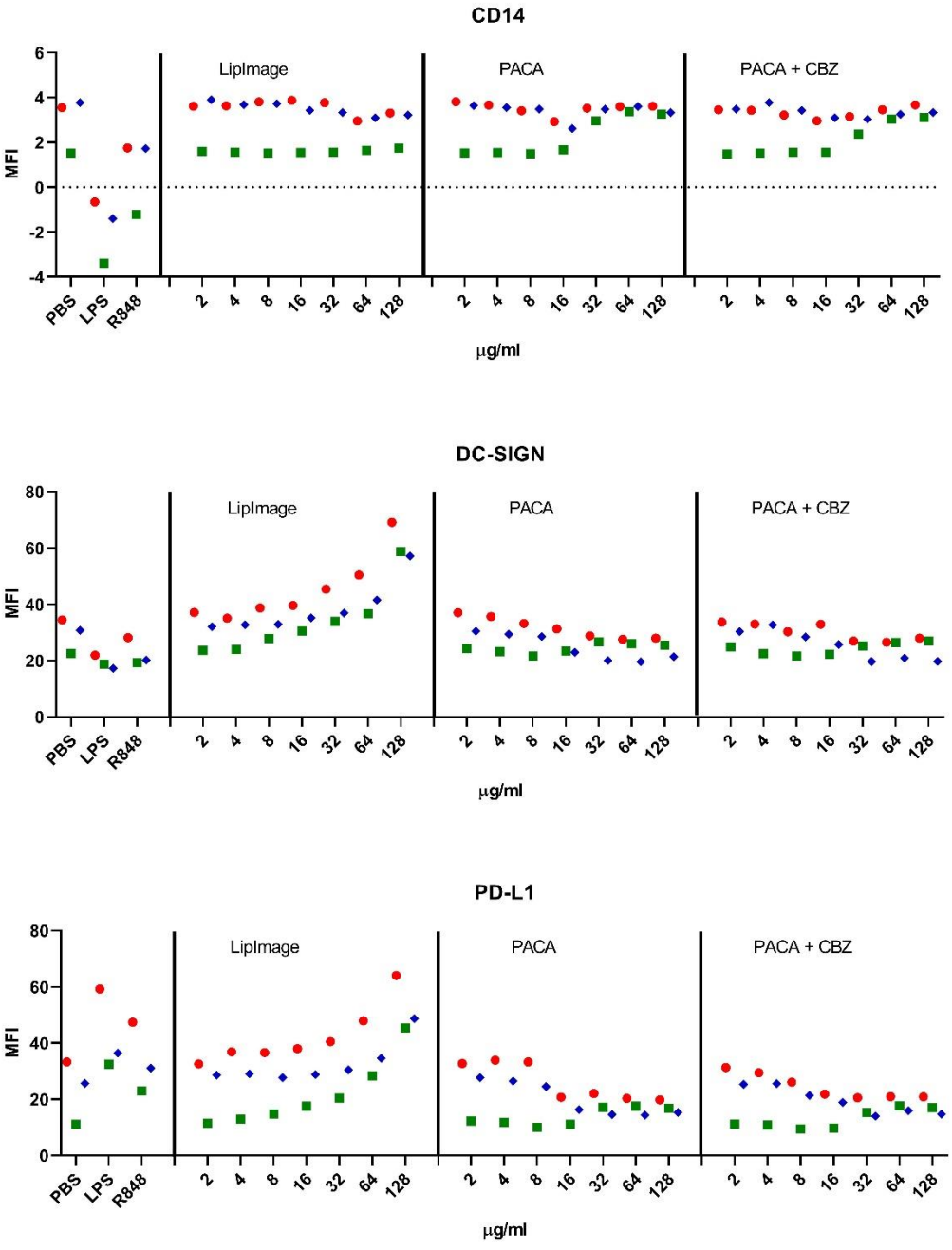


Fig. 6d Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD14, DC-SIGN, and PD-L1 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

We analysed DC maturation also by concentration-response modelling. The three experiments presented in Fig. 6 were analysed (together). When an EC_{30} could not be calculated (LipImage™ 815: WST-1, SSC, and CD14; PACA: FSC and PD-L1; PACA-CBZ: FSC, CD14, DC-SIGN, and PD-L1), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: Live/dead, DC-SIGN, and PD-L1; PACA: CD14), the data were not considered (CD80, CD83, and CD86 were not included). ~~This was the case for all measurements for LipImage™ 815, and for FSC, CD80, CD83, CD86, CD14, DC-SIGN, and PD-L1 measurements for PACA and PACA-CBZ.~~ For WST-1, Live/dead staining, and SSC (Table ~~6~~5), the EC_{30} values for PACA-CBZ were on average 1.5 times higher than for PACA, possibly suggesting that in these assays PACA-CBZ may be slightly less cytotoxic compared to PACA. ~~Possibly, the decreased SSC at concentrations slightly higher than increased Live/dead staining may suggest that reduced viability induced by PACA has decreased granularity as characteristic.~~

	PACA		PACA-CBZ	
	EC_{30}	**90% CI**	**EC_{30}**	**90% CI**
WST-1	51.0	35.0-67.7	72.3	66.3-88.8
Live/dead	10.3	5.34-11.6	17.5	15.2-18.9
SSC	17.5	13.0-17.9	25.9	18.1-35.0

Table ~~6~~5: EC_{30} and 90% CI values (µg/ml).

Inter-laboratory variance in DC parameters

To evaluate the inter-laboratory variance in all DC parameters including WST-1, we first normalized for each experiment the CCM control to 100%. From this, for each of the three pharmaceuticals, for each of the two partners, and for each individual concentration we calculated the mean and standard deviation over the three independent replicate experiments. After this, the inter-laboratory variance was calculated and expressed in a heat map (Fig. 7). For LipImage™ 815, the largest inter-laboratory variance was for Live/dead staining and, to a lesser extent, DC-SIGN and PD-L1. It should be mentioned that a larger inter-laboratory variance is to be expected when a concentration-dependent effect is seen. For LipImage™ 815, this is seen for DC-SIGN, PD-L1 (for University of Liverpool but not RIVM), but not for Live/dead staining. For PACA and PACA-CBZ, the largest inter-laboratory variance was for Live/dead staining and, to a lesser extent, CD86 and HLA-DR. Of notice, while both WST-1 and Live/dead staining show a rather similar concentration-response as evidenced by concentration-response modelling, the inter-laboratory variance of Live/dead staining is much higher, suggesting that this parameter is much more sensitive to differences between laboratories.

Data from RIVM (Supplementary Information S4A) and the University of Liverpool (Supplementary Information S4B) is included.

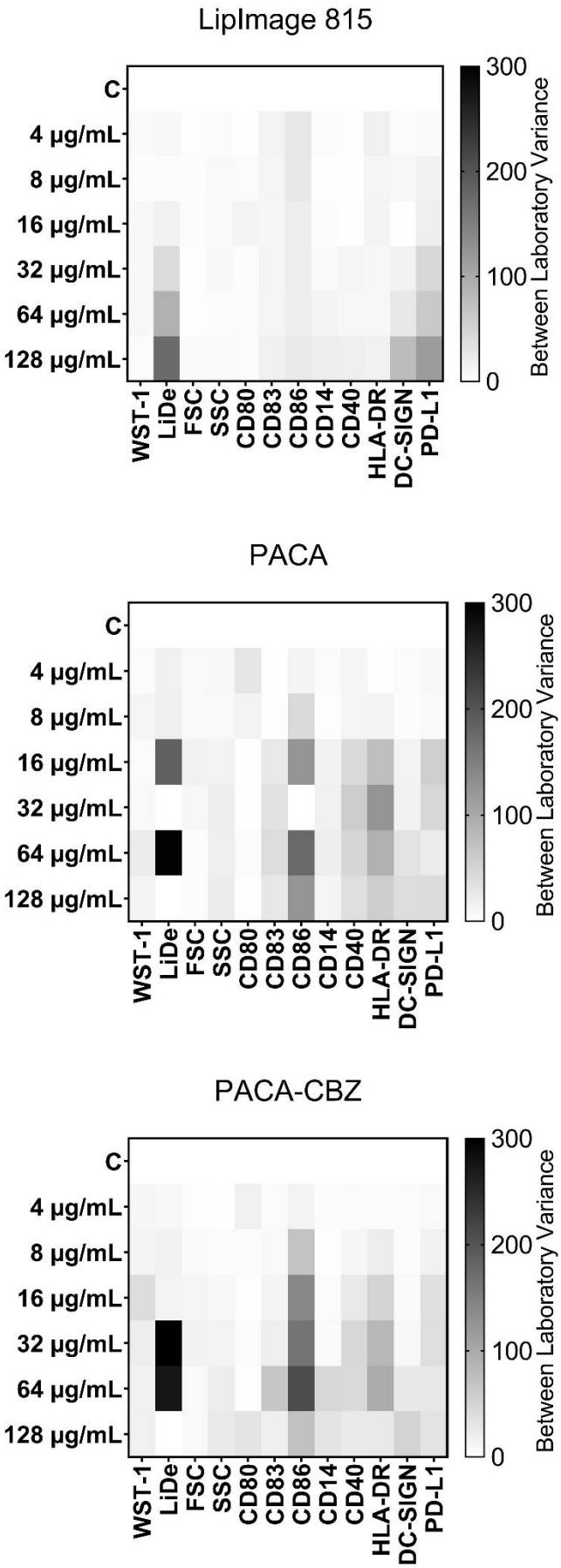

LipImage 815
C
4 µg/mL
8 µg/mL
16 µg/mL
32 µg/mL
64 µg/mL
128 µg/mL
WST-1
LiDe
FSC
SSC
CD80
CD83
CD86
CD14
CD40
HLA-DR
DC-SIGN
PD-L1
300
200
100
0
Between Laboratory Variance
PACA
C
4 µg/mL
8 µg/mL
16 µg/mL
32 µg/mL
64 µg/mL
128 µg/mL
WST-1
LiDe
FSC
SSC
CD80
CD83
CD86
CD14
CD40
HLA-DR
DC-SIGN
PD-L1
300
200
100
0
Between Laboratory Variance
PACA-CBZ
C
4 µg/mL
8 µg/mL
16 µg/mL
32 µg/mL
64 µg/mL
128 µg/mL
WST-1
LiDe
FSC
SSC
CD80
CD83
CD86
CD14
CD40
HLA-DR
DC-SIGN
PD-L1
300
200
100
0
Between Laboratory Variance

Fig. 7 Heat maps of the inter-laboratory variance for each of the three pharmaceuticals tested. For each of N = 3 independent experiments, the CCM control (C) was set at 100% and the mean and standard deviation was calculated for these experiments. This was done for each laboratory, after which the variance between the two laboratories was calculated and expressed in a heat map on a scale of 0-300.

Discussion

Here we evaluated the effects of two nanomedicines, representing nanostructured lipid carriers and polymers, on two *in vitro* assays. These assays, NLRP3 inflammasome activation and DC maturation, are among the ones listed to fulfil the information requirements for regulatory acceptance of nano-pharmaceuticals but are still remote from being a standardised assay [5]. Moreover, with a view to possible future standardization and regulatory application, these assays were subject to an inter-laboratory comparison study, using common SOPs. To this end, one laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. –Ttwo laboratories each performed ~~each of the assays in~~ three independent DC maturation experiments. While the nanostructured lipid nanocarrier only showed marginal effects, the polymers showed major cytotoxicity. No evidence for NLRP3 inflammasome activation or DC maturation was demonstrated. Intra- and inter-laboratory comparison showed clearly reproducible results.

NLRP3 inflammasome activation evaluated by RIVM showed a slight decrease in viability upon exposure to LipImage™ 815 and a sharp decrease in viability upon exposure to PACA and PACA-CBZ. This observation is underlined by concentration-response modelling that showed concentration-dependent cytotoxicity for PACA and PACA-CBZ but not LipImage™ 815. Next, this modelling showed a similar EC_{30} for PACA and PACA-CBZ suggesting that the cytotoxicity observed is caused by PACA and not CBZ. Moreover, the data show a high intra-laboratory reproducibility. Evaluation by the University of Liverpool showed similar results to those obtained by RIVM. No effect on IL-1β production was seen in either laboratory. This, ~~together with similar results in ASC- or NLRP3-deficient cells compared to wild type cells~~ suggests that that neither of the nanomedicines induced NLRP3 inflammasome activation. In any case, intra- and inter-laboratory reproducibility seem to warrant subsequent steps to standardisation of the assay. In such future studies, nanoparticles well-known to activate the NLRP3 inflammasome should be included, such as SiO_2 nanoparticles [21].

Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3 activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death.

In a separate inter-laboratory comparison study within the REFINE project the cytotoxicity of the same batches of the three materials LipImage™815, PACA, and PACA-CBZ was tested in four different cell lines using both the WST-8 and the LDH release assay. LipImage™ 815 was non-cytotoxic up to a concentration of 128 µg/ml, whereas PACA caused dose-dependent cytotoxic effects starting from 8 µg/ml. PACA-CBZ showed a less pronounced dose-dependent effect with the lowest concentration of 2 µg/mL causing cytotoxic effects (Eder et al. submitted for publication).

