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Development of a cell line-based *in vitro* assay for assessment of Diphtheria, Tetanus and acellular Pertussis (DTaP)-induced inflammasome activation

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ABSTRACT

Safety and potency assessment for batch release testing of established vaccines still relies partly on animal tests. An important avenue to move to batch release without animal testing is the consistency approach. This approach is based on thorough characterization of the vaccine to identify critical quality attributes that inform the use of a comprehensive set of non-animal tests to release the vaccine, together with the principle that the quality of subsequent batches follows from their consistent production. Many vaccine antigens are by themselves not able to induce a protective immune response. The antigens are therefore administered together with adjuvant, most often by adsorption to aluminium salts. Adjuvant function is an important component of vaccine potency, and an important quality attribute of the final product. Aluminium adjuvants are capable of inducing NLRP3 inflammasome activation. The aim of this study was to develop and evaluate an *in vitro* assay for NLRP3 inflammasome activation by aluminium-adjuvanted vaccines. We evaluated the effects of Diphtheria-Tetanus-acellular Pertussis combination vaccines from two manufacturers and their respective adjuvants, aluminium phosphate (AP) and aluminium hydroxide (AH), in an *in vitro* assay for NLRP3 inflammasome activation. All vaccines and adjuvants tested showed a dose-dependent increase in IL-1β production and a concomitant decrease in cell viability, suggesting NLRP3 inflammasome activation. The results were analysed by benchmark dose modelling, showing a similar 50% effective dose (ED50) for the two vaccine batches and corresponding adjuvant of manufacturer A (AP), and a similar ED50 for the two vaccine batches and corresponding adjuvant of manufacturer B (AH). This suggests that NLRP3 inflammasome activation is determined by the adjuvant only. Repeated freeze-thaw cycles reduced the adjuvant biological activity of AH, but not AP. Inflammasome activation may be used to measure adjuvant biological activity as an important quality attribute for control or characterization of the adjuvant.

1. Introduction

Vaccines are a very efficient tool for protection against many infectious diseases. They are biological products composed of antigens derived from whole microorganisms or components thereof. Therefore, antigen batches may have minor variations in composition. The variability of vaccines is further complicated by the fact that many are produced as combinations of antigens in the final product, and have adjuvants added that interact with the different antigens. Therefore, each batch must undergo extensive quality control testing. While manufacturers perform many tests at various stages throughout vaccine production, routine testing of each final product batch is a regulatory requirement to ensure vaccine safety and potency. For older vaccines, batch release testing often relies on animal models. The consistency approach considers each batch to be one of a series where the focus for testing is shifted from the final batch to the overall production process [1,2]. The consistency approach has been further adapted to include, in addition to the use of well-characterized production methods, analytical tools including *in vitro* assays to establish a product profile based on relevant characteristics [3]. Tests for these characteristics or Critical Quality Attributes (CQAs) are expected to be part of

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routine release testing and furthermore to be able to discriminate between batches of standard and substandard quality.

Many vaccines require the combination of antigen and adjuvant in order to induce a protective immune response in vaccinees. In order to unravel the contribution of each component, evaluating adjuvant activity independently of the antigen(s) may be beneficial. While it is generally accepted that aluminium adjuvants are capable of inducing NLRP3 inflammasome activation at the cellular level, there is disagreement on the necessity of this response in generating adjuvant effects *in vivo*. In a recent review [4] the requirement of NLRP3 inflammasome activation for aluminium adjuvant-based enhancing activity was suggested to depend on the type of aluminium adjuvant. Here we developed an *in vitro* quantitative assay to measure NLRP3 inflammasome activation and used this assay to evaluate diphtheria-tetanus-acellular pertussis (DTaP) vaccines (final product) from two different manufacturers along with their corresponding aluminium adjuvants. To evaluate whether the NLRP3 inflammasome activation assay is able to detect differences in the enhancing capacity of the adjuvants, they were subjected to repeated freezing/thawing. This procedure has been shown to reduce the immunogenicity of Hepatitis B vaccine formulated with freeze/thawed aluminium hydroxide adjuvant [5].

2. Methods

2.1. Vaccines and adjuvants

Two batches of diphtheria-tetanus-acellular pertussis vaccine (DTaP) and one batch of AP, all from manufacturer A were used for testing. Similarly, two batches of DTaP and one batch of AH, all from manufacturer B were used. The vaccines and their corresponding adjuvants were compared based on the content of aluminium).

