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Chapter 4

Transcriptional profiling of TBE vaccine-stimulated human PBMCs: a path from pattern recognition receptors to interferon signaling

Manuscript submitted

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Abstract

Tick-borne encephalitis virus (TBEV) causes an inflammation of the central nervous system. The disease can be effectively prevented by whole inactivated virus vaccines. Here, we investigated the innate immune profile induced in vitro by the main component of the vaccines, inactivated TBEV (I-TBEV), to gain insights into the mechanism of action of the TBE vaccine as compared to the live virus. To this end, we exposed human peripheral blood mononuclear cells (PBMCs) to inactivated and live TBEV and assessed cell responses by RNA sequencing. Both inactivated and live TBEV induced an interferon-dominated gene signature, and an increase in the expression of RIG-I-like receptors (RLRs). Using pathway inhibitors, we assessed the involvement of pattern recognition receptors in the sensing of inactivated or live TBEV. Only inhibition of the RLR signaling pathway suppressed the downstream cascade induced by I-TBEV, while responses to the replicating virus were impacted by the inhibition of both RIG-I-like and Toll-like receptors. Our results show that inactivated and live TBEV induced predominantly an interferon response in our in vitro PBMC platform, and indicate RLRs as the main pattern recognition receptors involved in I-TBEV sensing. These insights could be useful for the quality control of TBE vaccine batches and provide guidance for the design of other viral vaccines.

Keywords: Tick-borne encephalitis virus; TBE vaccine; peripheral blood mononuclear cells; RNA sequencing; interferon; RIG-I; TLR
Introduction

Tick-borne encephalitis is an inflammation of the central nervous system caused by the tick-borne encephalitis virus (TBEV), a flavivirus endemic in parts of Europe and Asia [1]. The virus is responsible for thousands of cases of human encephalitis every year, and its incidence in Western Europe has been growing in the past decades [2,3]. Currently, there are no specific treatments for TBE, but inactivated virus vaccines containing aluminum hydroxide as adjuvant are available and effectively prevent infection [4]. Their use has led to successful containment of the disease in countries with high vaccination coverage [5].

In our previous studies, we showed that the main component of the vaccines – chemically inactivated TBEV (I-TBEV) – induces interferon (IFN) responses in peripheral blood mononuclear cells (PBMCs) and plasmacytoid dendritic cells (pDCs), as well as B cell activation [6]. Moreover, the I-TBEV-induced antiviral state correlated well with the quality of the formulation, as non-conforming (heat-treated) I-TBEV failed to activate PBMCs to a similar degree as the conforming (high quality) I-TBEV [7]. While the development of adaptive responses has been investigated (showing the induction of envelope protein-directed antibodies and of TBEV-specific CD4+ T cells), a full picture of the innate immune responses induced by the TBE vaccine is yet to be presented [8]. A better overview of the molecular pathways involved could shed light on the mechanisms that contribute to a protective immune response, as well as delineate vaccine-induced innate immune functions associated with favorable vaccination outcome.

Several studies have highlighted how the specific innate immune pathways activated by certain vaccine components, such as viral RNA, can determine the efficacy of a vaccine [9–11]. Our earlier results revealed an increase in the expression of IFN-stimulated genes, but not of selected pro-inflammatory molecules, in I-TBEV-stimulated cells [7]. Here, we aimed to fully characterize the innate immune signature and identify the molecular factors – specifically the pattern recognition receptors (PRRs) and immune signaling pathways – involved in the stimulation of human primary cells by I-TBEV. To this end, we exposed human PBMCs to I-TBEV (of high or low quality) or live TBEV and assessed the response by RNA sequencing. Our results show that TBEV (live or inactivated) induced a distinct interferon-dominated signature in PBMCs, while inflammatory responses were regulated in a highly selective manner. Using pathway-specific inhibitors and reporter cell lines, we demonstrated the involvement of RIG-I-like receptors (RLRs) in the initiation of the antiviral response for I-TBEV and live TBEV, with the latter being able to also trigger selected Toll-like receptors (TLRs).
This study brings new insights into the innate immune response elicited and the signaling pathways engaged by live TBEV and by the inactivated virus – the primary constituent of the TBE vaccine.

Materials and Methods

Vaccine and virus

Vaccine formulations. K23 formalin-inactivated TBEV (“I-TBEV”; 60 μg/mL protein) was kindly provided by GlaxoSmithKline (GSK, Marburg, Germany). I-TBEV, the antigen-containing fraction of the Encepur vaccine, consists of whole, formalin-inactivated TBEV in a 42% sucrose solution. To produce non-conforming batches, a strategy that has been shown to reduce binding by TBEV-specific antibodies [12] was followed: I-TBEV was heat-treated at 42°C for 4 weeks in glass vials (“HT I-TBEV”). A 42% low-endotoxin sucrose (Sigma-Aldrich, St. Louis, USA) solution in DMEM medium (Gibco, Life Technologies; Paisley, UK) was used as control (“matrix”), per indications of GSK.

Culture and quantification of TBEV. Live TBEV (strain Neudörfl H2J) was obtained from the European Virus Archive (Marseille, France). 300 μL of the virus seed (10^4 TCID₅₀/mL) were expanded on Vero E6 cells (ATCC, Rockville, MD) for 21 days, transferring the cell culture supernatant from the inoculum in 0.3 * 10⁶ cells to 1 * 10⁸ cells, and then to 2.7 * 10⁶ cells – on days 7 and 14, respectively. The infectious particles in the supernatant were quantified by plaque assay on A549 cells (ATCC) which are highly susceptible to the virus cytopathic effect [13]. 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.

