Chapter 3

In vitro assessment of tick-borne encephalitis vaccine: suitable human cell platforms and potential biomarkers


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Abstract

Tick-borne encephalitis (TBE) virus causes a severe disease that can lead to permanent neurological complications. The whole inactivated TBE vaccine is highly effective, as proven by high seroconversion rates and near eradication of the disease in countries where vaccination programs have been implemented. TBE vaccine potency testing currently requires the use of in vivo methods that present issues of reproducibility as well as animal discomfort. As an alternative, public and private entities are currently exploring a batch-to-batch consistency approach which would demonstrate conformity of a newly produced vaccine batch with a batch of proven in vivo efficacy with respect to a range of in vitro measurable quality parameters. For the identification of a suitable cellular platform to be used in a panel of in vitro batch-to-batch assessments for the TBE vaccine, we exposed human cell-based systems, both of primary origin and cell line-derived, to vaccine formulations of high and low quality. Following stimulation, cell responses were evaluated by assessing the expression of selected genes by qPCR. Our findings show that the expression of interferon-stimulated genes differed after treatment with non-adjuvanted vaccine batches of different quality in peripheral blood mononuclear cells (PBMCs) and in monocyte-derived dendritic cells, but not in monocyte-free PBMC suspensions nor in cell line-derived immune cells. These results indicate suitable platforms and potential biomarkers for a cell-based assay that, together with other immunochemical analyses, could serve for batch-to-batch assessment of the TBE vaccine, reducing (and eventually replacing) in vivo methods for potency testing.

Keywords: Tick-borne encephalitis virus; TBE vaccine; peripheral blood mononuclear cells; THP-1; interferon-stimulated genes; qPCR; in vitro
In vitro assessment of the TBE vaccine

**Introduction**

Tick-borne encephalitis virus (TBEV) is an arthropod-borne flavivirus endemic in forested areas across Europe and Asia, and the etiological agent of a neuroinvasive illness that can lead to severe long-term sequelae [1,2]. Approximately 13,000 TBEV-related cases of human encephalitis and meningitis are reported annually worldwide [3,4]. Furthermore, the incidence of the disease has increased by more than 300% in Europe and Russia in the last 30 years, because of climate and socio-economical changes [5,6]. Existing TBE vaccines are highly immunogenic and provide at least 10 years of antibody persistence [7] through the induction of envelope protein-directed antibodies and of TBEV-specific CD4+ T cells [8]. Endemic countries where vaccination measures have been implemented have seen a dramatic decline in the disease, with a rate of protection of over 95% [9].

Currently, several licensed TBE vaccines are available across central Europe, Russia and Asia. All consist of formalin-inactivated whole virus formulations, and differ in the viral strains and the excipients used [10]. The European vaccines FSME-IMMUN (Pfizer) and Encepur (GlaxoSmithKline) must undergo, before release, mandatory batch validation by means of in vivo potency testing, as stated by the European Pharmacopoeia [11]. Each newly produced batch is compared to a reference batch of proven quality by assessing the protection rate of immunized mice infected with a lethal viral dose. The procedure presents conspicuous disadvantages, from causing significant pain and distress to a large number of animals, to showing potency variations of up to 300% [12]. Additionally, the phylogenetic distance between laboratory animals and humans may limit the predictive value of such in vivo tests [13].

In the context of vaccine potency testing, a “consistency approach” has been proposed to replace, reduce or refine the use of animal tests. This approach designs a lot-release strategy in which in vivo tests may only be needed for the reference lots; in vitro assays can then be used to assess the conformity of new vaccine batches to earlier lots of proven safety and efficacy [14]. Nowadays, several in vitro techniques provide the opportunity of investigating the functional features of vaccines on models for innate or adaptive immune responses [15,16]. Antigen-presenting cells (APCs), as the sentinels of the immune system and main targets and vectors of TBEV during primary viremia [17], represent an ideal candidate platform for testing vaccine-induced innate immune responses. APCs and APC-like cell lines have been regularly used to analyze vaccine functions in vitro through multiparametric techniques [18–20], and have been shown to respond to vaccines in characteristic and vaccine-specific ways [21].
In this study, we aimed to identify a cellular platform that could serve to reproducibly assess cellular responses induced by TBE vaccine batches. The cellular platform for such an assay should therefore satisfy two criteria: to display up- (or down-) regulation of biomarkers indicative of the vaccine quality - with distinctive responses to high-quality (conforming to product specification) and low-quality (non-conforming) vaccine batches - and to respond in a reproducible manner. We assessed a human cell line-based system, advantageous for relying on a consistent source of cells not subject to donor-dependent variation, and a primary cell-based system, that preserves important in vivo properties [22]. The cell activation following treatment with TBE vaccine formulations was evaluated in THP-1-derived cells - as candidates for cell line-based APC models [23,24] - and in cryopreserved human peripheral blood mononuclear cells (PBMCs) and subpopulations derived from these cells.

THP-1-derived cells did not demonstrate TBE vaccine-specific activation and thus proved unsuitable for our purposes. In contrast, primary cell-based platforms were able to respond specifically to the non-adjuvanted TBE vaccine through increased transcription of interferon-stimulated genes. Using selected interferon-stimulated genes (ISGs) as biomarkers in PBMCs, we were able to identify differential innate responses to conforming and non-conforming vaccine batches, in an assay that is highly sensitive to differences in the product formulation. Our results indicate that a primary cell-based system can successfully be included in an array of in vitro methods for the evaluation of the conformity of TBE vaccine batches.