2.2. Cell line maintenance

THP-1 cells (ATCC TIB-202, lot No. 59796015), THP1-defASC cells and THP1-defNLRP3 cells (InvivoGen) were used in this study. The cells were cultured in cell culture medium (CCM; 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 the presence of HygroGold (200 µg/ml, InvivoGen) to maintain the siRNA responsible for suppression of ASC or NLRP3. All cell lines were sub-cultured twice per week, seeded to a cell density of 2×10^5 cells/ml and not allowed to grow to a density greater than 1×10^6 cells/ml. Cells were not cultured beyond twenty passages to prevent genetic divergence.

2.3. Maturation of THP-1 cells

Song and co-workers showed that priming of the human monocyte cell line THP-1 by PMA resulted in the phosphorylation of NLRP3 [6]. This phosphorylation was shown to be required for NLRP3 inflammasome activation and IL-1β production. The THP-1 cells were matured into macrophage-like cells by culturing for 3 h 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 medium was replaced with fresh culture medium without PMA and the plates were incubated for 24 h at standard conditions (humidified incubator at 37 °C, 5 % CO2). After this incubation period, the cells were exposed to a twofold dilution series of DTaP batch 1, DTaP batch 2, and AP (manufacturer A), or DTaP batch 1, DTaP batch 2, and AH (manufacturer B), in 10 % PBS (Gibco) in CCM and incubated for 48 h at standard conditions.

Nanosized SiO_2 (NM202; JRC) and nigericin (InvivoGen) were used as positive controls. Treatment with either agent showed a clear activation of the NLRP3 inflammasome (Supplementary Information S1 and S2).

2.4. Viability

The viability of the cells after exposure to DTaP vaccine or adjuvant was assessed using the cell proliferation reagent WST-1 (Sigma-Aldrich). Exposed cells (and controls) were incubated for 2.5 h under standard conditions in the presence of 10 % (v/v) WST-1 reagent. After incubation, in each well the absorbance (A) was measured at 440 nm and corrected for background absorbance at 620 nm. 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 DTaP or adjuvant (in 10 % PBS in CCM) and C the negative control (10 % PBS in CCM). The viability was expressed as percentage of the control. For dose–response curve fitting (see below), the corrected absorbance values (A (cells in medium, X) – A (medium only, X) were used (and not percentages). It may be suggested that WST-1 was measuring (in part) mitochondrial disruption rather than cell death. A comparison between CellTiter-Blue and WST-1 reagents for cell viability following treatment with a concentration range of AP and AH, on wild-type, ASC-deficient, and NLRP3-deficient cells, showed a similar response between these read-out parameters (results not shown) suggesting that in our cells WST-1 was indeed measuring cell death.

2.5. IL-1β ELISA

The IL-1β concentration in the culture supernatant was determined using a human IL-1β ELISA kit (Thermo Fisher scientific, ref: 88-7261-88) according to the manufacturer's instructions. Exposures for the assessment of IL-1β secretion were performed in four wells per condition. Culture supernatants were frozen at −80 °C until further use. Before use, the supernatants were thawed and assessed in twentyfold dilution (to stay within the standard curve concentration range of the ELISA kit).

2.6. Dose-response curve fitting

Dose response curve fitting for IL-1β production and viability was performed with statistical software package PROAST [7] within the software environment 'R' [8].

2.7. Freeze/thaw of adjuvant

AP and AH were frozen at −20 °C for 16 h and then thawed at 4 °C. This was repeated 3 times, on consecutive days. A concentration range of freeze/thaw treated adjuvant was compared for NLRP3 inflammasome activation to adjuvant that had been kept at a constant 4 °C.

2.8. Statistics

Comparison between treatment groups was done using the Independent Samples T-Test (SPSS, IBM, version 28.0.1.0).

3. Results

3.1. Inflammasome specific response

In the wildtype cells (THP-1), a concentration-dependent increase in IL-β production is seen upon stimulation with AH con-

taining vaccine, together with a concomitant decrease in cell viability (Fig. 1). To confirm that the effects on viability and IL-1β production were NLRP3 inflammasome specific, the response of the wildtype cells was compared to the response of the ASC- and NLRP3- deficient cells. A clear difference in IL-1β production and viability was observed (Fig. 1).

A residual background observed in the ASC- and NLRP3-deficient cell lines can be explained by the fact that the silencing of the ASC and NLRP3 genes is only partial. Nonetheless, the results show a clear difference between the responses of the wildtype and deficient cells.