Outside the inter-laboratory comparison study presented here, but within the REFINE project and using the same SOP as in the study presented here, NLRP3 inflammasome activation by a commercially available liposome (Avanti, Birmingham, AL) was evaluated. The REFINE partners RIVM and CEA each performed 3 independent replicate experiments. Both partners did not observe effects on viability and IL-1β production over the entire concentration range tested (up to of 128 µg/ml) with an appropriate response by the positive control nigericin (Supplementary Information S5~~data not shown~~). This data suggests a lack of NLRP3 inflammasome activation by the liposome.

The DC maturation assays performed by RIVM showed no effect on viability of LipImage™ 815, whereas a clear~~sharp~~ decrease in viability by~~of~~ PACA and PACA-CBZ was seen. These findings are underlined by concentration-response modelling that showed concentration-dependent cytotoxicity for PACA and PACA-CBZ but not LipImage™ 815. Next, this modelling showed a 1.5-fold lower EC_{30} for PACA compared to PACA-CBZ, suggesting that the cytotoxicity observed is caused by PACA and not CBZ. These findings were similar for the WST-1 assay and Live/dead-staining, two orthogonal methods to assess cell viability. DC maturation evaluated by the University of Liverpool showed similar results: cytotoxicity induced by PACA and PACA-CBZ but not LipImage™ 815, a 1.5-fold lower EC_{30} for PACA compared to PACA-CBZ, and similar results for the WST-1 assay and Live/dead-staining. It should be noted, however, that for Live/dead staining the EC_{30} values themselves were rather different between RIVM and the University of Liverpool. For PACA and PACA-CBZ, ~~limiting to the concentration range where no or little cytotoxicity is seen (up to 32 µg/ml),~~ RIVM established as the most sensitive parameter ~~is a~~n increase in HLA-DR, seen from 16 and 32 µg/ml, respectively. ~~decrease in FSC and SSC, seen from 8-16 µg/ml and 8-32 µg/ml, respectively, as seen by T~~the University of Liverpool established a decrease in FSC and SSC as most sensitive parameters, seen from 16 and 32 µg/ml, respectively. ~~A decrease in FSC (from 32 µg/ml and 64 µg/ml, respectively), induction of CD14 expression (from 32 µg/ml and 64 µg/ml, respectively), CD40 expression (from 32 µg/ml and 64 µg/ml, respectively), and HLA-DR expression (from 16 µg/ml and 32 µg/ml, respectively) was seen by RIVM.~~ Although CD40 and HLA-DR are regarded as DC maturation markers, thi~~sese~~ data, especially the lack of effect on CD80, CD83, and CD86 expression, ~~do not~~ suggests that neither of the nanomedicines induce DC maturation. In any case, intra- and inter-laboratory reproducibility seem to warrant subsequent steps to standardisation of the assay. In such future studies, nanoparticles well-known to induce DC maturation should be included, such as TiO_2 nanoparticles [22].

The heat map shows a considerable difference in Live/dead staining between the two participating laboratories, whereas for WST-1 this difference was limited. This suggest that currently, the WST-1 assay, being the only assay not included in the flow cytometry measurement, should remain to be included in the evaluation of effects on DC maturation.

Flow cytometry is a powerful method for immune cell phenotyping. It is routinely used in clinical immunology laboratories around the world. Moreover, OECD guidelines and ISO standards include the use of flow cytometry, such as the h-CLAT [23]. Still, some of the flow cytometry characteristics may hamper acceptance by regulatory authorities of assays that rely on this method. After data collection, compensations are required to correct for the overlap between adjacent emission spectra of different fluorochromes. Next, to select a specific population of cells serial gating is required, which is done by visual inspection of 2D scatterplots. Both compensation and serial gating are often done manually and may differ between operators. Especially manual gating is subjective, not only because gate setting can be more or less strict, but also the sequence of gating to arrive at the desired cell population may

differ [24]. A promising way out is the use of computational flow cytometry, reviewed by Saeys et al. [24] and more recently by Lucchesi et al. [25].

Conclusions

An inter-laboratory comparison study was performed for two assays, NLRP3 inflammasome activation and~~or~~ DC maturation, using two nanomedicines, the nanostructured lipid carrier LipImage™ 815 and the polymer PACA, either loaded or not with CBZ. PACA and PACA-CBZ showed clear cytotoxicity whereas LipImage™ 815 did not. Neither of the nanomedicines induced NLRP3 inflammasome activation or DC maturation. Intra- and interlaboratory reproducibility seem to warrant subsequent steps to standardisation of these assays. In such future studies, nanoparticles well-known to activate the NLRP3 inflammasome resp. induce DC maturation should be included.

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Authors' contributions

RJV: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; CAWD: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing; JPV: Data curation, Investigation, Methodology, Validation, Writing – review & editing; NJL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing

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Data availability

Data are available upon request to the corresponding author (Rob J. Vandebriel, rob.vandebriel@rivm.nl).

Compliance with ethical standards

Competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethics approval and consent to participate

The use by RIVM of buffy coats for the current study was authorised by Sanquin (Amsterdam, the Netherlands) under agreement NVT0243.02. The use of, primary, human blood and blood products at the University of Liverpool is covered under Research Ethics Committee (REC) approval 2056.

Consent for publication

Written informed consent for publication was obtained from all authors.

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An inter-laboratory comparison of an NLRP3 inflammasome activation assay and dendritic cell maturation assay using a nanostructured lipid carrier and a polymeric nanomedicine, as exemplars

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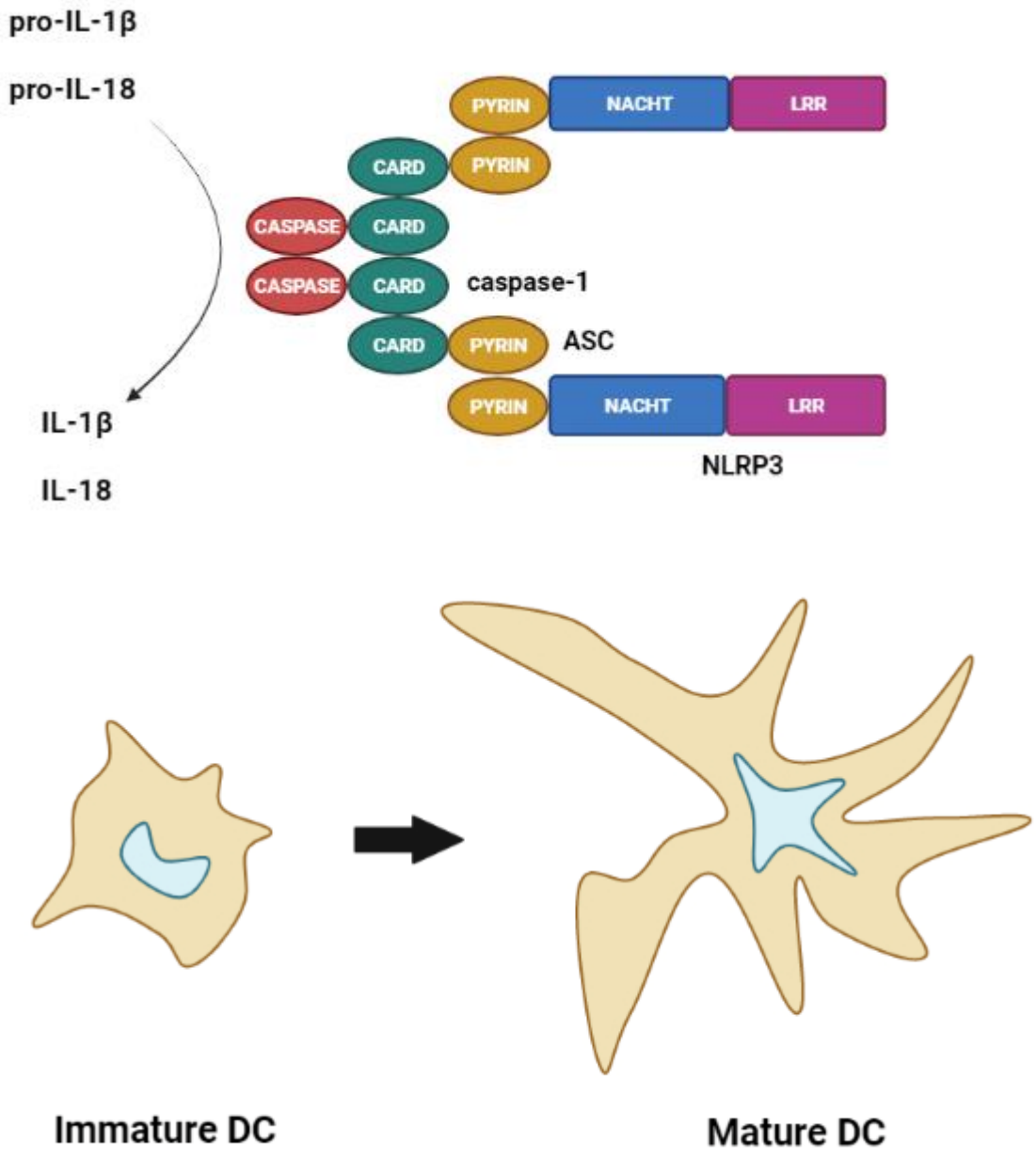
Abstract

Nanoparticles including nanomedicines, are known to be recognised by and interact with the immune system. As these interactions may result in adverse effects, for safety evaluation the presence of such interactions needs to be investigated. Nanomedicines in particular should not unintendedly interact with the immune system, since patient's exposure is not minimised as in the case of "environmental" nanoparticles, and repeated exposure may be required. NLRP3 inflammasome activation and dendritic cell (DC) maturation are two types of immune mechanisms known to be affected by nanoparticles including nanomedicines. NLRP3 inflammasome activation results in production of the pro-inflammatory cytokines IL-1β and IL-18, as well as a specific type of cell death, pyroptosis. Moreover, chronic NLRP3 inflammasome activation has been related to several chronic diseases. Upon maturation, DC activate primary T-cells; interference with this process may result in inappropriate activation and skewing of the adaptive immune response. Here we evaluated the effect of two nanomedicines, representing nanostructured lipid carriers and polymers, on these two assays. Moreover, with a view to possible future standardization and regulatory application, these assays were subject to an inter-laboratory comparison study using common SOPs. One laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. Two laboratories each performed three independent DC maturation experiments. While the nanostructured lipid carrier only showed marginal effects, the polymers showed major cytotoxicity. No evidence for inflammasome activation or DC maturation was demonstrated. Intra- and inter-laboratory comparison showed clearly reproducible results.



Graphical Abstract

Keywords

NLRP3 inflammasome activation; monocyte-derived dendritic cell; dendritic cell maturation; nanostructured lipid carrier; polymeric nanomedicine; inter-laboratory comparison

Introduction

Nanoparticles (NP) are known to interact with the immune system [1]. This also holds for nanomedicines [2]. Generally, effects on the immune system can be regarded as detrimental as it disturbs the intricate homeostasis of the system. Especially nanomedicines should not interact with the immune system, since patients are intentionally exposed and often so for a prolonged period. The degree and nature of NP interaction with the immune system depends on the NP's characteristics [3-4]. However, the relationship between these is still not completely understood meaning that prediction of effects on the immune system from these characteristics is limited. A series of (preferably *in vitro* and high throughput) assays is therefore required, to establish possible effects on the immune system. In a recent analysis we, among others, listed immune system endpoints for which, however required by regulatory authorities, no generally accepted assays exist [5]. From this list of endpoints two assays, measuring different immune mechanisms, are the subject of the current study. Both mechanisms are known to be affected by nanomaterials and are linked to adverse immune effects [6-8].

The NLR family, pyrin domain-containing 3 (NLRP3) inflammasome consists of a NLRP3 scaffold, an apoptosis-associated speck-like protein containing a CARD (ASC) adaptor, and pro-caspase-1. Upon activation, NLRP3 recruits ASC. ASC then binds to pro-caspase-1, resulting in auto-cleavage of this pro-enzyme to become the active enzyme caspase-1. Caspase-1 processes pro-IL-1β and pro-IL-18 to bioactive IL-1β and IL-18, respectively [9]. Please refer to this publication also for a clear representation of the pathways involved. These cytokines are potent mediators of inflammation. Next to host-derived molecules and a multitude of infectious agents [9], the NLRP3 inflammasome can be induced by a wide range of xenobiotics including NP [6]. Its activation is associated with various inflammatory diseases, including lung fibrosis, obesity and type-2 diabetes [7].

Dendritic cells (DCs) are sentinel cells that are pivotal in the initiation of adaptive immune responses [10]. Moreover, they integrate various stimuli, such as from different pathogen-associated molecular patterns (PAMPs) and the cytokine milieu. PAMPs are detected by pattern recognition receptors (PRRs) that are highly expressed by DCs. Important classes of PRR form the Toll-like receptors and Nod-like receptors. Importantly, the nature of the immune response following DC maturation is significantly influenced by the PRR (or combination of different PRR). In this way, DCs form an important link between the innate and adaptive immune response. DCs appear as immature DCs that are well capable of ingesting protein antigens and as mature DCs that are especially capable of presenting peptides to naive T cells. This process of DC maturation is central to the functioning of DC. Various types of NP can influence the process of DC maturation and by that immune function [8]. DC maturation can be readily measured by cell surface marker expression and cytokine production. The panel of cell surface markers used to measure DC maturation generally comprises HLA-DR (MHC class II) and the T-cell co-receptors CD40, CD80, and CD86, although additions to this panel such as CD83, PD-L1 and DC-SIGN, are sometimes included, while in other studies the panel used is more limited. The cytokines measured to evaluate DC maturation are mostly IL-12p40 or IL-12p70, but also IL-10 and TNF-α.