Materials and Methods

Vaccines and virus

Vaccine formulations. Non-adjuvanted TBE vaccine (“NAV”; 60 μg/mL protein) and the alum-adsorbed Encepur vaccine (“vaccine”; 3 μg/mL protein, 2 mg/mL aluminum hydroxide) were kindly provided by GlaxoSmithKline (GSK, Marburg, Germany). NAV, the antigen-containing fraction of the Encepur vaccine, consists of whole, formalin-inactivated TBEV in a 42% sucrose solution – thus contains virions including structural proteins and viral genome. Encepur is prepared from NAV by dilution of the inactivated virus and addition of the adjuvant [12]. To produce non-conforming batches, NAV was heat-treated at 42°C for 4 weeks or at 100°C for 15 min in glass vials (“HT-NAV 42C” and “HT-NAV 100C”, respectively). A 42% low-endotoxin sucrose (Sigma-Aldrich, St. Louis, USA) solution in DMEM medium (Gibco, Life Technologies; Paisley, UK) was used as control (“matrix”) for NAV, per indications of GSK. A vaccine excipient solution was provided by GSK and used as vaccine control (“excipient”).
Culture and quantification of TBEV. Live Tick-Borne Encephalitis virus (strain Neudörfl H2J) was obtained from the European Virus Archive (Marseille, France). 300 μL of the virus seed (10^4 TCID_{50}/mL) were expanded on Vero E6 cells (ATCC, Rockville, MD), grown in DMEM medium (Gibco) supplemented with 10% fetal calf serum (FCS; Life Science Production, Bedford, UK) and 1% penicillin/streptomycin (Gibco). The virus culture was performed over 21 days, transferring the cell culture supernatant from the inoculum in 0.3 * 10^6 cells to 1 * 10^6 cells, and then to 2.7 * 10^6 cells - on days 7 and 14, respectively. The infectious particles in the supernatant were quantified by plaque assay on A549 cells (ATCC), highly susceptible to the virus cytopathic effect [25]. Briefly, monolayers of A549 cells cultured in 12-well culture plates were inoculated with 10-fold dilutions of TBEV-containing cell supernatants for 4 h at 37°C. The cells were overlaid with 2% agarose in 2X MEM medium and incubated for 4 days at 37°C with 5% CO₂. The cells were then fixed with 10% formaldehyde for 1 h, the overlay was discarded and the cells stained with crystal violet to visualize the plaques. The virus titers were expressed as plaque-forming units (PFU) per mL.

Cellular platforms

THP-1 cells. The human monocytic cell line THP-1 (ATCC) was grown in RPMI-1640 medium (Gibco) supplemented with 10% FCS, 1% penicillin/streptomycin, 1mM sodium pyruvate (Gibco) and 50 μM β-mercaptoethanol (Gibco). The cells were either used as such or differentiated to a dendritic phenotype (medium supplemented with GM-CSF and interleukin-4 (IL-4) (both 1500 U/mL, ProsPec, Rehovot, Israel) added every 2 days over a period of 5 days), or a macrophage phenotype (medium supplemented with 100 nM phorbol 12-myristate 13-acetate (PMA, ThermoFisher Scientific, Waltham, USA) for 48 h, followed by 24 h in PMA-free culture medium). All cells were cultured at a density of 0.3 * 10^6 cells/mL in 24-well plates and incubated at 37°C, 5% CO₂.

PBMCs. Buffy coats were purchased from the Dutch blood bank (Sanquin, Groningen, The Netherlands) who had obtained consent of the donors to use the cells for scientific research. Peripheral blood mononuclear cells were isolated as previously described [21]. Briefly, buffy coats were mixed with RPMI-1640 and layered on Ficoll Paque (GE Healthcare, Uppsala, Sweden). After centrifugation, PBMC fractions were collected and red blood cells lysed with Ammonium-Chloride-Potassium (ACK) lysis buffer (ThermoFisher Scientific). PBMCs were then stored in cryopreservation medium (90% FCS, 10% DMSO) in liquid nitrogen until needed. PBMCs were thawed as previously described [21], and seeded at a density of 2 * 10^6 cells/mL in 24-well plates in RPMI-1640 supplemented with 10% FCS, 50 μM β-mercaptoethanol, and 1% penicillin/streptomycin. Cells were incubated at 37°C, 5% CO₂.

Monocyte-derived dendritic cells. Monocytes differentiated from thawed PBMCs were isolated using an immunomagnetic negative selection kit (MagniSort Human pan-
Monocyte Enrichment Kit, ThermoFisher Scientific). To obtain immature DCs, monocytes were seeded at a density of $1 \times 10^6$ cells/mL in 24-well plates and culture in medium supplemented with GM-CSF (450 U/mL) and IL-4 (500 U/mL). Fresh cytokines were added every 2 days over a period of 6 days.