3.2. Dose-response modelling

We addressed possible differences in inflammasome activation between two batches of DTaP vaccine and the corresponding adjuvant by dose–response modelling. Materials from two manufacturers were investigated. For two independent vaccine batches and the corresponding adjuvant AH, all from manufacturer B, three experiments were conducted, and the data analysed by dose–response modelling (Fig. 2). A significant difference was observed in the background between replicate experiments, resulting in shifts of the curves along the Y-axis. The biological activity of the vaccine or adjuvant is calculated based on the concentration that induces a 50 % effect compared to the maximum response (50 % effective dose or ED_{50}). For both parameters tested (IL-1β production and viability), the ED_{50} was similar for each of the nine curves. This suggests there is no difference in inflammasome activation between the two vaccine batches, or between the vaccine and the adjuvant, both expressed as the amount of aluminium added to the cells.

Similarly, for each of the two vaccine batches and the corresponding adjuvant (AP), all from manufacturer A, three experiments were conducted, and the data analysed by dose–response modelling (Fig. 3). Again, a significant difference was observed in the background of each experiment. For both parameters tested (IL-1β production and viability), the ED_{50} was similar for each of the nine curves. This suggests that also for manufacturer A material there is no difference in inflammasome activation between the two vaccine batches, or between the vaccine and the adjuvant.

Next to the ED_{50} value, the dose–response modelling software PROAST allows for generation of a 90 % confidence interval (CI).

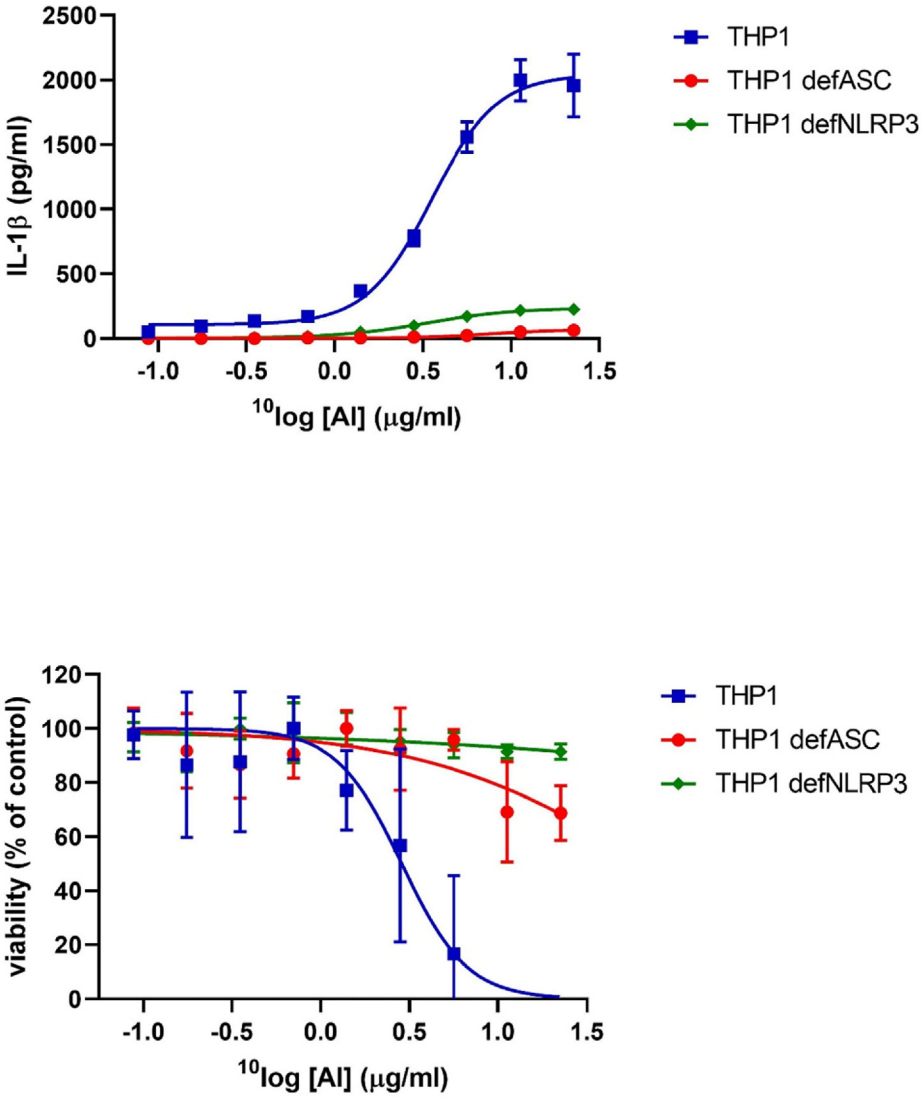

Fig. 1. Wildtype and ASC- and NLRP3-deficient THP-1 derived macrophage response to a dilution series of DTaP vaccine containing AH (manufacturer B). Top panel, IL-1β production; bottom panel, viability. Mean ± SD. Results from a representative (of n = 2) experiment are shown. Both batches of DTaP were tested. X-axis: ^{10}log [Al] (μg/ml). Y-axis top figure: IL-1β (pg/ml). Y-axis bottom figure: viability (%).