Although DC harbour a fully functional NLRP3 inflammasome, for the NLRP3 inflammasome activation assay we chose to use macrophages derived from THP-1 human monocytes, since (1) the NLRP3 inflammasome is more strongly expressed in macrophages compared to DC, (2) a monocyte cell line

is likely to provide more reproducible data than primary monocytes, and (3) there is no possible interference by DC maturation on NLRP3 inflammasome activation. DC obtained from cell lines have limited functionality compared to those obtained from primary monocytes, justifying primary monocytes as a source for the DC maturation assay.

Here we present the results of an inter-laboratory comparison study of two assays, NLRP3 inflammasome activation (using macrophages derived from THP-1 monocytes), and DC maturation (using DC derived from primary monocytes). The assays were performed by two laboratories using common SOPs. While RIVM performed three independent replicate experiments of the NLRP3 inflammasome activation assay, the University of Liverpool performed one experiment. RIVM and the University of Liverpool each performed the DC maturation assay in three independent replicate experiments. Two types of nanomedicines were tested: the nanostructured lipid carrier LipImage™ 815, and a nanocarrier composed of poly (alkyl cyanoacrylate) polymer. The latter was tested both empty and loaded with Cabazitaxel.

Materials and methods

Nanomedicines

Two types of nanomedicines were tested: (i) the nanostructured lipid carrier LipImage™ 815 [11], (ii) the nanocarrier composed of the poly (alkyl cyanoacrylate) (PACA) polymer: poly (2-ethylbutyl cyanoacrylate) (PEBCA). PEBCA was tested both empty and loaded with Cabazitaxel (CBZ) [12]. In the present paper, these polymer nanocarriers are designated PACA and PACA-CBZ, respectively.

LipImage™ 815 synthesis and characterisation

Batches of LipImage™ 815 were prepared by high-pressure homogenization (HPH). The lipid phase comprised 19.125 g of soybean oil, 6.375 g of Suppocire™ NB, 4.875 g of lecithin, and 150 mg of IR-870 oleyl (molar mass: 986.29 g/mol), which was synthetized as previously described [11]. The aqueous phase comprised 25.875 g of Myrj™ S40 and 110 ml NaCl 154 mM. Mixtures of lipid and aqueous phases were pre-emulsified using a mechanical disperser (Ultra-T25 Digital Turrax, IKA) operated at 15,000 rpm for 5 minutes. The emulsion was then processed with a High-Pressure Homogenizer (Panda Plus 2000, GEA Niro Soavi, Italy) operated for 16 cycles with a total pressure of 1250 bars, the pressure of the second stage chamber being set at 50 bars and the cooling system at 30°C. Batches of 200 g of particles were then purified by 5 µm filtration followed by tangential flow filtration (Labscale TFF system, Millipore) against NaCl 154 mM through a Pellicon XL Biomax™ cassette (Merck) operated at a trans-membrane pressure of 1 bar at a flow rate of 2 ml/min. The nanoparticle dispersion was adjusted to a concentration of 100 mg/ml and filtered through a 0.22 µm Millipore membrane for sterilization before storage and use.

Dynamic light scattering (DLS) was used to determine the particle hydrodynamic diameter and zeta potential (Zeta Sizer Nano ZS, Malvern Instrument, Orsay, France). Particle dispersions were diluted to 2 mg/ml of lipids in 0.22 µm filtered 0.1 X PBS and transferred in Zeta Sizer Nano cells (Malvern Instrument) before each measurement, performed in triplicate. Results (Z-average diameter, dispersity index, ζ-potential) were expressed as mean and standard deviation of three independent measurements performed at 25 °C. The encapsulation efficiency and payload of IR780-oleyl dye in the

LipImage™ 815 were determined by high-performance liquid chromatography (HPLC WATERS Alliance 2695 / Fluorescence 2475 detector) and compared with a calibration curve established from the reference fluorophore IR780-Oleyl alone, as previously described [13]. The theoretical amount of IR780-Oleyl encapsulated in a batch of LipImage™ 815 at 100 mg/ml lipid nanoparticles is 266 µM. The size, polydispersity index, ζ-potential, and dye loading of LipImage™ 815 is shown in Table 1.

PACA synthesis

PACA nanoparticles were synthesized under aseptic conditions at SINTEF (Trondheim, Norway) by mini-emulsion polymerization. Prior to synthesis, all solutions were sterile filtered, and all equipment was autoclaved. An oil phase consisting of poly(ethyl butyl cyanoacrylate) (PEBCA) (Cuantum Medical Cosmetics) containing 2 wt % Miglyol 812 (Cremer) and 10 wt % vanillin was prepared. For drug-loaded particles, 12 wt % CBZ (Shanghai Biochempartner Co., Ltd. (Shanghai, China).) was added to the oil phase and only 2 wt % vanillin was used. For dye-loaded particles, either 0.4 wt % IR-780-Oleyl (custom synthesis at CEA LETI) or NR668 (modified Nile Red, custom synthesis at SINTEF [14]) was added to the oil phase.

An aqueous phase consisting of 0.1 M HCl containing the two PEG stabilisers (Brij®L23 and Kolliphor®HS15, both Sigma-Aldrich, 5 wt % of each) was added to the oil phase. The water and oil phases were mixed and immediately sonicated for 3 min on ice (6 × 30 s intervals, 60% amplitude, Branson Ultrasonics digital sonifier). The solution was rotated (15 rpm) at room temperature (RT) overnight. The pH was then adjusted to 5.0 to allow further polymerisation at RT for 5 hours. The dispersions were dialyzed (Spectra/Por dialysis membrane MWCO 100.000 Da) against 1 mM HCl to remove unreacted PEG. The size (z-average), polydispersity index (PDI) and the ζ-potential of the NPs in phosphate buffer (10 mM, pH 7.0) were measured by DLS and laser Doppler Micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments).

To calculate the amount of encapsulated drug, the drug was extracted from the particles by dissolving them in acetone (1:10), and quantified by liquid chromatography coupled to mass spectrometry (LC-MS/MS) using an Agilent 1290 HPLC system coupled to an Agilent 6490 triple quadrupole mass spectrometer. The size, polydispersity index, ζ-potential, and drug loading of PACA and PACA-CBZ is shown in Table 1.

	Size (nm)	PDI	ζ-pot (mV)	Drug loading (wt %)	Dye loading (wt %)
LipImage™815	53 ± 1	0.15	-1.5 ± 1	-	0.35%
PACA	136.2	0.11	-4.8	-	-
PACA-CBZ	121.8	0.14	-5.5	10.8%	-

Table 1. Size, polydispersity index, ζ-potential, and dye and drug loading of the nanomedicines tested. Characteristics as measured by the producers CEA and SINTEF.

NLRP3 inflammasome activation

Cell line maintenance

THP-1 cells (ATCC TIB-202) were cultured in complete cell culture medium (CCM), that is: RPMI 1640 (Gibco) supplemented with foetal calf serum (10% v/v, Greiner-Bio), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco). The cells were sub-cultured twice per week, seeded to a cell density of $2x10^5$ cells/ml, and not allowed to grow to a density beyond $1x10^6$ cells/ml. Cells were not cultured for more than twenty passages to prevent genetic divergence.

Differentiation of THP-1 cells

The THP-1 cells were differentiated into macrophage-like cells by culturing for 3 hours in the presence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) in 96-well format at a cell density of $5x10^5$ cells/ml, 100 µl/well. After this incubation the cells were adherent. The cells were visually inspected for macrophage-like appearance. The medium was replaced with fresh culture medium without PMA and the plates were incubated for 24 hours at standard conditions (humidified incubator at 37°C, 5% CO2). After this incubation period, the cells were exposed to a two-fold dilution series of LipImage™ 815, PACA, or PACA-CBZ (2, 4, 8, 16, 32, 64, and 128 µg/ml), for 48 hours at standard conditions. As positive control for NLRP3 inflammasome activation, nigericin (InvivoGen) was used (0.625 and 1.25 µg/ml). CCM was used as negative control. Cells were used for viability testing; culture supernatants were frozen at -80°C until further use (ELISA).

Viability of THP-1 cells

The viability of the cells after exposure was assessed using the cell proliferation reagent WST-1 (Sigma-Aldrich). Exposed cells (and controls) were incubated for 2.5 hours under standard conditions in the presence of 10% (v/v) WST-1 reagent. After incubation, the absorbance (A) was measured in each well at 440 nm (A_{440}) and corrected for background absorbance at 620 nm (A_{620}). Exposures for viability assessment were performed in triplicate and the viability was calculated as follows: (A (cells in medium, X) – A (medium only, X))/A (cells in medium, C) – A (medium only, C), where X is a specific concentration nanomedicine or positive control and C the CCM control. The viability was expressed as percentage of the control. As a control, for each nanomedicine at the highest exposure concentration (in CCM), the A_{440}-A_{620} signal was measured and found not to interfere with the read-out signal of the WST-1 assay.

IL-1β ELISA

The IL-1β concentrations in the culture supernatant were determined using ELISA (eBioscience) according to the manufacturer's instructions. An 8-point, 2-fold dilution series of a cytokine standard was prepared, diluent was used as blank. A calibration curve was calculated using 5-parameter curve fitting. Exposures for the assessment of IL-1β secretion were performed in four wells per condition. The supernatants were tested in a 2-fold dilution, except nigericin (0.625 µg/ml in a 5-fold dilution and 1.25 µg/ml in a 30-fold dilution) to stay within the standard curve concentration range.

Concentration-response modelling

Concentration-response modelling for viability and IL-1β production was performed with the statistical software package PROAST [15] (version 70.3) within the software environment 'R' [16] (version 4.1.0).

In this approach, a concentration-response dataset is evaluated as a whole by fitting a concentration-response model over the entire concentration range studied. Having fitted a concentration-response model to the data, this curve is used to assess the benchmark concentration (BMC) associated with the benchmark response (BMR) of 50%. The choice of the model for deriving the BMC follows from a procedure of applying likelihood ratio tests to the five members of the following two nested families of models:

Exponential family	Hill family
E1: $y = a$	H1: $y = a$
E2: $y = a \exp(b\,x)$	H2: $y = a\,(1 - x / (b + x))$
E3: $y = a \exp(b\,x^d)$	H3: $y = a\,(1 - x^d / (b^d + x^d))$
E4: $y = a\,(c - (c - 1)) \exp(b\,x)$	H4: $y = a\,(1 + (c - 1)\,x / (b + x))$
E5: $y = a\,(c - (c - 1)) \exp(b\,x^d)$	H5: $y = a\,(1 + (c - 1)\,x^d / (b^d + x^d))$

where y is any continuous endpoint and x denotes the concentration. In these models, the parameter "a" represents the background response and the parameter "b" can be considered as the parameter reflecting the efficacy of the chemical (or the sensitivity of the subject). First, the likelihood ratio test was used to establish whether extension of a model by increasing the number of parameters resulted in a statistically significant improvement of the fit. The model that could not be significantly improved was considered as the most appropriate member (which adequately fits but does not overfit the data) within each family. In addition, a goodness of fit test ($P > 0.05$) was applied by comparing the log-likelihood of the fitted model to that associated with the so-called "full model." The full model simply consists of the observed (mean) responses at each applied concentration. The model is accepted when the log-likelihood value of the fitted model is not significantly worse than that of the full model. Subsequently, the BMCs are derived from the different models and the 90% confidence intervals (CIs) surrounding the BMCs are calculated using the profile-likelihood method. The BMC used in the analysis was the geometric average of the BMCs derived for the different models. The 90% CI surrounding this BMC comprised the BMCL and the BMCU found for the BMC estimates derived from the different models.

The Standard Operating Procedure is included (Supplementary Information S1).

Dendritic cell maturation

Isolation of $CD14^+$ cells

Human buffy coats were purchased from the Dutch blood bank (Sanquin, Amsterdam) and obtained the night before isolation of the cells. They were kept at RT until starting cell isolation the next morning. At the University of Liverpool, blood was obtained from healthy volunteers on the day of the experiment. The buffy coat was diluted 1:1 with PBS. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat by centrifugation (1000g, 30 min, 20°C) over Ficoll (Lymphoprep; Axis Shield, Oslo, Norway). After washing with PBS, red blood cells were lysed by resuspending the cell pellet in ACK buffer (156 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.3) and a subsequent wash with PBS. $CD14^+$ cells were positively selected from the cell suspension using a magnetic-activated cell sorting (MACS) kit with CD14-specific antibodies (MACS, Miltenyi Biotec, Leiden, the Netherlands) according to the manufacturer's instructions. To achieve a high purity, a lower amount of antibody was used than recommended (5 µl per 10^7 cells). The positive and negative fractions from the MACS were analysed by flow cytometry to determine $CD14^+$ purity (see Table 2 for antibody panel).

Marker	Label	Dilution	Manufacturer
CD14	PE	1:50	Becton Dickinson
Live/dead	Aqua	1:400	Invitrogen

Table 2: Antibody panel for the assessment of $CD14^+$ purity of cell samples by flow cytometry.

Staining was done in two consecutive steps. First, the cells were stained with Live/dead (in PBS, 0.2 mM EDTA) at 4 °C for 30 min. Second, the cells were washed with FACS buffer (PBS pH 7.2, 0.2 mM EDTA, 0.5% BSA), and stained with anti-CD14 antibody in FACS buffer at 4 °C for 30 min.