**Cell stimulation**

Cells were stimulated for 24 or 48 h with the vaccine formulations (or their control solutions) at dilutions varying from 1:16 to 1:4000 (equivalent to concentrations from 4 to 0.015 μg/mL for NAV and from 0.192 to 0.00075 μg/mL for the adjuvanted vaccine). Incubation with live TBEV was performed for 24 or 48 h at a multiplicity of infection (MOI) of 1, 5, or 10. The TLR7 stimulant R848 (10 μg/mL; Invivogen, Toulouse, France) and human IFNα2a (1000 U/mL; ProsPec) were used as positive controls.

**Toxicity determination, surface marker staining and flow cytometry analysis**

To determine the effect of the treatments on cell viability, PBMCs and THP-1 cells were stained with Viability Fixable Dye (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the cells were washed with PBS and resuspended in cold washing buffer (1× PBS supplemented with 2% FCS and 1 mM EDTA), then incubated for 15 min at room temperature with the dye. Cells were then washed, fixed in 200 μL of 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in PBS for 30 min, washed again and finally resuspended in 200 μL of washing buffer for flow cytometry analysis. In order to examine the expression levels of selected surface markers in THP-1 cells by flow cytometry analysis, APC-labeled recombinant anti-human antibodies (CD11c, CD14, CD32, CD299 and CD120c, all from Miltenyi Biotec) were used for staining according to the manufacturer’s instructions. All flow cytometry analyses were performed on a FACSVerse flow cytometer (BD Pharmingen, San Diego, CA, USA). Data were analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

**Cell lysis, RNA isolation and RT-qPCR**

To detect changes in the gene expression of stimulated cells, cell lysates of different cell subsets were collected and the mRNA levels of selected genes analyzed through RT-qPCR.

**Cell lysates.** Non-adherent cells in culture supernatants were collected, centrifuged (300 g, 10 min) and lysed by adding 350 μL RLT buffer (Qiagen, Hilden, Germany) + 1% β-mercaptoethanol. The adherent cells were lysed in-well. These lysates were then combined and stored at -20°C until further analysis. For what concerns the evaluation of gene expression in adherent vs non-adherent PBMC subsets, the lysates were instead kept separate.
**RNA isolation and RT-qPCR.** RNA isolation was performed using the RNeasy Mini Kit (Qiagen) following the instructions of the manufacturer. cDNA from the isolated RNA was generated using the Primerscript RT Reagent kit (Takara, Saint-Germain-en-Laye, France) according to the manufacturer’s instructions. The cDNA was then analyzed by qPCR: the reaction [10 μL 2x ABsolute qPCR SYBR Green Mix (ThermoFisher Scientific), 1 μL 10 mM forward primer, 1 μL 10 mM reverse primer, 1.5 μL cDNA and 6.5 μL H2O] was carried out for 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C in a CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, CA). The gene expression levels of the target genes were normalized against the housekeeping gene GAPDH and quantified relatively to the expression levels in non-treated cell cultures (primer sequences shown in Table S1). Data were analyzed according to the comparative Ct method [26] and are expressed as fold change.

**Statistical Analysis**

Significant differences between the responses to the vaccine formulations and to their respective negative controls were determined using the unpaired Student’s t-test. Significant differences across multiple groups were determined using two-way ANOVA, applying correction for multiple testing. A p-value of p < 0.05 was considered significant and indicated by *; ** stand for 0.01 and *** for 0.001. Statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).
Results

THP-1 cells do not show specific responses to TBE vaccine formulations

To develop an *in vitro* system suitable for assessing the conformity of batches in the context of commercial TBE vaccine production, we initially turned to THP-1 cells, a human monocytic cell line extensively used to study monocyte or macrophage functions [27]. We first determined the viability of THP-1 cells upon stimulation with TBE vaccine and non-adjuvanted vaccine (NAV), along with their respective negative controls - excipient and matrix (Fig. S1). Cells incubated for 24 hours with equal dilutions of vaccine or NAV (and their controls) showed a dose-dependent decrease in viability. The antigen concentrations at which vaccine and NAV were tolerated by the cells differed – likely due to the presence, in the former, of aluminum hydroxide. Even when used at final concentrations as low as 0.2 µg/mL (corresponding to the 1:16 dilution), the vaccine (and the excipient) induced cell death in >30% of the cells. In contrast, NAV (and matrix) was well tolerated even at 5-10 times higher concentrations.

To assess the activation of THP-1 cells by the treatments, the gene expression levels of *TNF-α* and *IL-12p40* (indicators of an inflammatory response) and of *MxA* and *ISG56* (antiviral genes; transcription of *ISG56* has been shown to increase upon incubation with live TBEV [28]). While THP-1 cells responded to positive controls R848 and IFNα2a, the TBE vaccine and NAV did not induce stronger stimulation than their respective controls (Fig. S2A-D). Responses were also evaluated after a longer incubation period, yielding similar results (Fig. S3). When exposed to live virus, THP-1 cells did however show increased expression of *TNF-α*, *ISG56* and *MxA* (Fig. S4). Thus, while the cells do possess the receptor for the entry of TBEV and the machinery necessary for responding to the replicating virus, vaccine and NAV were unable to trigger these responses.