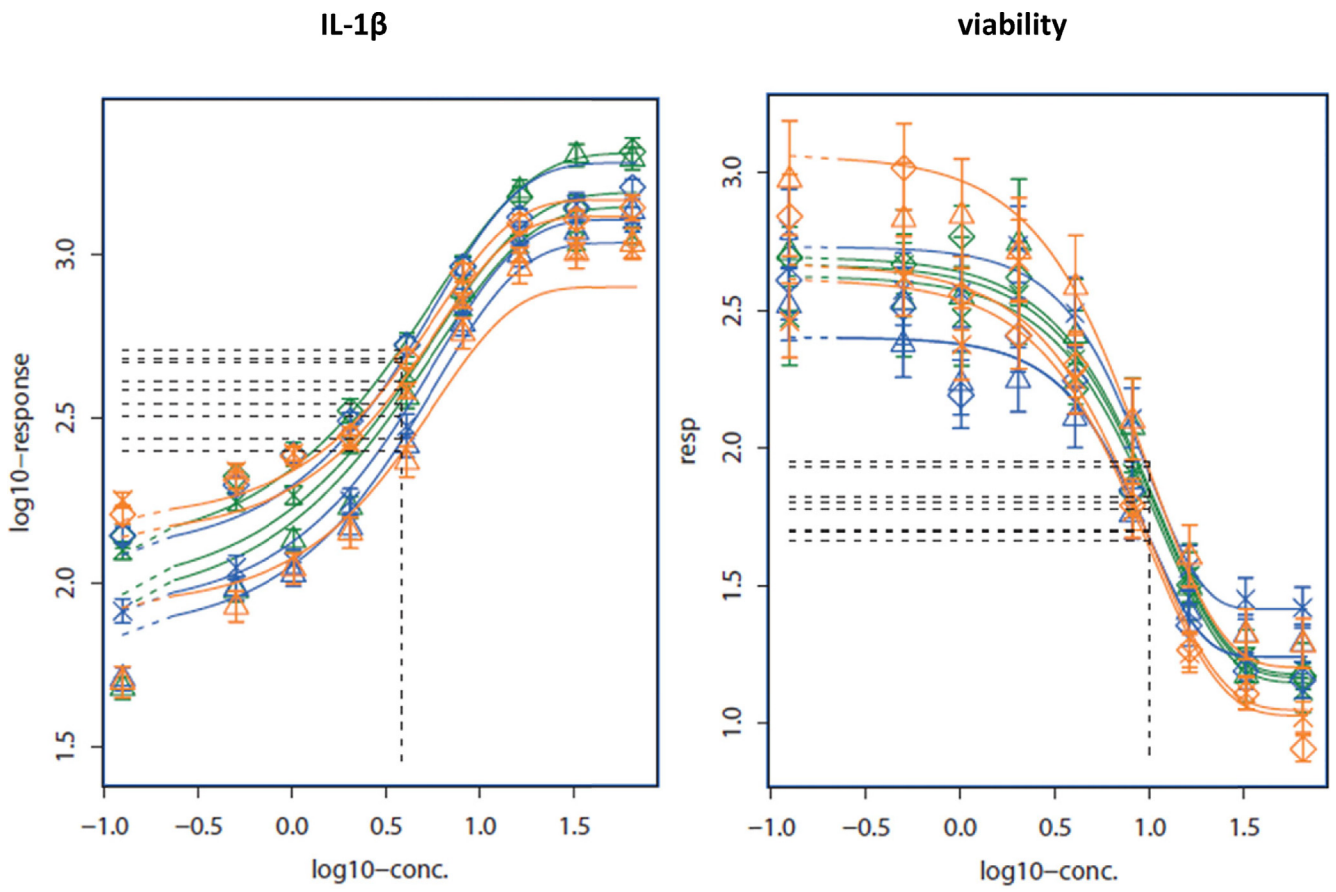

Fig. 2. Dose-response data and optimal fitted model for IL-β production (left panel) and viability (right panel). Two batches of DTaP containing adjuvant and one batch of adjuvant alone (N = 3) were tested, all from manufacturer B. Blue, DTaP batch 1; green, DTaP batch 2; orange: adjuvant. Horizontal dashed line: 50 % effect level, and vertical dashed line: associated concentration. X-axis: ^{10}log [Al] (μg/ml). Y-axis left figure: ^{10}log [IL-1β] (pg/ml). Y-axis right figure: ^{10}log [viability] (%). Overlapping dashed lines may cause less than 9 to be visible. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In Table 1, the ED_{50} values and corresponding 90 % CIs are shown, expressed on a molar basis. The data shows that for IL-1β production the ED_{50} value of the vaccine/adjuvant from manufacturer B is higher than that of the vaccine/adjuvant from manufacturer A (no overlapping 90 % CI's), whereas for viability the ED_{50} values are rather similar between manufacturers A and B. It should be noted that the ED_{50} values of the two vaccine batches and their corresponding adjuvant was tested in a separate series of experiments for each manufacturer individually. Therefore, the enhancing capacity of the adjuvant cannot be directly compared between the two manufacturers. This was, however, not the aim of the study.

In summary, dose–response modelling of inflammasome activation is a tool to monitor biological activity of vaccine adjuvants.

3.3. Effects of freeze/thaw cycles on adjuvant activity

For the AP, repeated (3 times) freezing/thawing (FT) of adjuvant did not show an effect on NLRP3 inflammasome activation (Fig. 4). In contrast, for AH, repeated FT showed a clearly decreased NLRP3 inflammasome activation, seen as a higher Al concentration to result in an increase in IL-1β production and a decrease in viability.