The $CD14^+$ cell fraction was resuspended in complete culture medium (CCM): RPMI 1640 GlutaMAX (Gibco), 10% Foetal Calf Serum (FCS; Hyclone; GE Healthcare), 1% pen/strep (Gibco), 450 U/ml GM-CSF (PeproTech) and 350 U/ml IL-4 (Active Bioscience). For a flow diagram see Fig. 1.

Differentiation of $CD14^+$ cells to immature DC, and exposure to nanomedicines

The $CD14^+$ cells were seeded in 12-well plates, 1 ml/well, $3x10^5$ cells/ml and incubated at 37°C and 5% CO_2 in a humidified incubator. After 3 days, 100 µl RPMI 1640 GlutaMAX containing 10% FCS, 4500 U/ml GM-CSF and 3500 U/ml IL-4 was added to each well to a final concentration of approximately 450 U/ml GM-CSF and 350 U/ml IL-4. After 6 days, 750 µl culture medium was removed and spun down. The pellet was resuspended in CCM and seeded back into the wells with a dilution series of LipImage™ 815, PACA, or PACA-CBZ. LPS (100 ng/ml) and R848 (5 µg/ml) were used as positive controls, 10% PBS as negative control. The plates were placed back in the incubator for 44-48 hours until harvest for analysis. For harvesting, from each well the culture medium was collected and spun down. Each supernatant was individually transferred to a fresh tube and stored at -80°C for ELISA. In the meantime, cold PBS was put on the cells that were attached to the wells. After detaching the cells by gentle scraping and pipetting, they were collected and added to the tube in which already part of the cells was collected. These cells were divided over two wells for staining with the two separate antibody panels.

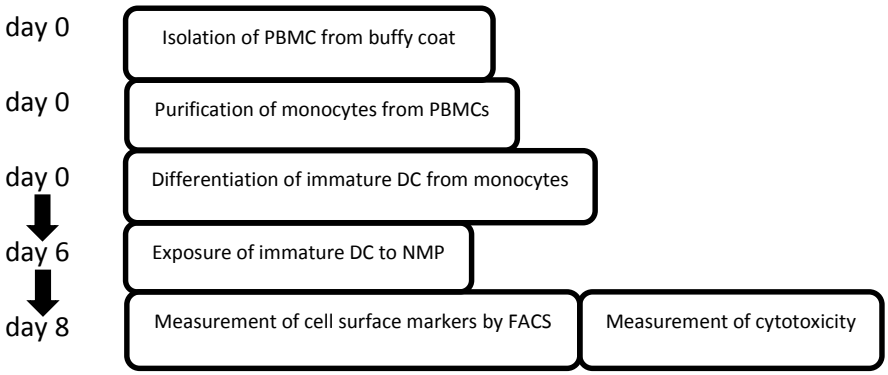


Fig. 1: Flow diagram of DC maturation assay.

Flow cytometric analysis of cultured cells

Maturation of the DCs was assessed by flow cytometry (FACS) using two antibody panels (Table 3). In addition, Forward Scatter (FSC; a measure of cell size) and Side Scatter (SSC; a measure of internal complexity (i.e. granularity)) were measured.

Panel 1

Marker	Label	Dilution	Manufacturer
CD80	FITC	1:40	Becton Dickinson
CD14	PE	1:50	Becton Dickinson
PD-L1	APC	1:400	eBioscience
HLA-DR	Pacific Blue	1:1000	Biolegend
Live/dead	Aqua	1:1000	Invitrogen

Panel 2

Marker	Label	Dilution	Manufacturer
CD83	FITC	1:20	Becton Dickinson
CD40	PE	1:10	Becton Dickinson
DC-SIGN	APC	1:200	Becton Dickinson
CD86	Pacific Blue	1:800	Biolegend
Live/dead	Aqua	1:1000	Invitrogen

Table 3: Antibody panels used for the assessment of DC maturation by flow cytometry.

First, the cells were washed twice with PBS. Second, the cells were stained with Live/dead in PBS, 0.2 mM EDTA at 4 °C for 30 min. Third, the cells were washed once FACS buffer (PBS pH 7.2, 0.2 mM EDTA, 0.5% BSA). To 100 µl of these cells, 100 µl of panel 1 or panel mix 2 (see Table 3) was added. After incubation at 4 °C for 30 min, the cells were washed twice, spun down, and included in FACS buffer. Data was acquired using the FACS Canto II (Becton Dickinson Biosciences) using the settings: (1) FSC: 150; SSC: 350; PE: 488 nm laser (blue), 585/42 filter; Aqua: 405 nm laser (violet), 510/50 filter. (2) Sample flow rate 3 µl/sec; sample volume 170 µl; mixing volume 70 µl; mixing speed 180 µl; number of mixes 3; washing volume 800 µl. (3) Compensations were set using beads and DC, on a population of 50% living and 50% dead cells. To obtain dead cells, the living cells were heat-shocked.

Data were analysed using FlowJo software (Becton Dickinson). Gating was done according to Fig. 2.

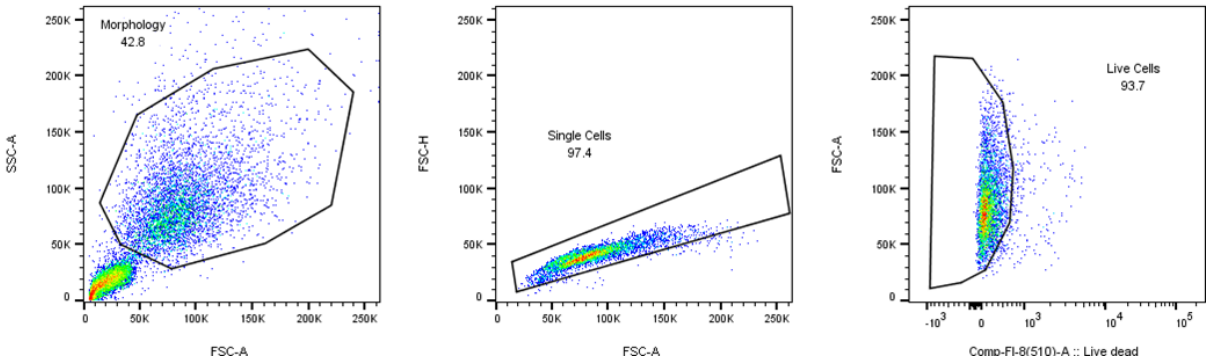


Fig. 2: Gating procedure. (1) Gating was done based on the morphology of the cells (left graph). The lower left corner (<50K FSC-A (X-axis) and <50 K-SSC-A (Y-axis)) is excluded. (2) Within the cell population gated under (1), the single cells were gated (middle graph). In the FSC-A (X-axis) vs. FSC-H (Y-axis) plot, doublet cells form a population below the diagonal. (3) Within the cell population gated under (2), the live cells were gated (right graph). In the Live/dead staining (X-axis) vs. FSC-A (right axis), the dead cells scatter to the right.

Determination of cultured dendritic cell viability

The $CD14^+$ cells were seeded in 96-well plates, 200 µl/well, $3x10^5$ cells/ml and incubated at 37°C and 5% CO_2 in a humidified incubator. The protocol as described above "Differentiation of CD14+ cells to immature DC, and exposure to nanomedicines" was used (and done concurrently). For viability assessment, the protocol as described above "Viability of THP-1 cells" was used.

Concentration-response modelling

The method as described above "Concentration-response modelling" was used.

The Standard Operating Procedure is included (Supplementary Information S2).

Results

Inflammasome activation

RIVM

PMA-activated THP-1 cells were incubated for 48 hr with the positive control nigericin at two concentrations (0.625 and 1.25 µg/ml) and a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, 4, and 2 µg/ml, plus a medium control (0)). After this, viability was evaluated using the WST-1 assay and the IL-1β concentration using an ELISA. The results shown are from three independent experiments (Fig. 3).

The positive control for NLRP3 inflammasome activation, nigericin, showed a strong reduction in viability (25% and 8% of the medium control for the low and high concentration, respectively) and a strong increase in IL-1β production (720 and 3200 pg/ml for the low and high concentration, respectively, where the medium control amounted 110 pg/ml). This data of concentration-dependent

reduction in viability and concomitant concentration-dependent increase in IL-1β production suggests a proper functioning of the NLRP3 inflammasome activation assay.

Exposure to LipImage™ 815 resulted in a 30% decrease in viability at the highest concentration tested (128 µg/ml). A 50% increase in IL-1β production (from 110 pg/ml to 165 pg/ml) was seen at the highest concentration tested. Although a decrease in viability and a concomitant increase in IL-1β production is seen, a hallmark of NLRP3 inflammasome activation, the effects observed are too small to suggest that LipImage™ 815 activates the NLRP3 inflammasome.

Exposure to PACA resulted in a clear decrease in viability within a 2-fold concentration range, from 70% viability at 8 µg/ml to 9% viability at 16 µg/ml. At these same concentrations, only a small increase in IL-1β production was seen, from 50 pg/ml to 70 pg/ml. Exposure to PACA-CBZ also resulted in a clear decrease in viability, albeit within a 4-fold concentration range, from 85% viability at 8 µg/ml, 37% viability at 16 µg/ml, to 8% viability at 32 µg/ml. At these same concentrations, only a small increase in IL-1β production was seen, being 50 pg/ml at 8 µg/ml, and 60 pg/ml at 16 µg/ml and 32 µg/ml. This data suggests a strong cytotoxic effect of PACA, both with and without CBZ, and no evidence for NLRP3 inflammasome activation.

Comparison of the results between the individual experiments shows a high reproducibility, with some quantitative differences in IL-1β production throughout individual concentration-response curves, but a highly similar shape of both the viability and the IL-1β production concentration-response curves.

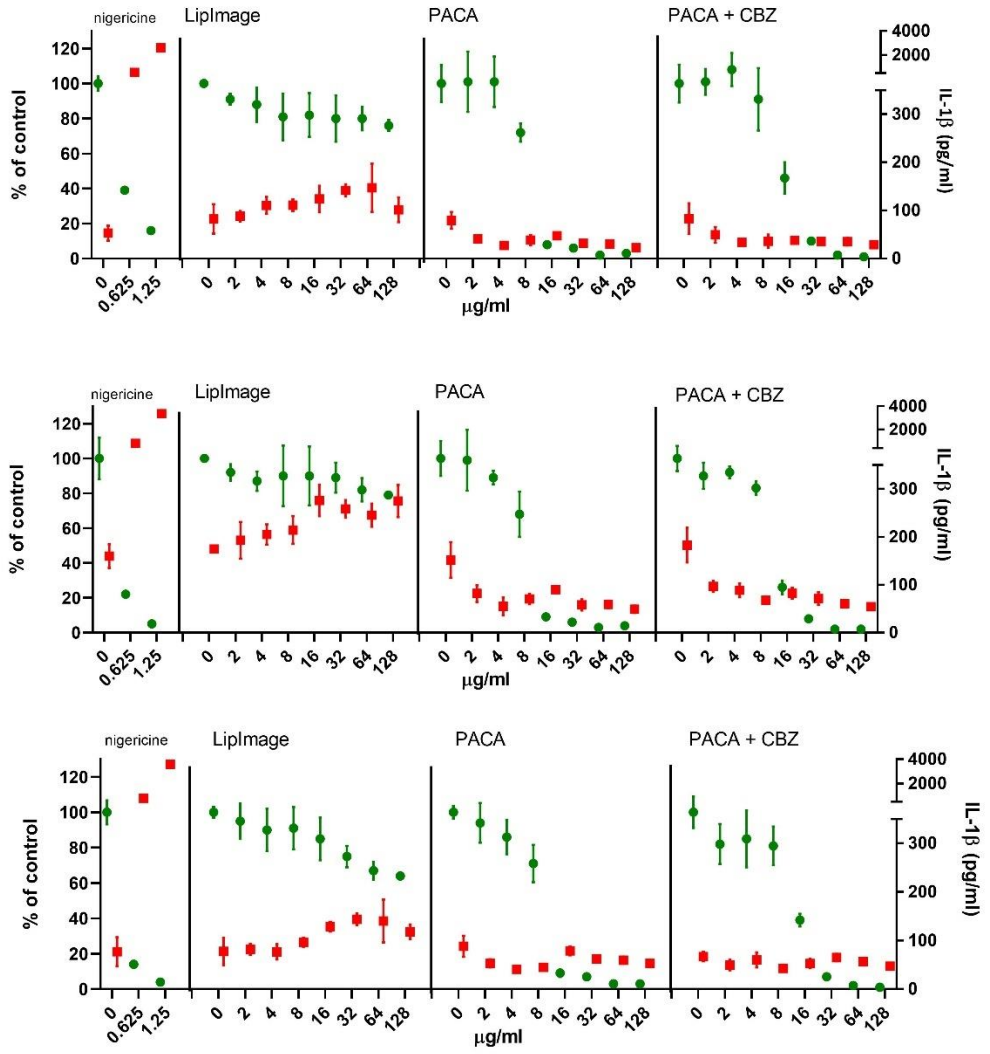


Fig. 3 NLRP3 inflammasome activation by LipImage™ 815, PACA, and PACA-CBZ. Nigericin: positive control. Green (plotted to the left Y-axis): viability (percentage of untreated control). Red (plotted to the right Y-axis): IL-1β production (pg/ml). Three independent experiments are shown. Mean ± SD, with N = 4 replicates per experiment.