To increase the cells’ sensitivity [29–31] and improve the specificity of the responses to the TBE vaccine, THP-1 cells were differentiated to dendritic-like (DC) and macrophage-like (MΦ) cells (Fig. S5) [23,31,32]. Yet, the responses remained not specific: no significant differences between the responses to NAV and matrix were found (Fig. S6A, B), nor between the vaccine and excipient (data not shown). From these results we concluded that, regardless of their differentiation status, THP-1 cells were not suitable for assessing TBE vaccine-induced responses based on the chosen markers.
Human PBMCs show NAV-specific upregulation of interferon-stimulated genes

As THP-1 cells proved to be an unsuitable platform for assessing TBE vaccine-induced changes in expression of the selected genes, we next turned to primary immune cells. The viability of peripheral blood mononuclear cells (PBMCs), isolated from a healthy donor and cryopreserved, was evaluated after an incubation of 24 hours with TBE vaccine and NAV. Of note, the TBE vaccination status of the donors in this study was unknown; however, given the absence of a governmental recommendation and the very low incidence of TBE in the Netherlands (with a total of only 12 cases reported so far), it is highly unlikely that the donors had been previously exposed to the virus or the vaccine [33]. Similar to the results obtained in THP-1 cells, the tolerability of the vaccine was lower than that of NAV for the same antigen content. The vaccine reduced the cell viability by more than 20% when used at concentrations higher than 0.012 µg/mL (corresponding to a 1:250 dilution), while NAV was well tolerated up to about 1 µg/mL (1:63 dilution in the cell suspension). On average, the primary cells exhibited a slightly higher loss in viability as compared to THP-1 cells undergoing the same treatment (Fig. S7).

We next analyzed the effect of vaccine and NAV on the expression of the previously selected genes. The vaccine increased the expression levels of IL-12p40, TNF-α, and MxA considerably (Fig. 1). However, similarly strong effects were observed for the excipient, indicating that the responses were most likely due to the adjuvant rather than to the vaccine. In contrast, NAV did exert responses far exceeding those to the corresponding matrix control, indicated by a NAV-specific increase in expression levels of type I interferon-stimulated genes (ISG) such as ISG56, MxA (Fig. 1C, D) and Viperin (data not shown). In contrast, inflammatory responses were not consistently induced in a NAV-specific manner. Given the high toxicity – at high antigen concentrations – and non-specificity (at low concentrations) of the responses to the adjuvanted vaccine, our experiments hereafter focused on the responses induced by NAV. To verify the reproducibility of these findings, cryopreserved PBMCs isolated from 10 healthy donors were analyzed for their responses to NAV by RT-qPCR analysis of the candidate biomarkers ISG56 and MxA (Fig. 2A, B). Even though we observed donor-to-donor variability in the degree of cell activation, all donors displayed significantly higher ISG56 responses to NAV than to the matrix – with the exception of donor #3. Notably, cells from the unresponsive donor also showed absent or very limited activation by TLR ligands and IFNα2a, used as positive controls (Fig. S8). These results convinced us that, despite the inherent variability in the extent of responses, a platform based on cryopreserved primary PBMCs could be used to evaluate cell activation by the non-adjuvanted vaccine for tick-borne encephalitis.
Figure 1. Gene expression levels of human PBMCs from a healthy donor exposed to TBE vaccine or NAV. (A-D) After 24 h of incubation with the indicated stimuli, expression levels of IL-12p40, TNF-α, ISG56 and MxA were analyzed by RT-qPCR. Concentration of vaccine and NAV is indicated as the dilution factor (v/v) in the medium, and corresponds to 0.75, 3, 12, 48 and 192 ng/mL for the vaccine and to 0.015, 0.06, 0.24, 1 and 4 μg/mL for NAV. Bars represent the mean fold change in gene expression as compared to the non-treated (NT) control +/- standard deviation of 3 independent experiments. Levels of significance: *: p ≤ 0.05; **: p ≤ 0.01 and ***: p ≤ 0.001.

In order to identify the most responsive subpopulation(s) of PBMCs, we assessed the upregulation of ISG56 in different cell subsets obtained from two of the pre-screened donors (donors #1, medium responder, and #7, high responder; Fig. 2C). First, we evaluated the activation of the adherent cells in the PBMC cultures. These cells, which largely consist of monocytes [34], displayed upregulated transcriptional levels of ISG56 in response to stimulation with NAV, similar to unfractionated PBMC cultures. Non-adherent cells present in the culture supernatant, on the other hand, did not show NAV-specific IFN responses. Differentiating PBMC-derived monocytes into DCs resulted in even stronger upregulation of ISG56 by NAV, compared to unfractionated PBMCs or adherent cells. Thus, the capability to respond to NAV resides in the myeloid rather than the lymphoid cell population.
**Figure 2. Responses to non-adjuvanted TBE vaccine in human immune cells from multiple donors.** (A, B) Expression levels of *ISG56* and *MxA* in 10 donors following NAV (1:250 v/v; 0.24 μg/mL) and matrix stimulation were analyzed by RT-qPCR. (C) Several cell subsets obtained from the PBMCs of donors #1 and #7 were stimulated with NAV (1:250 v/v; 0.24 μg/mL) and matrix, and the expression levels of *ISG56* were analyzed by RT-qPCR. Results represent the mean fold change in gene expression as compared to the non-treated control +/− standard deviation of 3 independent experiments. Levels of significance: ns: p > 0.05; *: p ≤ 0.05; **: p ≤ 0.01 and ***: p ≤ 0.001.