4. Discussion

Our results show that increased IL-1β production and decreased viability of THP-1 derived macrophages exposed to DTaP and adjuvant is due to inflammasome activation (wild type vs deficient cells). Dose-response modelling shows a similar ED_{50} for the two batches of vaccine and the corresponding adjuvant from manufacturer A (AP), and a similar ED_{50} for the two batches of vaccine and the corresponding adjuvant from manufacturer B (AH). Therefore, the NLRP3 inflammasome activation is determined by the adjuvant only. Dose-response modelling of inflammasome activation is a tool to monitor adjuvant activity of different vaccine batches, contributing to consistency testing.

It may be suggested that for measuring adjuvant activity, it is sufficient to simply measure Al content rather than evaluate adjuvant biological activity via an assay such as NLRP3 inflammasome activation. It should, however, be noted that aluminium adjuvants are particulate in nature and properties of these particles are dependent not only on the presence of elemental aluminium but on the type of aluminium salt. For example, $AlPO_4$ was found to be composed of 50 nm plate-like particles, while $Al(OH)_3$ was composed of 4.5 × 2.2 × 10 nm fibrous particles [9]. The NLRP3 inflammasome responds to a wide range of nanosized materials [10], suggesting the importance of the nanosized structures of aluminium adjuvants. This hypothesis is supported by studies by Sun et al. [11] and Orr et al. [12] who showed by manipulating the size of aluminium oxyhydroxide adjuvant, size is important for NLRP3 inflammasome activation. In conclusion, the NLRP3 inflammasome activation capacity of aluminium is defined by more than solely the Al content.

While it is generally accepted that aluminium adjuvants are capable of inducing NLRP3 inflammasome activation at the cellular