Inflammasome activation was also assessed using concentration-response modelling. The three experiments presented in Fig. 3 were analysed (together). Since ISO [17] takes 70% viability as a threshold for cytotoxicity, 30% reduction was chosen as effect size. The concentration at which a 30%

effect is obtained is designated here as the Effective Concentration $(EC)_{30}$. Since no guidance exists on an effect size for markers of NLRP3 inflammasome activation, by default we chose a 30% effect (in this case an increase), similar in size to viability. Next to establishing the EC, the software tool PROAST provides a 90% confidence interval (CI) around a specific EC (here EC_{30}). In Table 4, the EC_{30} values and corresponding 90% CIs are shown.

When an EC_{30} could not be calculated (LipImage™ 815: viability; PACA-CBZ: IL-1β production), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: IL-1β production; PACA: IL-1β production) the data were not considered. The data in Table 3 show that the effects of PACA and PACA-CBZ on viability are highly similar, suggesting that in this assay the reduced viability is only due to PACA and not to CBZ.

	viability	
	EC_{30}	**90% CI**
PACA	8.15	6.31-10.1
PACA-CBZ	8.00	6.41-9.83

Table 4: EC_{30} and 90% CI values of viability (µg/ml).

University of Liverpool

Exposure to LipImage™ 815 resulted in a 26% increase at the highest concentration tested (128 µg/ml). A 10% decrease in IL-1β production was seen at the highest concentration tested (from 50 pg/ml to 45 pg/ml). The effects observed do not suggest that LipImage™ 815 activates the NLRP3 inflammasome.

Exposure to PACA resulted in a clear decrease in viability within a 2-fold concentration range, from 79% viability at 8 µg/ml to 19% viability at 16 µg/ml, so at the same concentrations as seen in the experiments performed at RIVM. At these same concentrations no effect on IL-1β production (48 pg/ml) was seen. Exposure to PACA-CBZ also resulted in a decrease in viability, albeit within a 4-fold concentration range, from 97% viability at 8 µg/ml, 78% viability at 16 µg/ml, to 44% viability at 32 µg/ml. Thus, similar to the findings at RIVM, PACA-CBZ showed a more gradual decrease in viability compared to PACA alone. At these same concentrations, no effect on IL-1β production (48 pg/ml for PACA and 45 pg/ml for PACA-CBZ) was seen. This data suggests a strong cytotoxic effect of PACA, both with and without CBZ, and no evidence for NLRP3 inflammasome activation.

Data from RIVM (Supplementary Information S3A) and the University of Liverpool (Supplementary Information S3B) is included.

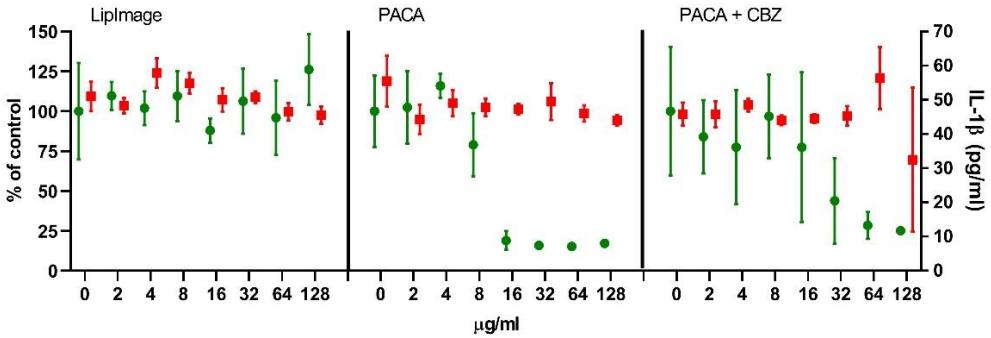


Fig. 4 NLRP3 inflammasome activation by LipImage™ 815, PACA, and PACA-CBZ. Green (plotted to the left Y-axis): viability (percentage of untreated control). Red (plotted to the right Y-axis): IL-1β production (pg/ml). Mean ± SD, with N = 4 replicates per experiment.

Dendritic cell maturation

RIVM

Monocytes were isolated from buffy coats and differentiated to immature DC. These were incubated for 48 hr with a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, and 4 µg/ml, plus a medium control (0)). After this, viability was evaluated using the WST-1 assay and surface marker expression using a FACS. The results shown are from three independent experiments (Fig. 5).

Viability

The positive controls for DC maturation, LPS and R848, did not affect viability. Incubation with LipImage™ 815 marginally affected cell viability, averaging 70% at the highest concentration tested (128 µg/ml). PACA showed a clear decrease in viability within a 4-fold concentration range, from 103% at 32 µg/ml, 14% at 64 µg/ml, and 3% at 128 µg/ml. PACA-CBZ, curiously, showed a biphasic viability curve, averaging 114%, 69%, 14%, 133%, 42%, and 2% for the entire concentration range. It should be noted that the results are obtained from three independent experiments using DCs cultured from monocytes of different donors, performed on different weeks. A complete loss of viability at 16 µg/ml does not fit the viability seen for PACA and PACA-CBZ as evaluated by Live/dead staining (see below), or for PACA and PACA-CBZ in the experiments performed by the University of Liverpool (see below). However, in a study dedicated to evaluate the cytotoxicity of LipImage™ 815, PACA, and PACA-CBZ, in four different cell lines using two different viability assays, both PACA and PACA-CBZ showed a clear reduction in viability from 2 µg/ml, depending on the cell line and the assay (Eder et al. submitted).

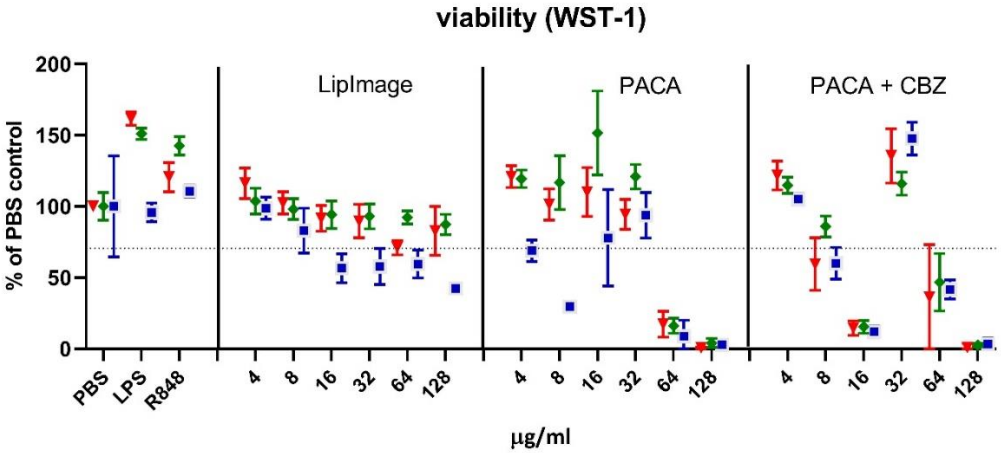


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Fig. 5a Viability of DC after incubation with LipImage™ 815, PACA, and PACA-CBZ. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation.

Live/dead, FSC, and SSC

The positive controls for DC maturation, LPS and R848, had a minor effect on Live/dead staining, and did not affect FSC (a measure of cell size) and SSC (a measure of internal complexity (i.e. granularity)). No exposure effects of LipImage™ 815 on Live/dead staining, FSC and SSC were seen. PACA and PACA-CBZ induced a clear increase in Live/dead staining from 32 µg/ml and from 64 µg/ml, respectively. PACA and PACA-CBZ decreased FSC from 32 and 64 µg/ml, respectively. PACA and PACA-CBZ did not affect SSC.

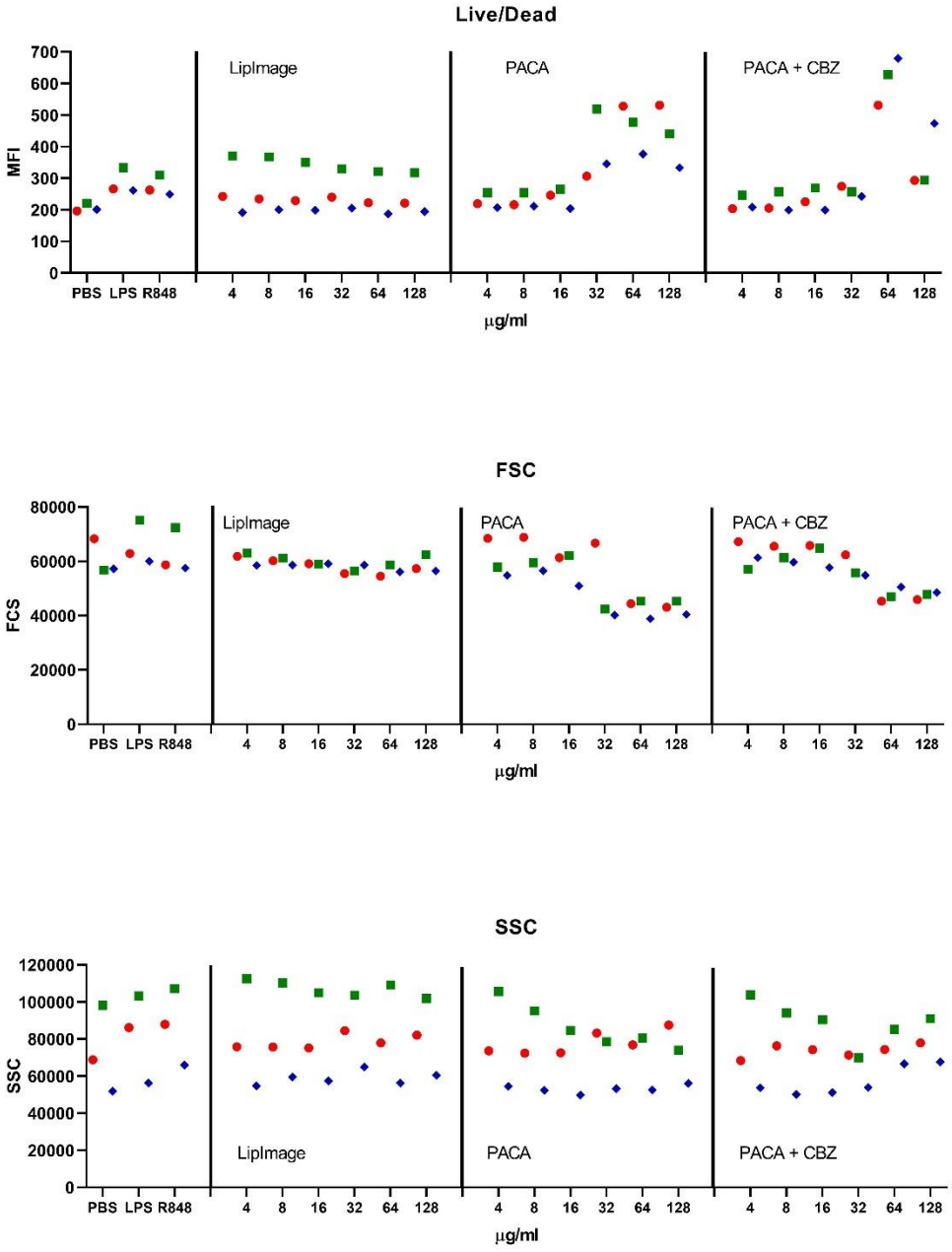

Fig. 5b Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on Live/dead staining, FSC, and SSC. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD80, CD83, and CD86

The positive controls LPS and R848 clearly induced CD80, CD83, and CD86 expression (5- and 3.5-fold for CD80; 7.5- and 6-fold for CD83; 30- and 23-fold for CD86, for LPS and R848, respectively), strongly suggestive of DC maturation. LipImage™ 815, PACA, and PACA-CBZ failed to do so, suggesting that none of the three nanomedicines induced DC maturation.