**NAV-specific responses in primary cells are consistent across batches, distinctive of unaltered TBEV antigens and can be used in a highly sensitive assay**

We next aimed to determine whether the identified responses were consistent across different conforming batches of NAV, and whether the PBMC platform was sufficiently sensitive in distinguishing high- and low-quality TBE NAV batches.
Seven different batches of NAV induced almost identical levels of upregulation of *ISG56* and *MxA* expression in PBMCs from two different donors, indicating high consistency of the *in vitro* responses to conforming products (Fig. 3A, B). We then artificially produced, from 4 NAV batches, ‘non-conforming’ batches using two different heat treatments, i.e. a 42°C/4 weeks treatment and a 100°C/15 min treatment, simulating poor NAV handling. These treatments are being investigated for their effect on the binding of TBE vaccine-specific antibodies to the antigens [35]. Exposure of PBMCs to heat-treated non-conforming NAV batches resulted in significantly reduced expression of *ISG56* as compared to exposure to conforming batches. Changes in the expression of *MxA* followed a similar trend but were less consistent, with non-significant differences between the responses to some conforming and heat-treated batches.

![Figure 3. Expression of interferon-stimulated genes in human PBMCs treated with conforming and non-conforming NAV. Changes in gene expression of *ISG56* (A) and *MxA* (B) in donor #4 and #9 following 24 h stimulation with conforming and non-conforming (heat-treated) NAV batches (1:250 v/v; 0.24 μg/mL) were analyzed by RT-qPCR. NAV batches 1-7 were used as conforming batches; batches 1-4 were also used to generate two sets of heat-treated NAV (42°C/4 weeks, 100°C/15 min). Bars represent the mean fold change in gene expression as compared to the non-treated control +/− standard deviation of 4 independent experiments. Levels of significance: ns: p > 0.05; *: p ≤ 0.05; **: p ≤ 0.01 and ***: p ≤ 0.001.](image-url)
To evaluate the sensitivity of the PBMC platform in identifying differences between conforming and non-conforming batches, we created mixtures of the two formulations at varying ratios. As a non-conforming formulation, for this assay we used a naturally aged (24 months) batch that elicited poor antiviral responses in PBMCs of a highly responsive donor (#2), to more closely mimic normally occurring non-potent batches. The changes in gene expression of *ISG56* show that, at a very low concentration (0.06 μg/mL, corresponding to a 1:4000 dilution v/v), the NAV-induced responses increase linearly with the percentage of conforming batch present in the mixtures (Fig. 4). Similar results were obtained assessing the expression of *CXCL10* (a chemokine found to be upregulated upon stimulation with live TBEV or NAV stimulation [28,36]), which was however not evaluated across multiple donors and batches. The responses to mixtures with less than 100% conforming NAV content were all significantly different to those obtained with the optimal formulation (Table S2 and S3). These promising results, if confirmed across multiple donors, could deliver an assay able to identify losses of active compound as low as 20%.

![Figure 4](image_url)

**Figure 4. Responses in PBMCs treated with mixtures of conforming and non-conforming NAV.** (A, B) Changes in the gene expression of *ISG56* and *CXCL10* in cells from donor #2 following 24 h stimulation with mixtures of conforming and non-conforming (naturally aged) NAV batches (1:4000 v/v; 0.06 μg/mL) were analyzed by RT-qPCR. % indicates the ratio of conforming to non-conforming batch mixture (i.e. 40% = 40% conforming NAV, 60% non-conforming NAV). Bars represent the mean fold change in gene expression as compared to the non-treated control +/- standard deviation of 3 independent experiments.

In conclusion, using the expression of *ISG56* as readout, the PBMC-based *in vitro* system here presented responds in a consistent way to multiple conforming NAV batches and across multiple (responsive) donors, showing significantly reduced responses to non-conforming batches.
Discussion

In this study, we aimed to identify a cellular platform that could be used for assessing the conformity of TBE vaccine batches \textit{in vitro}. We assessed several cell-based systems for their capacity to discriminate between altered and non-altered TBEV antigens, and to display low variability in their responses. Using RT-qPCR analysis, we showed that human peripheral blood mononuclear cells (PBMCs) stimulated with non-adjuvanted TBE vaccine (NAV) displayed changes in the expression of several ISGs, which were: 1) donor-independent in their upregulation, 2) consistent across different batches and 3) significantly (in the case of \textit{ISG56}) lower in magnitude after stimulation with non-conforming batches. These results support the suitability of a PBMC-based system for the comparative evaluation of TBE NAV batches \textit{in vitro}.