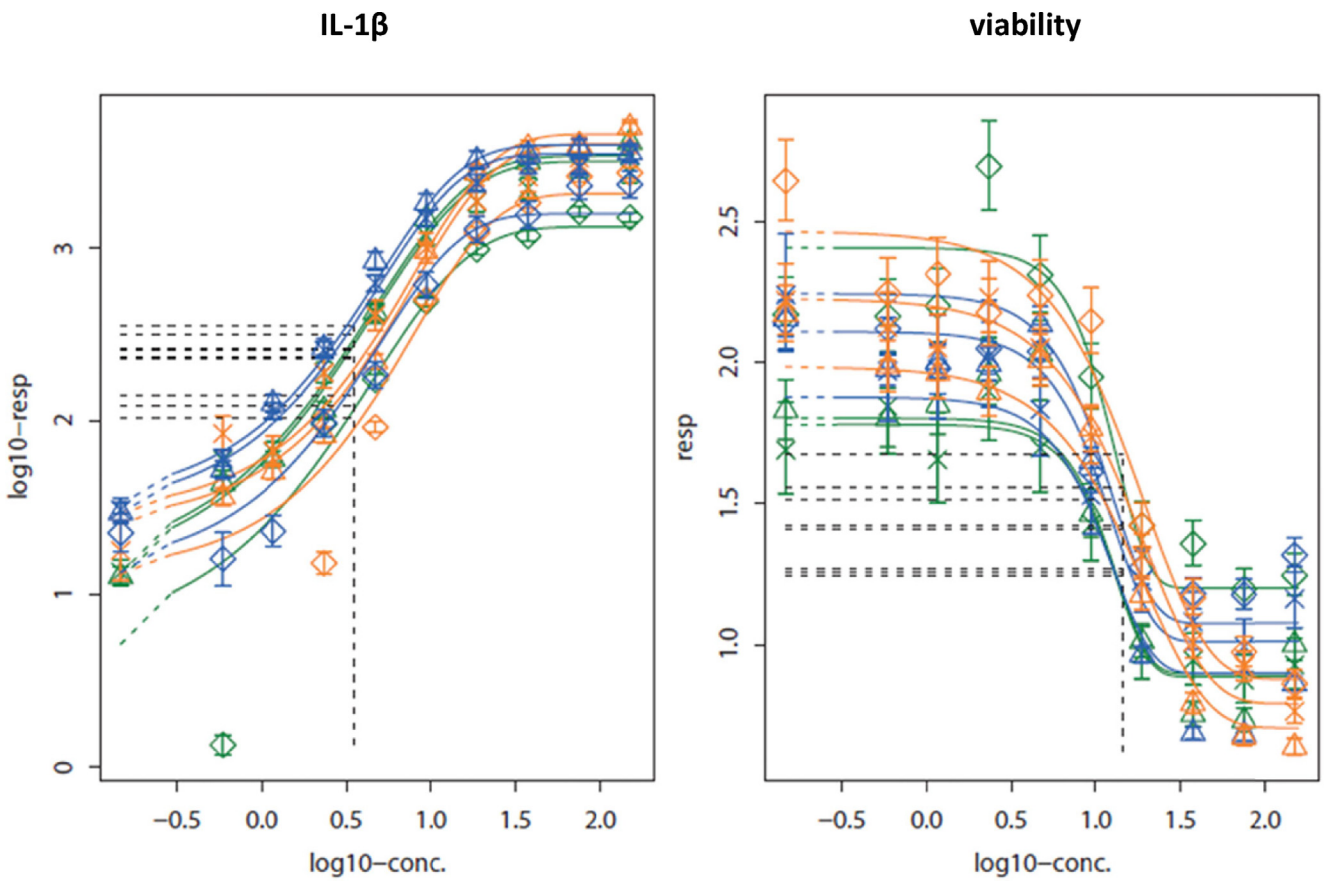

Fig. 3. Dose-response data and optimal fitted model for IL-β production (left panel) and viability (right panel). Two batches of DTaP containing adjuvant and one batch of adjuvant alone (N = 3) were tested, all from manufacturer A. Blue, DTaP batch 1; green, DTaP batch 2; orange: adjuvant. Horizontal dashed line: 50 % effect level; vertical dashed line: associated concentration. X-axis: ^{10}log [Al] (μg/ml). Y-axis left figure: ^{10}log [IL-1β] (pg/ml). Y-axis right figure: ^{10}log [viability] (%). Overlapping dashed lines may cause less than 9 to be visible. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
ED_{50} and 90 % CI values of IL-1β production and viability (μM of $AlPO_4$ (A) or $Al(OH)_3$ (B)).

	IL-1β		viability	
	ED_{50}	90 % CI	ED_{50}	90 % CI
A	26.0	24.0–28.2	121.0	111.5–131.2
B	47.8	44.7–51.2	125.4	113.7–137.2

level, there is disagreement on the necessity of this response in generating adjuvant effects *in vivo*. In a recent review [4] the requirement of NLRP3 inflammasome activation for aluminium-based enhancing activity is suggested to depend on the type of aluminium adjuvant, with studies showing NLRP3 inflammasome dependency [13,14] using the Imject adjuvant, and other studies showing NLRP3 inflammasome activation to be dispensable [15,16] using aluminium hydroxide (Brenntag) adjuvant. The Imject and Brenntag adjuvants were subsequently shown to have different capacities to induce immune responses in animals against the same model antigen [17]. We propose that the assay presented in the present study is an important quality attribute for adjuvants of which the enhancing activity depends on NLRP3 inflammasome activation.