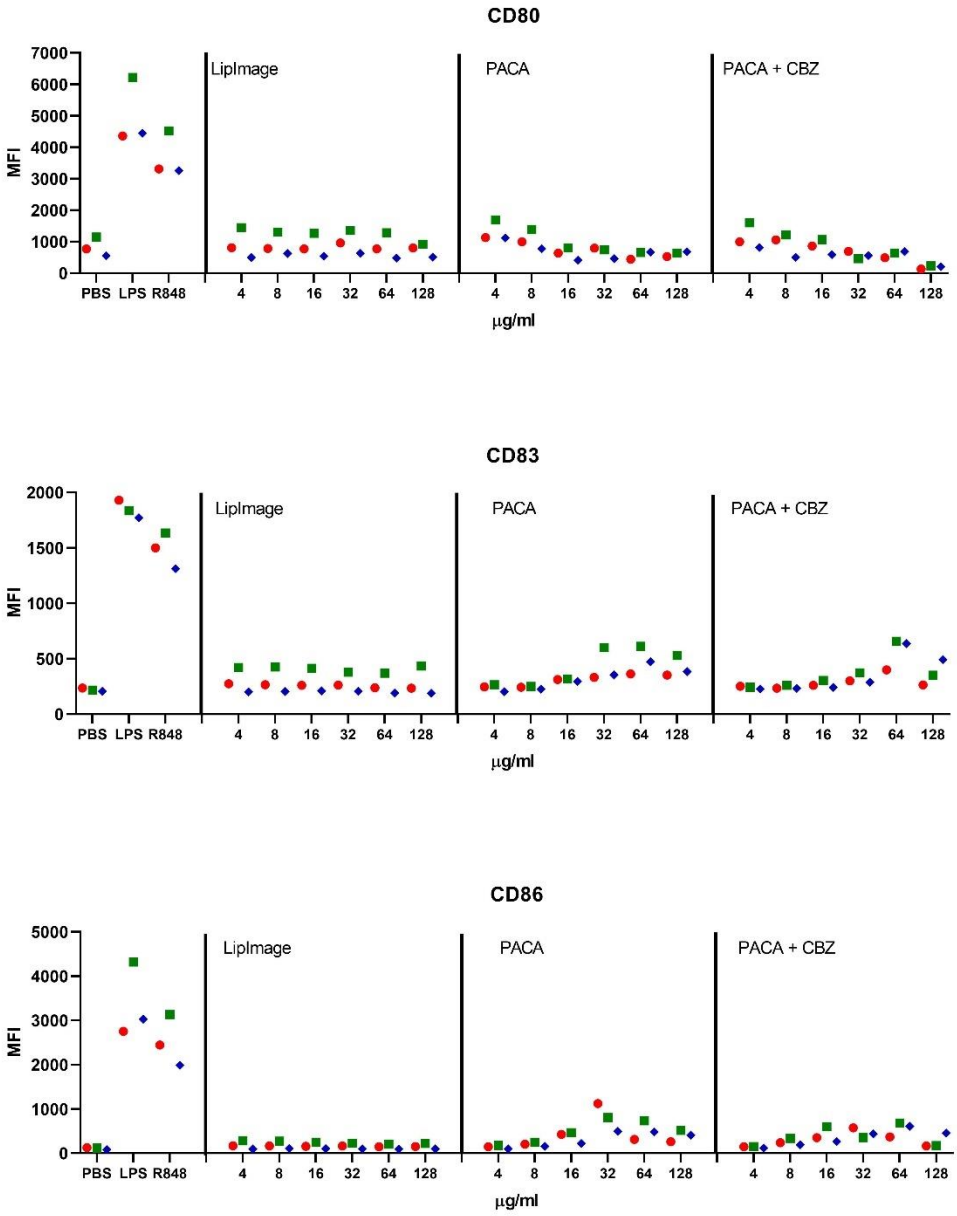

Fig. 5c Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD80, CD83, and CD86 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD14, DC-SIGN, and PD-L1

LPS and R848 rather similarly downregulated CD14 expression, by 40%. CD14 downregulation by LPS is in line with previous data showing combined endocytosis of LPS, TLR4, and CD14 [18]. DC-SIGN expression was reduced by 30% and 20% by LPS and R848, respectively. PD-L1 expression was induced 11-fold and 7-fold by LPS and R848, respectively. Decreased DC-SIGN expression and increased PD-L1 expression both suggest DC maturation. DC-SIGN is typically downregulated in DC upon maturation [19]. PD-L1 is upregulated in DC upon maturation [20].

LipImage™ 815 did not affect CD14 expression. For PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml and 64 µg/ml, respectively. LipImage™ 815 slightly increased DC-SIGN expression at

the highest concentration tested (128 µg/ml), while PACA and PACA-CBZ slightly decreased DC-SIGN expression at this concentration. LipImage™ 815, PACA, and PACA-CBZ did not affect PD-L1 expression, in line with a lack of effect on the maturation markers CD80, CD83, and CD86.

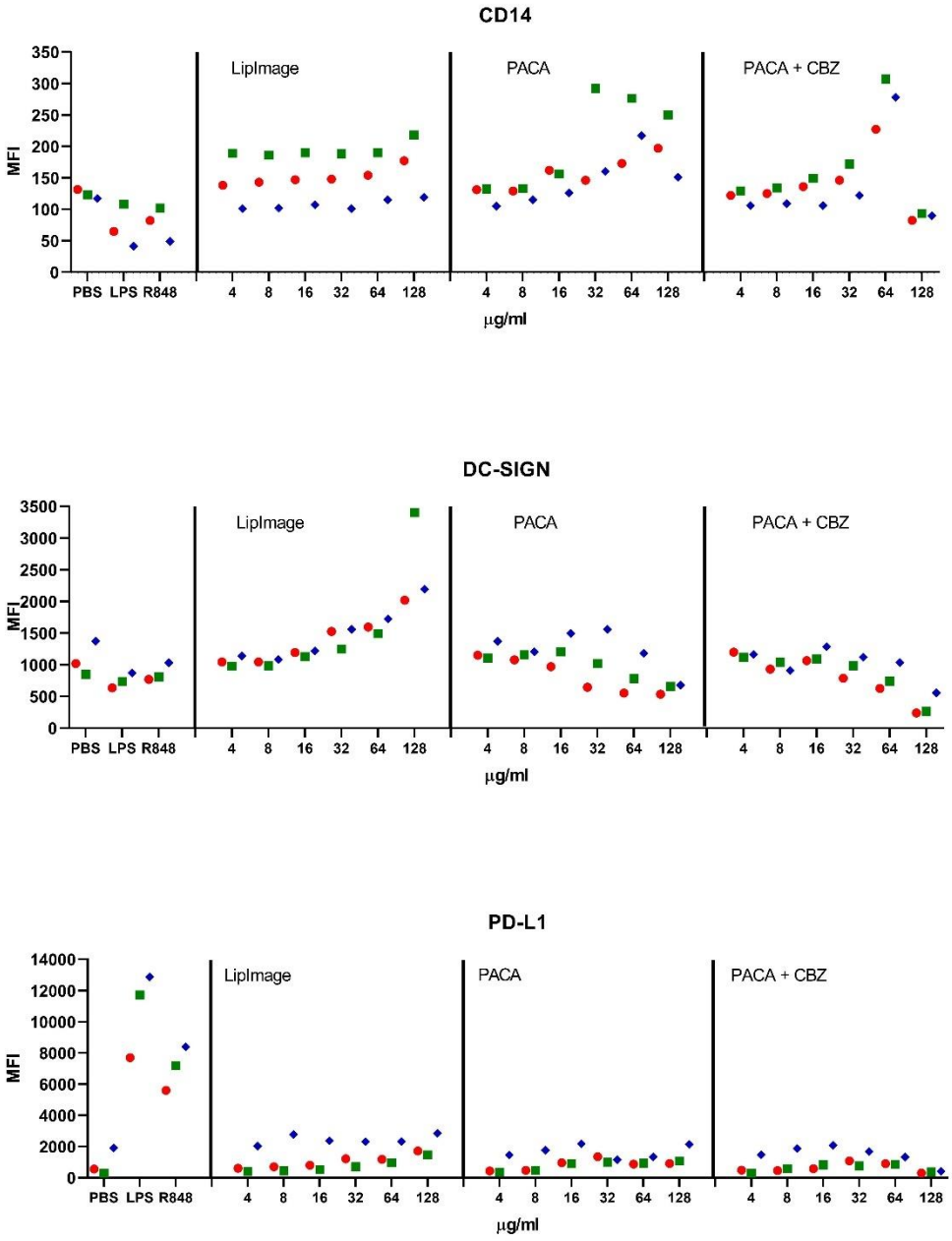

Fig. 5d Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD14, DC-SIGN, and PD-L1 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD40 and HLA-DR

The positive controls LPS and R848 clearly induced CD40 expression (3.5- and 3-fold, respectively) and to a lesser extent HLA-DR expression (1.6- and 2-fold, respectively), suggestive of DC maturation. LipImage™ 815 failed to induce expression of either CD40 or HLA-DR. PACA and PACA-CBZ induced a

2-fold CD40 expression from 32 µg/ml and 64 µg/ml, respectively, and a 2-fold HLA-DR expression from 16 µg/ml and 32 µg/ml, respectively.

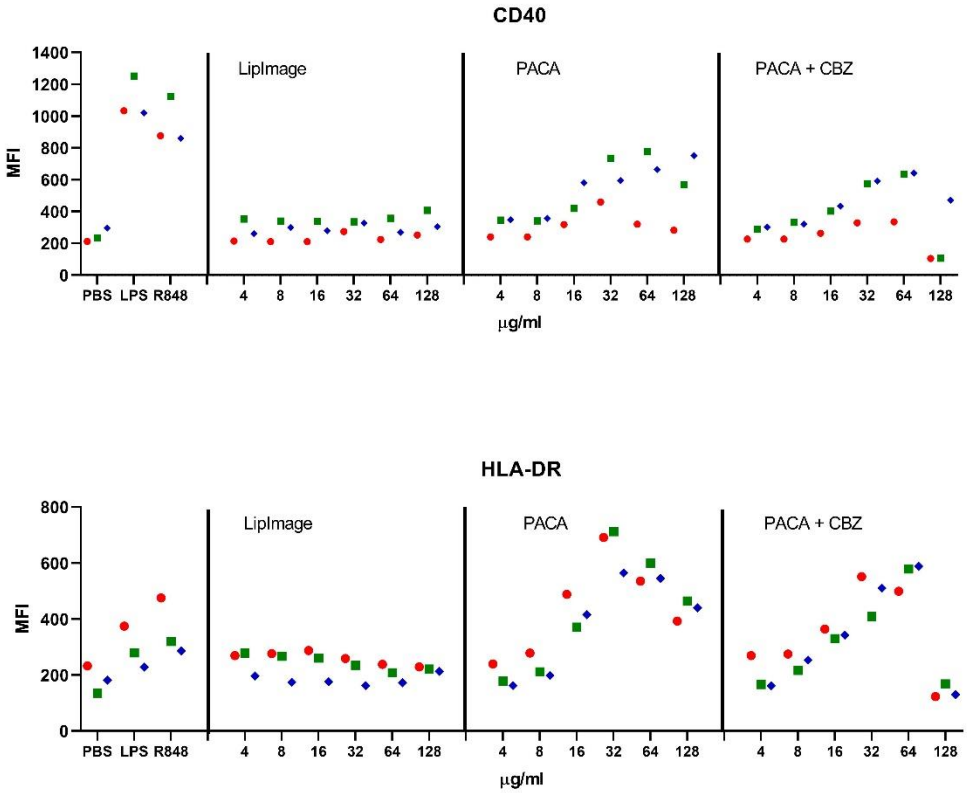

Fig. 5e Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD40 and HLA-DR surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

We analysed DC maturation also by concentration-response modelling, using the PROAST software tool. The three experiments presented in Fig. 5 were analysed (together). Since ISO [17] takes 70% viability as a threshold for cytotoxicity, 30% was chosen as effect size. The concentration at which a 30% effect is obtained is designated here as the Effective Concentration $(EC)_{30}$. Since no guidance exists on an effect size for markers of DC maturation, by default we chose a 30% effect, similar to viability. Next to establishing the ED, the software tool PROAST allows for generation of a 90% confidence interval (CI) around a specific ED (here EC_{30}). In Table 5, the EC_{30} values and corresponding 90% CIs are shown.

When an EC_{30} could not be calculated (LipImage™ 815: all parameters except DC-SIGN and PD-L1; PACA: FSC; PACA-CBZ: CD14, CD40), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: DC-SIGN and PD-L1; PACA: all parameters except FSC, WST-1 and Live/dead; PACA-CBZ: all parameters except WST-1, Live/Dead, CD14, and CD40), the data were not considered. For WST-1 and Live/dead staining (Table 4), the EC_{30} values for PACA-CBZ were 1.5 times higher than for PACA, possibly suggesting that in these assays PACA-CBZ may be slightly less cytotoxic compared to PACA.

	PACA		PACA-CBZ	
	EC_{30}	90% CI	EC_{30}	90% CI
WST-1	43.1	28.7-46.8	63.7	34.1-84.6
Live/dead	24.8	15.3-29.7	36.2	25.7-46.3

Table 5: EC_{30} and 90% CI values (µg/ml).

University of Liverpool

Monocytes were isolated from buffy coats and differentiated to immature DC. They were incubated for 48 hr with a 2-fold dilution series of LipImage™ 815, PACA, and PACA-CBZ (128, 64, 32, 16, 8, 4, and 2 µg/ml, plus a medium control (0)). After that, viability was evaluated using the WST-1 assay and surface marker expression using a FACS. The results shown are from three independent experiments (Fig. 6).

Viability

The positive controls for DC maturation, LPS and R848, did not affect viability. Incubation with LipImage™ 815 marginally affected cell viability, averaging 80% at the highest concentration tested (128 µg/ml). PACA showed a clear concentration-dependent decrease in viability within a 4-fold concentration range, from 91% at 32 µg/ml, 58% at 64 µg/ml, to 27% at 128 µg/ml. PACA-CBZ showed a similar decrease in viability, from 91% at 32 µg/ml, 74% at 64 µg/ml, and 37% at 128 µg/ml.