\textit{In vitro} cell-based systems have been studied extensively for the purpose of vaccine assessment, and proposed as a valid replacement method for \textit{in vivo} potency tests [18,21,37–41]. Most of these systems use cell lines, such as MUTZ-3 and THP-1, to achieve high reproducibility of the results, a desirable characteristic in a quality control setting. Due to earlier discouraging results with MUTZ-3 cells [21], here we focused on THP-1 cells as a cell line-based platform for the assessment of vaccine batches. These cells have long been used as an \textit{in vitro} macrophage model and can also be differentiated to a DC-like phenotype [31,42–45]. Yet, despite our efforts in exploring a variety of stimulation and differentiation conditions, we could not identify any TBE vaccine-specific responses in THP-1 cells. The cell line was, however, able to display antiviral responses after incubation with live TBEV. Indeed, previous studies showed that THP-1 cells are permissive to infection with the virus, but mount lower responses to live TBEV than other \textit{in vitro} platforms [46,47]. Thus, while being a valuable antigen-presenting cell model, THP-1 cells appear to be a weak platform for investigating live or inactivated TBEV.

The inability to exert vaccine-specific responses in THP-1 cells prompted us to turn to PBMCs as primary cell platform for vaccine screening. We showed that PBMCs, frozen immediately after isolation and preserved in liquid nitrogen for extended times, after thawing readily responded to exposure to NAV by upregulation of \textit{ISG56} and \textit{MxA} expression. In a vaccine screening setting, cryopreserved PBMCs are much more convenient than freshly isolated ones, as they do not present the disadvantages of limited and time-restricted availability or repeated isolation. Contrasting results have been reported regarding the effects of cryopreservation on primary cells’ responses, with some studies showing retainment of functionality and population frequency [48,49] and others indicating differences in cytokine production and gene expression [50,51].
Thus, responses identified in freshly-isolated cells should be validated when using cryopreserved PBMCs, with particular attention to the cell subset(s) necessary for the designed assay. In a head-to-head comparison of monocyte-derived DCs differentiated from fresh or frozen PBMCs, Tapia-Calle et al. indicated that cryopreservation does not affect the cells’ ability to respond consistently to vaccine candidates [21]. The retained functionality of monocytes in cryopreserved PBMCs proved to be essential for our assay, as the activation induced by NAV was found to be dependent on the responses of the myeloid cell fraction. Cryopreserved PBMCs are therefore an advantageous and reliable platform for the in vitro test here described.

A possible problem of using a primary cell-based platform is inter-individual variability. In our study, we found that while the responses to the non-adjuvanted vaccine differed quantitatively among donors, the changes in expression of ISGs were qualitatively consistent and significant compared to non-treated or matrix-treated cells. The presence of non-responding donors (in our case, 1 out of the 10 we screened) can be an issue when assessing vaccine responses in vitro. However, non-responders can be identified by the lack of cell activation upon treatment with pattern recognition receptor ligands. For using a PBMC-based assay in an industrial setting – which requires quantitatively consistent results – donor cells could be pre-screened, selected based on their responsiveness to reference compounds (such as LPS and IFN) and to reference vaccine batches, and then cryopreserved. To further mitigate donor-dependent variability, pooling of PBMCs from several donors could be implemented, as it has been shown to reduce inter-assay variation in other in vitro assays [52,53]. The data presented shows that, among the biomarkers investigated, ISG56 performed best with respect to specificity and sensitivity. Further analyses focusing on CXCL10 or other interferon-stimulated genes (e.g. ISG15 and ISG54, highly upregulated in response to live TBEV [54]) might reveal more promising markers for this purpose.

Our results demonstrate the ability of TBE NAV batches to consistently induce the transcription of selected interferon-stimulated genes in human primary cells. Indeed, pathway enrichment analysis of microarray data has shown that type I IFN-related genes are common early markers in PBMCs stimulated with several viral vaccines [55]. In contrast, selected inflammatory markers were not associated with the response to NAV, which was unexpected as pro-inflammatory cytokines are often upregulated in PBMCs stimulated with whole inactivated vaccines [20,56]. As the molecular mechanisms involved in cell activation by TBEV (live or inactivated) are still poorly understood [57], the described PBMC platform could be a valuable tool for the identification of relevant immune pathways.
A limitation of our *in vitro* system is that it could only assess the quality of the non-adjuvanted vaccine, and not that of the final vaccine product. The evaluation of the adjuvanted TBE vaccine by the PBMC-based platform proved to be unfeasible due to the low concentration of viral antigens and, more importantly, the high alum content in the adjuvanted formulation. While the adjuvant is safe for human use [58], a considerable amount of evidence indicates that aluminum can interfere with *in vitro* assays, and that the toxicity of aluminum hydroxide in cell-based assays can affect the viability of the cellular platform [59–62]. Our results show that the PBMC-based system could not tolerate the adjuvanted formulation at antigen concentrations required for vaccine-specific responses. WHO guidelines indicate that, for nonclinical and initial clinical evaluation of aluminum-adjuvanted vaccines, the potency assessment may require multiple tests, including potency tests prior to adsorption with the adjuvant [63]. The assay here described could therefore be used to evaluate the quality of the TBEV antigen in the pre-adsorption product, thereby allowing identification of inferior batches for commercial purpose but also for process performance qualification before they enter expensive and ethically problematic animal testing.