So far, inactivating mutations in the NLRP3 gene of humans have not been reported, nor people that lack NLRP3 expression [18]. Evaluating a role of the NLRP3 inflammasome in the response to DTaP in humans is therefore not straightforward. Moreover, the requirement for and functionality of adjuvants in specific vaccines intended for humans must be demonstrated by clinical immunogenicity and efficacy trials.

Another potential application for the inflammasome assay may be for assessment of biological activity of aluminium adjuvant with altered formulations aimed to improve its effect and Th1 steering capacity, such as oxyhydroxide nanorods [11] and PAA-nanoalum [12]. Other novel adjuvants with NLRP3 inflammasome activating activity such as rod-shaped gold nanoparticles [19] may also be assessed. Moreover, the assay may be used to assess the biological activity of cationic liposomes, a class of nanomedicines that activates the NLRP3 inflammasome [20,21].

A recent study by Kooijman et al. [22] investigated differences in the response of PMA-primed THP-1 cells between $Al(OH)_3$ adjuvated DTaP (Infanrix) and $Al(OH)_3$ adjuvant (Brenntag). The authors observed a >10-fold higher IL-1β production by DTaP compared to adjuvant, whereas we did not observe a difference. This difference may be explained, in part, by differences in the $Al(OH)_3$ manufacturer (Brenntag vs GSK). In our study, DTaP and AH were from the same manufacturer. Of note, while we measured approximately 2000 pg/ml IL-1β after incubation with AH, Kooijman et al. measured <10 pg/ml.

We showed that repeated freezing/thawing (FT) of AH resulted in a reduction of the enhancing effect of the adjuvant. This is in line with the study by Clapp et al. [5] who showed a reduction by FT of the immunogenicity of Alhydrogel, an aluminium hydroxide adjuvant. Interestingly, Clapp et al. [5] showed reduced hepatitis B surface antigen (HBsAg)-specific IgG levels after vaccination with HBsAg plus FT-treated adjuvant compared to HBsAg plus control