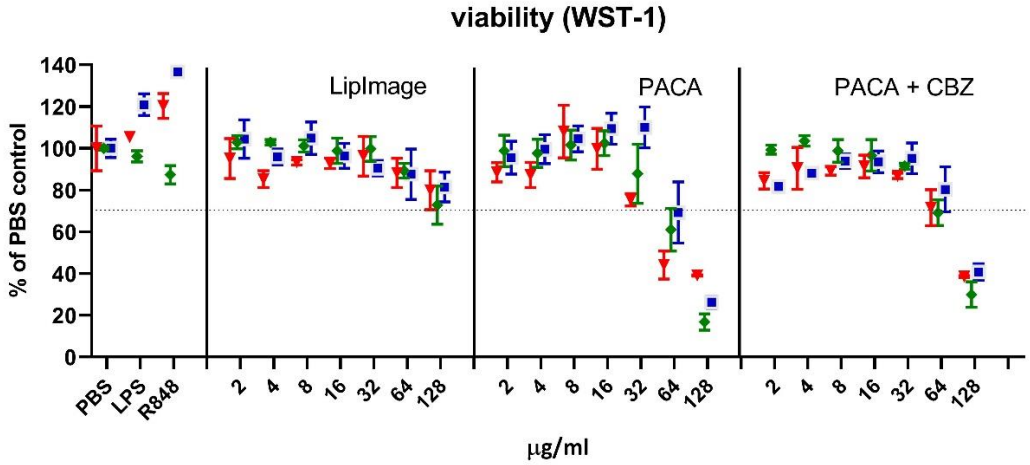

Fig. 6a Viability of DC after incubation with LipImage™ 815, PACA, and PACA-CBZ. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation.

Live/dead, FSC, and SSC

The positive controls for DC maturation, LPS and R848, did not affect Live/dead staining, FSC, and SSC. LipImage™ 815 induced a concentration-dependent increase in Live/dead staining, while no exposure effects on FSC and SSC were seen. PACA induced a clear increase in Live/dead staining from 16 µg/ml in two out of three independent experiments. PACA-CBZ induced a clear increase in Live/dead staining from 32 µg/ml. PACA decreased FSC and SSC from 16 µg/ml, while PACA-CBZ affected FSC and SSC from 32 µg/ml.

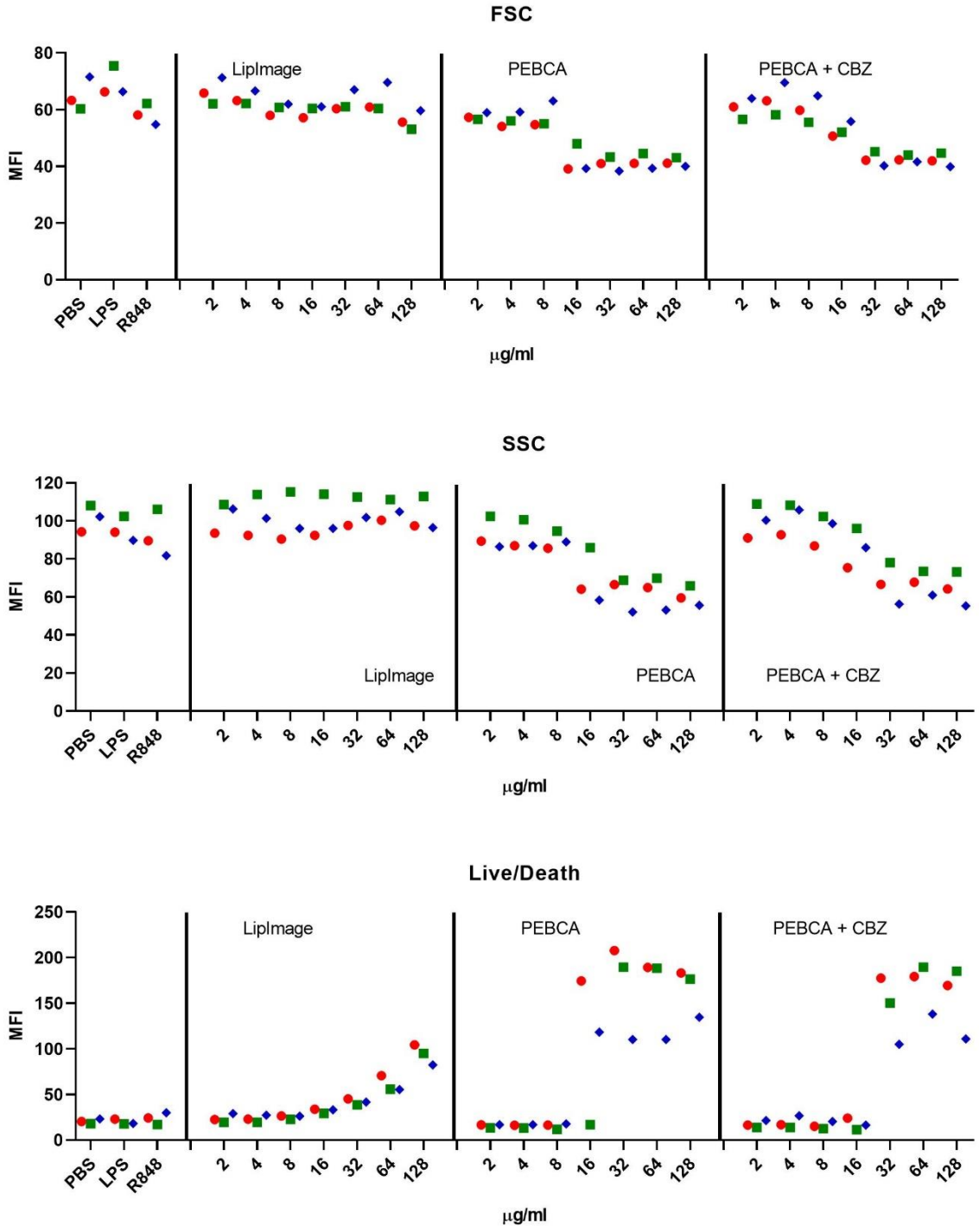

Fig. 6b Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on Live/dead staining, FSC, and SSC. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD80, CD83, and CD86

While LPS and R848 clearly induced CD80, CD83, and CD86 expression showing DC maturation, LipImage™ 815, PACA, and PACA-CBZ failed to do so. This suggests that none of the three nanomedicines induced DC maturation.

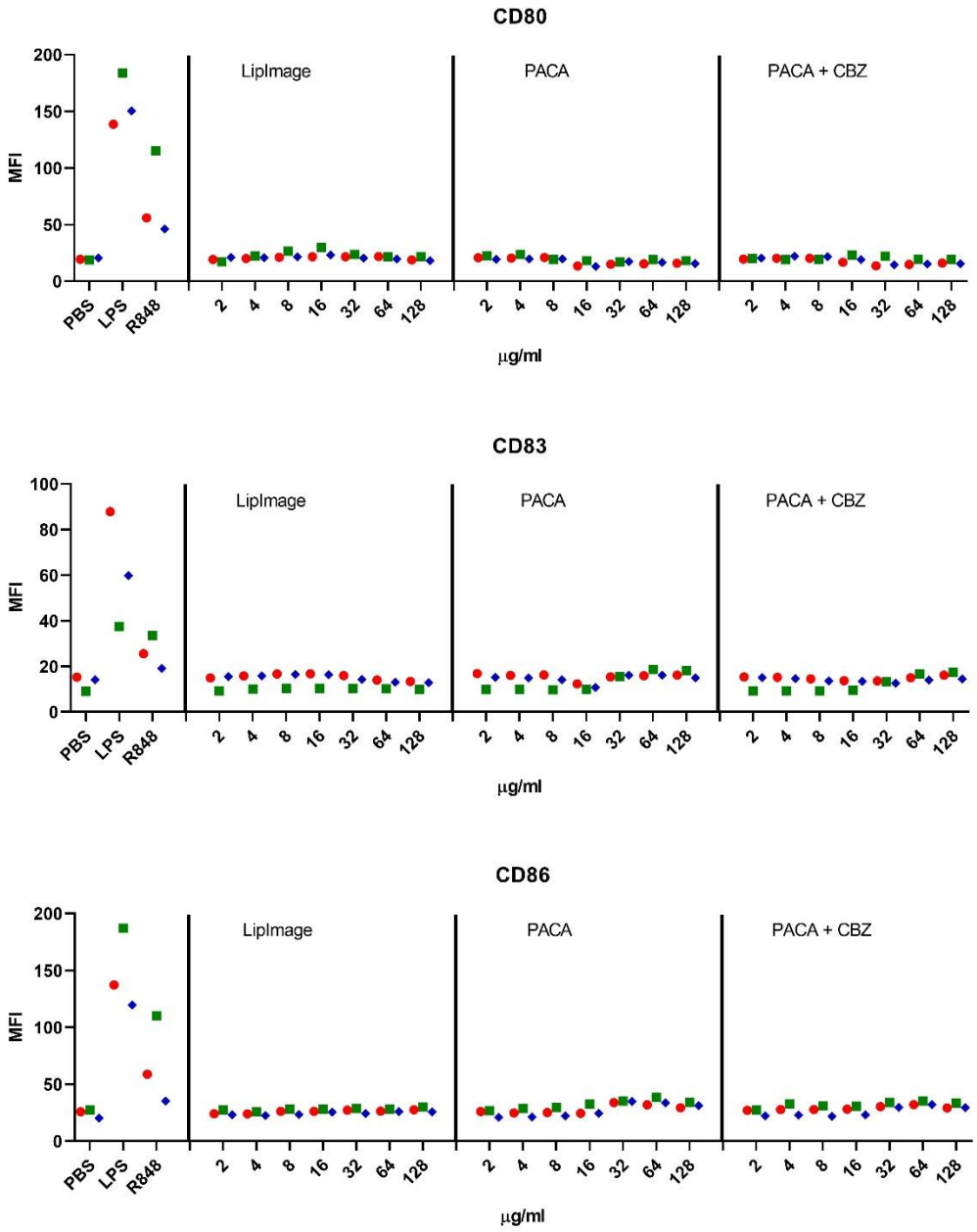

Fig. 6c Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD80, CD83, and CD86 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

CD14, DC-SIGN, and PD-L1

LPS clearly downregulated CD14 expression, in line with previous data showing combined endocytosis of LPS, TLR4, and CD14 [18]. R848 decreased CD14 to a lesser extent than LPS did. DC-SIGN expression was not affected by LPS or R848. PD-L1 expression was induced by LPS and R848.

LipImage™ 815 did not affect CD14 expression. For one of the three independent experiments, for both PACA and PACA-CBZ, CD14 expression is increased from 32 µg/ml. LipImage™ 815 induced the expression of DC-SIGN and PD-L1 from 64 µg/ml. DC-SIGN is typically downregulated in DC upon maturation [19]. No effects on DC-SIGN and PD-L1 expression by PACA and PACA-CBZ were seen.

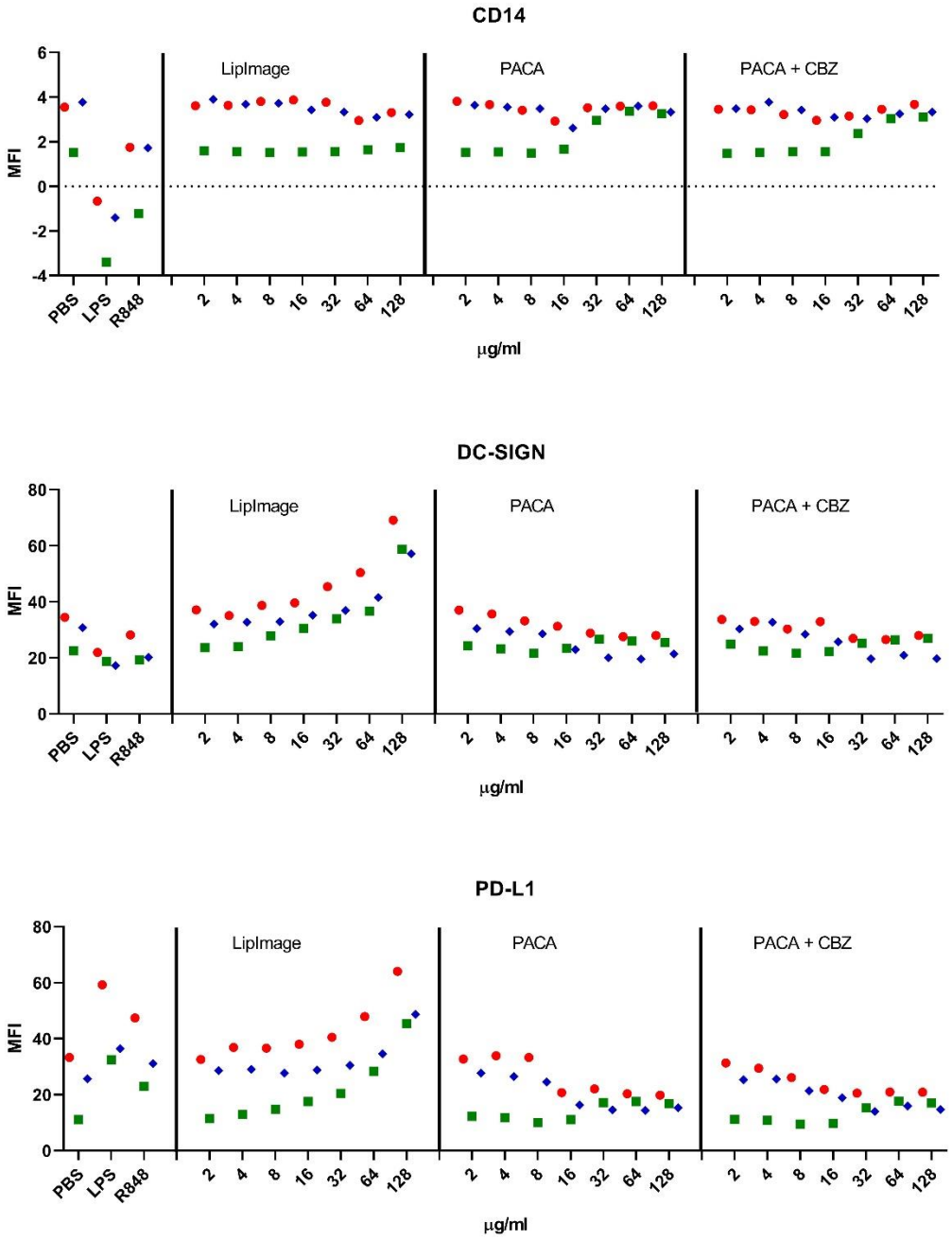

Fig. 6d Effects of incubation with LipImage™ 815, PACA, and PACA-CBZ on CD14, DC-SIGN, and PD-L1 surface marker expression. Red, blue, green: three independent experiments. LPS and R848 were used as positive controls for DC maturation. MFI, mean fluorescence intensity.