The importance of cell-based approaches for the quality control of established vaccines has been emphasized in recent years by various international organizations [64–66]. The concerted efforts of many research groups and consortia (e.g. VAC2VAC, a collaborative research project funded by the Innovative Medicine Initiative) has resulted in several studies examining replacement, reduction and refinement (3Rs) principles in the context of vaccine production [37,40,67]. Indeed, there is growing scientific evidence that 3Rs strategies are applicable in vaccine development, evaluation and release. The consistency approach facilitates this transition, as it designs a lot testing strategy that requires animal tests only for the profile definition of the reference product. Then, verification of the conformity between the newly produced and the reference batches could be performed through several *in vitro* assays [68]. In this context, our assay could be included in a panel of *in vitro* analyses [35] which can eventually replace animal testing for batch release control.

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## Supplementary data

### Table S1. Primer list for the genes assessed through RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Source</th>
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<td>IL-12p40</td>
<td>CTGCCAGAGGAAGATGTGTC</td>
<td>CATTTCAGGGGGCATCCG</td>
<td>Own design</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ATGAGCAGAAAGCATGATCC</td>
<td>GAGGGCTGATTAGAGAGGTC</td>
<td>Rajput et al., MolCanther (2013)</td>
</tr>
<tr>
<td>ISG56</td>
<td>CCTGGAGTACTATGAGGGGC</td>
<td>TGGGTGCTAAGGACCTTGTC</td>
<td>Holzinger et al., JVirol (2007)</td>
</tr>
<tr>
<td>MxA</td>
<td>TTCAGACCTGATGGCTATC</td>
<td>TGGATGATCAAAGGATGTTG</td>
<td>Holzinger et al., JVirol (2007)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGGGCTTTAATCTCTGTT</td>
<td>CCCCAGGATTTTGGAGGGA</td>
<td>Abubaker et al., PLOS One (2013)</td>
</tr>
</tbody>
</table>
Figure S1. Viability of vaccine-stimulated THP-1 cells. THP-1 cells were assessed for their viability following 24 h incubation with TBE vaccine or non-adjuvanted TBE vaccine (and their respective controls, excipient and matrix) by flow cytometry using a Viability Fixable Dye. Concentration of vaccine and NAV is indicated as the dilution factor (v/v) in the medium, and corresponds to 3, 12, 48 and 192 ng/mL for the vaccine and to 0.06, 0.24, 1 and 4 μg/mL for the NAV. Results represent the mean percentage of viable cells +/- standard deviation of 4 independent experiments.
Figure S2. Gene expression levels in THP-1 cells exposed to TBE final vaccine or non-adjuvanted vaccine (NAV). (A-D) THP-1 cells were exposed to the indicated stimuli for 24 h and expression levels of 4 genes representative of inflammatory and antiviral responses (IL-12p40, TNF-α, ISG56 and MxA) were analyzed by reverse transcription polymerase chain reaction (RT-qPCR). Concentration of vaccine and NAV is indicated as the dilution factor (v/v) in the medium, and corresponds to 3, 12, 48 and 192 ng/mL for the vaccine and to 0.06, 0.24, 1 and 4 μg/mL for the NAV. Bars represent the mean fold change in gene expression as compared to the non-treated (NT) control +/- standard deviation of 4 independent experiments.
Figure S3. Gene expression levels of THP-1 cells following 24 h stimulation. Expression levels of IL-12p40, ISG56 and MxA were analyzed by RT-qPCR following 48 h incubation with the indicated stimuli. Concentrations of vaccine and NAV are indicated as the dilution factor (v/v) in the medium, and corresponds to 0.06 and 0.24 μg/mL. Bars represent the mean fold change in gene expression as compared to the non-treated control +/- standard deviation of 3 independent experiments. Pastel-colored bars represent the responses to positive controls and NAV, grey bars to the matrix.

Figure S4. Gene expression levels of THP-1 cells stimulated with live TBEV. Expression levels of IL-12p40, TNF-α, MxA and ISG56 were analyzed by RT-qPCR following 24 and 48 h incubation with live TBEV at different multiplicities of infection (MOI). Bars represent the mean fold change in gene expression as compared to the non-treated control +/- standard deviation of 3 independent experiments.
**Figure S5. Differentiation of THP-1 cells.** THP-1 cells were differentiated to DC-like and MΦ-like cells and differentiation was assessed via flow cytometry by analyzing the expression of the surface markers associated with the DC-or MΦ-like phenotype. The graph depicts the mean percentages of positive cells for the different markers +/- standard deviation of 3 independent experiments.
Figure S6 (in previous page). Gene expression levels of NAV-stimulated dendritic-like and macrophage-like THP-1 cells. (A, B). Differentiated THP-1 cells were exposed to the indicated stimuli for 24 h and expression levels *IL-12p40*, *TNF-α*, *ISG56* and *MxA* were analyzed by RT-qPCR following 24 h incubation with the cell treatments. Concentration of NAV is indicated as the dilution factor (v/v) in the medium, where 1:1000, 1:250, 1:63 and 1:16 correspond to 0.06, 0.24, 1 and 4 μg/mL. Bars represent the mean fold change in gene expression as compared to the non-treated control +/- standard deviation of 3 independent experiments. Pastel-colored bars represent the responses to positive controls and NAV, grey bars to the matrix.