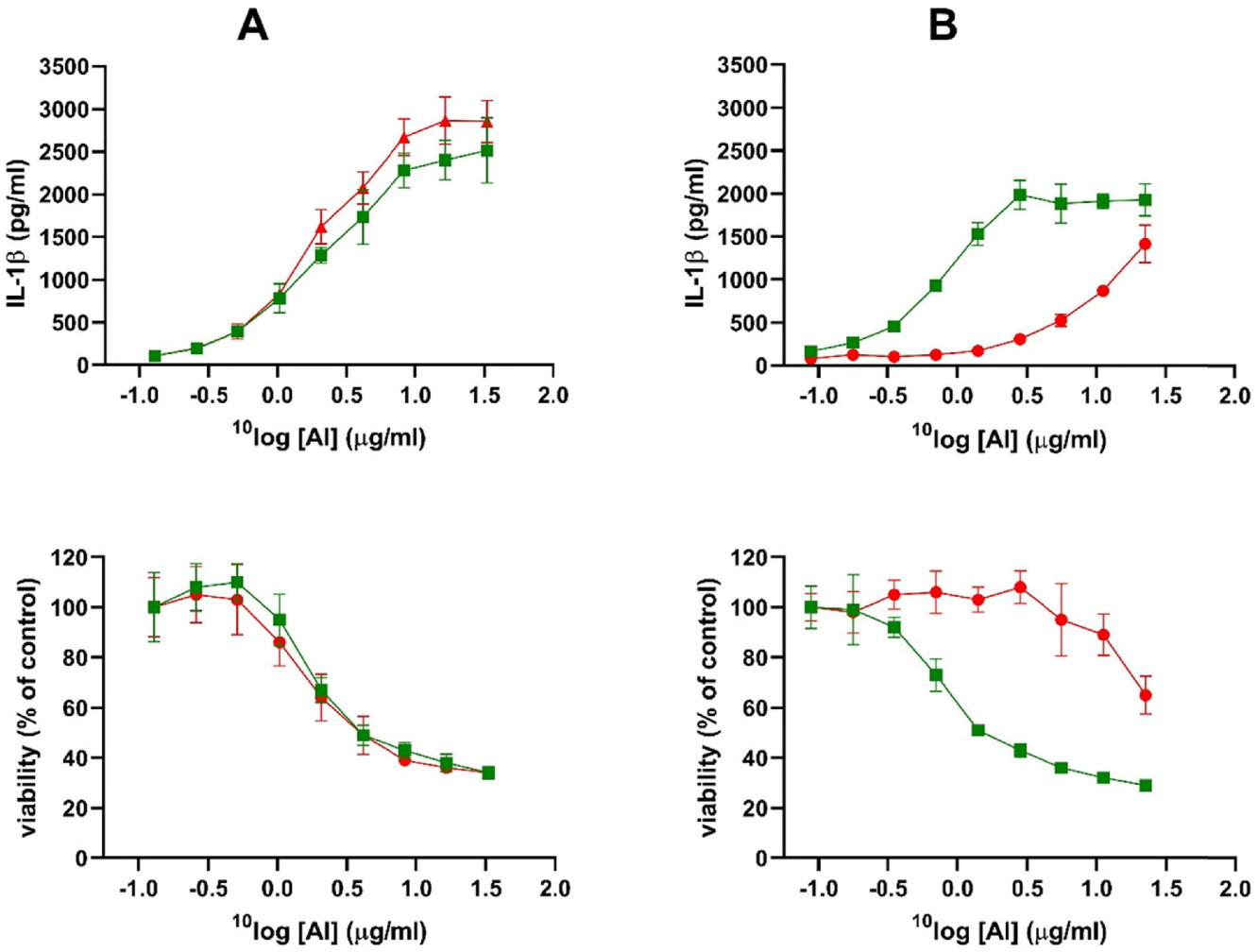

Fig. 4. Effect of repeated (3 times) freezing/thawing of the adjuvants of manufacturers A and B (AP and AH, respectively) on NLRP3 inflammasome activation of PMA-activated THP-1 cells. Left: manufacturer A, right: manufacturer B. Green: control (left at 4 °C), red: freezing/thawing. X-axis: ^{10}log [Al] (μg/ml). Y-axis top figures: IL-1β (pg/ml). Y-axis bottom figures: viability (%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adjuvant (HBsAg itself was not FT-treated). In contrast to AH, NLRP3 inflammasome activation by AP was not affected by FT. While AP and AH are known to have different particulate structures as discussed above, the underlying mechanism(s) for this observed difference in the response to FT is unknown.

Nanosized SiO_2 and nigericin were used as positive controls for NLRP3 inflammasome activation. In wild-type cells, SiO_2 showed a dose-dependent decrease in viability and a concomitant increase in IL-1β production (Supplementary Information S1). In ASC- and NLRP3-deficient cells [23], viability is 60 % at the highest SiO_2 concentration, whereas in the wild-type cells viability is 40 % at the same concentration. In contrast to the wild-type cells, the deficient cells show an almost complete lack of IL-1β production. In conclusion, the SiO_2 data supports the notion that the assay used in the current study is indeed measuring NLRP3 inflammasome activation. In wild-type cells, nigericin showed a strong decrease in viability and a strong increase in IL-1β production (Supplementary Information S2). In ASC- and NLRP3-deficient cells, viability is significantly higher compared to the wild-type cells (30 % vs 6 %; $P < 0.01$). In contrast to the wild-type cells, the deficient cells show an almost complete lack of IL-1β production. In conclusion, this nigericin data supports the notion that the assay used in the current study is indeed measuring NLRP3 inflammasome activation. Poly dA/dT stimulates the AIM2 inflammasome, an innate immune pathway that, similar to the NLRP3 inflammasome, involves ASC, caspase-1, and IL-1β, but lacks involvement of NLRP3 [24]. In wild-type cells, poly dA/dT showed a clear decrease in viability and increase in IL-β production, in line with AIM2 inflammasome activation (Supplementary Information S3). Given the involvement of ASC in AIM2 inflammasome activation, ASC-deficient cells were expected to show a higher viability compared to wild-type cells, and this is not seen. In addition, given the lack of involvement of NLRP3 in AIM2 inflammasome activation, NLRP3-deficient cells were expected to show IL-1β production similar to the wild-type cells. Together, this data question to some extent the adequate performance of the deficient cell lines in our hands. Still, we feel that the adjuvant data, supported by the SiO_2 and nigericin data, support a role for NLRP3 inflammasome activation by AP and AH.

The ability to discriminate between batches of standard and substandard quality is an important prerequisite for an assay to be included in a battery of *in vitro* assays to monitor batch-to-batch consistency. Future studies using the NLRP3 inflammasome assay may include testing of adjuvants after extended storage.

In conclusion, we have developed a quantitative *in vitro* assay to measure NLRP3 inflammasome activation as a readout for vaccine adjuvant biological activity. NLRP3 inflammasome activation was induced only by the adjuvant, the presence of the DTaP vaccine antigens did not alter the response.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.08.022.

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