We analysed DC maturation also by concentration-response modelling. The three experiments presented in Fig. 6 were analysed (together). When an EC_{30} could not be calculated (LipImage™ 815: WST-1, SSC, and CD14; PACA: FSC and PD-L1; PACA-CBZ: FSC, CD14, DC-SIGN, and PD-L1), or the ratio between the upper (95%) and lower (5%) limit around the EC_{30} was > 5 (LipImage™ 815: Live/dead, DC-SIGN, and PD-L1; PACA: CD14), the data were not considered (CD80, CD83, and CD86 were not included). For WST-1, Live/dead staining, and SSC (Table 5), the EC_{30} values for PACA-CBZ were on average 1.5 times higher than for PACA, possibly suggesting that in these assays PACA-CBZ may be slightly less cytotoxic compared to PACA.

	PACA		**PACA-CBZ**	
	EC_{30}	**90% CI**	**EC_{30}**	**90% CI**
WST-1	51.0	35.0-67.7	72.3	66.3-88.8
Live/dead	10.3	5.34-11.6	17.5	15.2-18.9
SSC	17.5	13.0-17.9	25.9	18.1-35.0

Table 5. EC_{30} and 90% CI values (µg/ml).

Inter-laboratory variance in DC parameters

To evaluate the inter-laboratory variance in all DC parameters including WST-1, we first normalized for each experiment the CCM control to 100%. From this, for each of the three pharmaceuticals, for each of the two partners, and for each individual concentration we calculated the mean and standard deviation over the three independent replicate experiments. After this, the inter-laboratory variance was calculated and expressed in a heat map (Fig. 7). For LipImage™ 815, the largest inter-laboratory variance was for Live/dead staining and, to a lesser extent, DC-SIGN and PD-L1. It should be mentioned that a larger inter-laboratory variance is to be expected when a concentration-dependent effect is seen. For LipImage™ 815, this is seen for DC-SIGN, PD-L1 (for University of Liverpool but not RIVM), but not for Live/dead staining. For PACA and PACA-CBZ, the largest inter-laboratory variance was for Live/dead staining and, to a lesser extent, CD86 and HLA-DR. Of notice, while both WST-1 and Live/dead staining show a rather similar concentration-response as evidenced by concentration-response modelling, the inter-laboratory variance of Live/dead staining is much higher, suggesting that this parameter is much more sensitive to differences between laboratories.

Data from RIVM (Supplementary Information S4A) and the University of Liverpool (Supplementary Information S4B) is included.

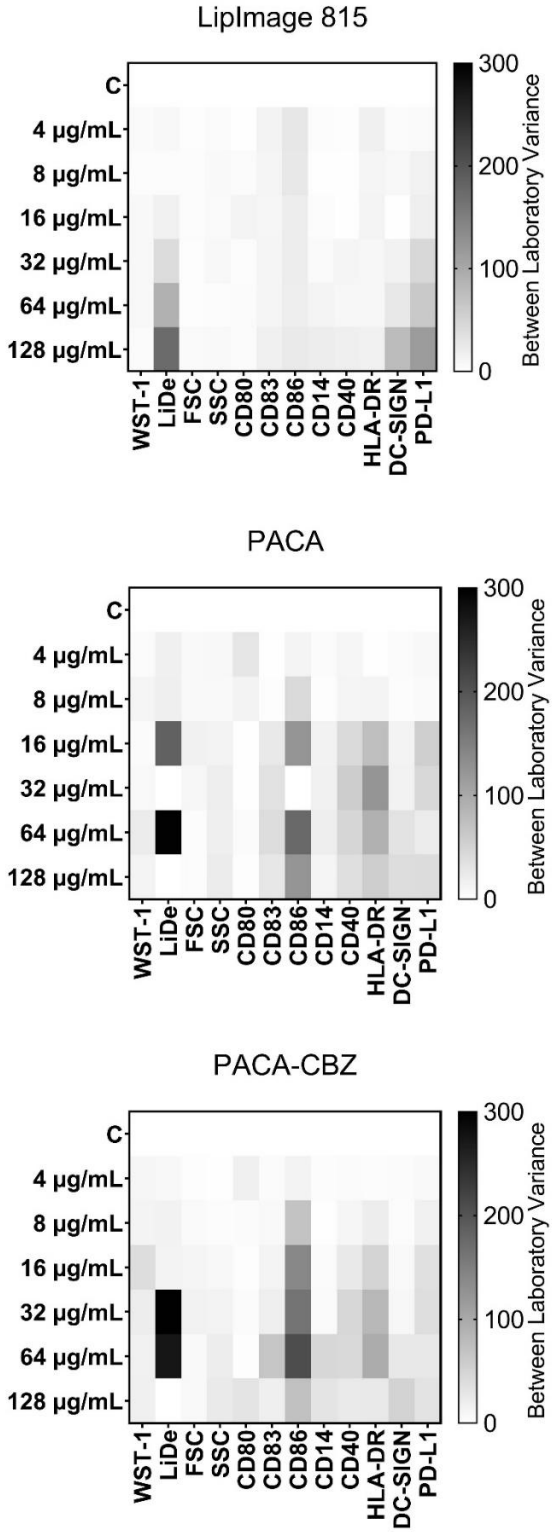

Fig. 7 Heat maps of the inter-laboratory variance for each of the three pharmaceuticals tested. For each of N = 3 independent experiments, the CCM control (C) was set at 100% and the mean and standard deviation was calculated for these experiments. This was done for each laboratory, after which the variance between the two laboratories was calculated and expressed in a heat map on a scale of 0-300.

Discussion

Here we evaluated the effects of two nanomedicines, representing nanostructured lipid carriers and polymers, on two *in vitro* assays. These assays, NLRP3 inflammasome activation and DC maturation, are among the ones listed to fulfil the information requirements for regulatory acceptance of nano-pharmaceuticals but are still remote from being a standardised assay [5]. Moreover, with a view to possible future standardization and regulatory application, these assays were subject to an inter-laboratory comparison study, using common SOPs. To this end, one laboratory performed three independent NLRP3 inflammasome activation experiments, while the other performed a single experiment. Two laboratories each performed three independent DC maturation experiments. While the nanostructured lipid nanocarrier only showed marginal effects, the polymers showed major cytotoxicity. No evidence for NLRP3 inflammasome activation or DC maturation was demonstrated. Intra- and inter-laboratory comparison showed clearly reproducible results.

NLRP3 inflammasome activation evaluated by RIVM showed a slight decrease in viability upon exposure to LipImage™ 815 and a sharp decrease in viability upon exposure to PACA and PACA-CBZ. This observation is underlined by concentration-response modelling that showed concentration-dependent cytotoxicity for PACA and PACA-CBZ but not LipImage™ 815. Next, this modelling showed a similar EC_{30} for PACA and PACA-CBZ suggesting that the cytotoxicity observed is caused by PACA and not CBZ. Moreover, the data show a high intra-laboratory reproducibility. Evaluation by the University of Liverpool showed similar results to those obtained by RIVM. No effect on IL-1β production was seen in either laboratory. This, suggests that that neither of the nanomedicines induced NLRP3 inflammasome activation. In any case, intra- and inter-laboratory reproducibility seem to warrant subsequent steps to standardisation of the assay. In such future studies, nanoparticles well-known to activate the NLRP3 inflammasome should be included, such as SiO_2 nanoparticles [21].

Measuring only IL-1β (with or without IL-18) may be too limited to establish NLRP3 inflammasome activation. We propose to also include measurement of (1) caspase-1 activity, to better connect IL-1β secretion to NLRP3 inflammasome activation, and (2) caspase-3 activity, to discriminate between pyroptosis resulting from NLRP3 inflammasome activation, and apoptosis as mechanism of cell death.

In a separate inter-laboratory comparison study within the REFINE project the cytotoxicity of the same batches of the three materials LipImage™815, PACA, and PACA-CBZ was tested in four different cell lines using both the WST-8 and the LDH release assay. LipImage™ 815 was non-cytotoxic up to a concentration of 128 µg/ml, whereas PACA caused dose-dependent cytotoxic effects starting from 8 µg/ml. PACA-CBZ showed a less pronounced dose-dependent effect with the lowest concentration of 2 µg/mL causing cytotoxic effects (Eder et al. submitted for publication).

Outside the inter-laboratory comparison study presented here, but within the REFINE project and using the same SOP as in the study presented here, NLRP3 inflammasome activation by a commercially available liposome (Avanti, Birmingham, AL) was evaluated. The REFINE partners RIVM and CEA each performed 3 independent replicate experiments. Both partners did not observe effects on viability and IL-1β production over the entire concentration range tested (up to of 128 µg/ml) with an appropriate response by the positive control nigericin (Supplementary Information S5). This data suggests a lack of NLRP3 inflammasome activation by the liposome.

The DC maturation assays performed by RIVM showed no effect on viability of LipImage™ 815, whereas a clear decrease in viability by PACA and PACA-CBZ was seen. These findings are underlined by concentration-response modelling that showed concentration-dependent cytotoxicity for PACA and PACA-CBZ but not LipImage™ 815. Next, this modelling showed a 1.5-fold lower EC_{30} for PACA compared to PACA-CBZ, suggesting that the cytotoxicity observed is caused by PACA and not CBZ. These findings were similar for the WST-1 assay and Live/dead-staining, two orthogonal methods to assess cell viability. DC maturation evaluated by the University of Liverpool showed similar results: cytotoxicity induced by PACA and PACA-CBZ but not LipImage™ 815, a 1.5-fold lower EC_{30} for PACA compared to PACA-CBZ, and similar results for the WST-1 assay and Live/dead-staining. It should be noted, however, that for Live/dead staining the EC_{30} values themselves were rather different between RIVM and the University of Liverpool. For PACA and PACA-CBZ, RIVM established as the most sensitive parameter an increase in HLA-DR, seen from 16 and 32 µg/ml, respectively. The University of Liverpool established a decrease in FSC and SSC as most sensitive parameters, seen from 16 and 32 µg/ml, respectively. Although CD40 and HLA-DR are regarded as DC maturation markers, this data, especially the lack of effect on CD80, CD83, and CD86 expression, suggests that neither of the nanomedicines induce DC maturation. In any case, intra- and inter-laboratory reproducibility seem to warrant subsequent steps to standardisation of the assay. In such future studies, nanoparticles well-known to induce DC maturation should be included, such as TiO_2 nanoparticles [22].

The heat map shows a considerable difference in Live/dead staining between the two participating laboratories, whereas for WST-1 this difference was limited. This suggest that currently, the WST-1 assay, being the only assay not included in the flow cytometry measurement, should remain to be included in the evaluation of effects on DC maturation.

Flow cytometry is a powerful method for immune cell phenotyping. It is routinely used in clinical immunology laboratories around the world. Moreover, OECD guidelines and ISO standards include the use of flow cytometry, such as the h-CLAT [23]. Still, some of the flow cytometry characteristics may hamper acceptance by regulatory authorities of assays that rely on this method. After data collection, compensations are required to correct for the overlap between adjacent emission spectra of different fluorochromes. Next, to select a specific population of cells serial gating is required, which is done by visual inspection of 2D scatterplots. Both compensation and serial gating are often done manually and may differ between operators. Especially manual gating is subjective, not only because gate setting can be more or less strict, but also the sequence of gating to arrive at the desired cell population may differ [24]. A promising way out is the use of computational flow cytometry, reviewed by Saeys et al. [24] and more recently by Lucchesi et al. [25].

Conclusions

An inter-laboratory comparison study was performed for two assays, NLRP3 inflammasome activation and DC maturation, using two nanomedicines, the nanostructured lipid carrier LipImage™ 815 and the polymer PACA, either loaded or not with CBZ. PACA and PACA-CBZ showed clear cytotoxicity whereas LipImage™ 815 did not. Neither of the nanomedicines induced NLRP3 inflammasome activation or DC maturation. Intra- and interlaboratory reproducibility seem to warrant subsequent steps to standardisation of these assays. In such future studies, nanoparticles well-known to activate the NLRP3 inflammasome resp. induce DC maturation should be included.

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Authors' contributions

RJV: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; CAWD: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – review & editing; JPV: Data curation, Investigation, Methodology, Validation, Writing – review & editing; NJL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing

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Data availability

Data are available upon request to the corresponding author (Rob J. Vandebriel, rob.vandebriel@rivm.nl).

Compliance with ethical standards

Competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethics approval and consent to participate

The use by RIVM of buffy coats for the current study was authorised by Sanquin (Amsterdam, the Netherlands) under agreement NVT0243.02. The use of, primary, human blood and blood products at the University of Liverpool is covered under Research Ethics Committee (REC) approval 2056.

Consent for publication

Written informed consent for publication was obtained from all authors.

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