![Viability after 24h](image)

Figure S7. Viability of TBE vaccine-stimulated human PBMCs. Cells were assessed for their viability following 24h incubation with TBE vaccine or NAV (and their respective negative controls, excipient and matrix) by flow cytometry. Concentration of vaccine and NAV is indicated as the dilution factor (v/v) in the medium, and corresponds to 0.75, 3, 12, 48 and 192 ng/mL for the vaccine and to 0.015, 0.06, 0.24, 1 and 4 μg/mL for the NAV. Results represent the mean percentage of viable cells +/- standard deviation of 3 independent experiments.
Figure S8. Gene expression levels in PBMCs stimulated with positive controls. PBMCs from a non-responder (donor 3) and a responder (donor 7) were analyzed for changes in expressions of *IL-12p40* and *ISG56* by RT-qPCR, following 24 h incubation with the positive controls R848, LPS and IFNα2a. Bars represent the mean fold change in gene expression as compared to the non-treated (NT) control $+/−$ standard deviation of 3 independent experiments. Supplementary Table 2. Tukey’s multiple comparisons test for assessing the sensitivity of the assay using *ISG56* as biomarker.
Table S2. Tukey’s multiple comparisons test for assessing the sensitivity of the assay using ISG56 as biomarker.

<table>
<thead>
<tr>
<th>Tukey’s multiple comparisons test</th>
<th>Mean Diff.</th>
<th>95.00% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT vs. 0%</td>
<td>-2</td>
<td>-8,418 to 4,418</td>
<td>No</td>
<td>ns</td>
<td>0,9194</td>
</tr>
<tr>
<td>NT vs. 20%</td>
<td>-6,74</td>
<td>-13,16 to -0,3222</td>
<td>Yes</td>
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<td>0,0374</td>
</tr>
<tr>
<td>NT vs. 40%</td>
<td>-12,5</td>
<td>-18,92 to -6,085</td>
<td>Yes</td>
<td>***</td>
<td>0,0003</td>
</tr>
<tr>
<td>NT vs. 60%</td>
<td>-16,43</td>
<td>-22,85 to -10,01</td>
<td>Yes</td>
<td>****</td>
<td>&lt;0,0001</td>
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<tr>
<td>NT vs. 80%</td>
<td>-25,25</td>
<td>-31,67 to -18,83</td>
<td>Yes</td>
<td>****</td>
<td>&lt;0,0001</td>
</tr>
<tr>
<td>NT vs. 100%</td>
<td>-32,03</td>
<td>-38,44 to -25,61</td>
<td>Yes</td>
<td>****</td>
<td>&lt;0,0001</td>
</tr>
<tr>
<td>0% vs. 20%</td>
<td>-4,74</td>
<td>-11,16 to 1,678</td>
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<td>0% vs. 40%</td>
<td>-10,5</td>
<td>-16,92 to -4,085</td>
<td>Yes</td>
<td>**</td>
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<td>-20,85 to -8,012</td>
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<td>0% vs. 80%</td>
<td>-23,25</td>
<td>-29,67 to -16,83</td>
<td>Yes</td>
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<td>&lt;0,0001</td>
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<td>0% vs. 100%</td>
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<td>-36,44 to -23,61</td>
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<td>-12,18 to 0,6545</td>
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<td>20% vs. 60%</td>
<td>-9,69</td>
<td>-16,11 to -3,272</td>
<td>Yes</td>
<td>**</td>
<td>0,0027</td>
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<td>20% vs. 80%</td>
<td>-18,51</td>
<td>-24,93 to -12,09</td>
<td>Yes</td>
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<td>&lt;0,0001</td>
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<td>20% vs. 100%</td>
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<td>-31,70 to -18,87</td>
<td>Yes</td>
<td>****</td>
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<td>40% vs. 60%</td>
<td>-3,927</td>
<td>-10,34 to 2,491</td>
<td>No</td>
<td>ns</td>
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<td>40% vs. 80%</td>
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<td>-19,16 to -6,329</td>
<td>Yes</td>
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<td>-25,94 to -13,11</td>
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<td>60% vs. 80%</td>
<td>-8,82</td>
<td>-15,24 to -2,402</td>
<td>Yes</td>
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<td>60% vs. 100%</td>
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<td>-22,01 to -9,179</td>
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<td>-13,19 to -0,3588</td>
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Table S3. Tukey’s multiple comparisons test for assessing the sensitivity of the assay using CXCL10 as biomarker.

<table>
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<th>Tukey’s multiple comparisons test</th>
<th>Mean Diff.</th>
<th>95,00% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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<td>-38,01 to 22,78</td>
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<td>-61,49 to -0,7025</td>
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<td>NT vs. 40%</td>
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<td>-85,48 to -24,69</td>
<td>Yes</td>
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<td>NT vs. 60%</td>
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<td>-117,4 to -56,66</td>
<td>Yes</td>
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<td>&lt;0,0001</td>
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<tr>
<td>NT vs. 80%</td>
<td>-129,8</td>
<td>-160,2 to -99,42</td>
<td>Yes</td>
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<td>&lt;0,0001</td>
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<tr>
<td>NT vs. 100%</td>
<td>-184,9</td>
<td>-215,3 to -154,5</td>
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<td>-53,87 to 6,914</td>
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<td>-77,86 to -17,07</td>
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<td>-109,8 to -49,04</td>
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<td>0% vs. 80%</td>
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<td>-152,6 to -91,81</td>
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<td>-86,35 to -25,56</td>
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<td>-160,2 to -99,39</td>
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