Cells

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. It should be noted that 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 [14]. Peripheral blood mononuclear cells were isolated as previously described [15].
Briefly, buffy coats were mixed with RPMI-1640 (Gibco, Life Technologies; Paisley, UK) 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, Waltham, USA). PBMCs were then stored in cryopreservation medium (90% FCS, 10% DMSO) in liquid nitrogen until needed. For the experiments, PBMCs were thawed as previously described [15], and seeded at a density of $2 \times 10^6$ cells/mL in 24-well plates in RPMI-1640 supplemented with 10% fetal calf serum (FCS; Life Science Production, Bedford, UK), 50 µM β-mercaptoethanol, and 1% penicillin/streptomycin (all from Gibco). Cells were incubated at 37°C, 5% CO₂.

**Reporter cells.** HEK-Blue™ cells (InvivoGen, Toulouse, France) co-express PRRs and an NF-κb-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene that can be monitored using the detection medium QUANTI-Blue™. Human HEK-Blue™ TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, NOD2 cells (and parental Null1 and Null2 cells) were cultured at 37 °C in 5% CO₂ according to the manufacturer’s instructions. 50,000 cells/well were plated in a 96-well plate and stimulated. After 48 h of incubation, 50 µl of supernatant were added to 150 µl of QUANTI-Blue™. After 30 min of incubation at 37°C, the plates were read in an ELISA reader (630 nm). Results are expressed as relative activation of cells in comparison to the activation level obtained upon stimulation with 2.5 µg/mL of TNF-α (ProsPec, Rehovot, Israel), which was set as 100%.

**Cell stimulation**

PBMCs and reporter cell lines were stimulated for 24 or 48 h with I-TBEV, HT I-TBEV (or the matrix control) at dilutions from 1:4000 to 1:16 (equivalent to antigen concentrations from 0.015 – 4 μg/mL). Incubation with live TBEV was performed for 24 or 48 h at a multiplicity of infection (MOI) from 1 to 100.

**Inhibitors.** Amlexanox and BX795, inhibitors for TBK1/IKKe (kinases involved in the RLR pathway), and Pepinh-MYD, an inhibitor peptide for MyD88 (signal transducer for TLR pathways), were used to pre-treat the cells for 1 or 6 h at 37 °C in 5% CO₂ before subsequent stimulation with I-TBEV or positive controls. All inhibitors were purchased from Invitrogen, and used at a concentration of 50 µg/mL (Amlexanox), 2 µM (BX795) and 10 µM (Pepinh-MYD).

**Positive controls.** TLR7 ligand R848 (10 µg/mL) and poly(I:C)-HMW/LyoVec (0.5 µg/mL) (both from Invitrogen) were used as controls to assess the stimulation of the cell platforms.
Cell lysis and RNA isolation

To detect changes in the gene expression of stimulated cells, cell lysates of PBMCs were collected and the mRNA isolated for subsequent analysis by RT-qPCR or RNA sequencing. Cells were lysed by adding 350 μL RLT buffer (Qiagen, Hilden, Germany) + 1% β-mercaptoethanol. The lysates were then stored at −20°C until further analysis. RNA isolation was performed using the RNeasy Mini Kit (Qiagen) following the instructions of the manufacturer.

RT-qPCR

cDNA from the isolated RNA was generated using the Primescript 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 [16] and are expressed as fold change.

RNA Sequencing

Library preparation and next generation sequencing (NGS). All NGS experiments (performed on RNA isolated from PBMCs of a healthy donor) were conducted by QIAGEN Genomic Services, using the QIAseq UPX 3’ Transcrintome Kit. Sequence reads were mapped to the human genome (version: hg38, annotation: NCBI RefSeq GRCh38.p11) using CLC Genomics Workbench (version 12.0.4) and CLC Genomics Server (version 11.0.3). This resulted in a table with gene count data for 54,362 genes and 24 samples.

Data analysis. Gene count data were further analyzed in R statistical software (version 3.6.2, www.r-project.org) using the following packages: DESeq2, limma, gplots, and rgl. Genes that had zero counts in all samples were considered unexpressed and discarded from further analysis. Gene count data were normalized using a variance stabilizing transformation (VST) on the remaining genes. Differentially expressed genes were selected by one-way ANOVA. p-values were corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR). Genes were considered differentially expressed if they had an FDR ≤ 5% and a Fold Change (FC) ≥ 2 (determined as the maximum vs minimum average group value across the groups).
Differences in gene expression compared to the control group were visualized by a heatmap combined with hierarchical clustering (using Euclidean distance and Ward.D linkage) as well as by Principal Component Analysis (PCA). Functional annotation and over-representation analysis of DEGs was carried out by using DAVID [17]. Additional functional analyses were conducted using the software packages Cytoscape plug-in ClueGO [18,19], Ingenuity Pathway Analysis (IPA, QIAGEN Inc. [https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis]) and Reactome [20].

Quantification of cytokines and chemokines

Culture supernatants were harvested after 24 hours of PBMCs stimulation with I-TBEV, HT I-TBEV, matrix (all diluted at 1:250 v/v) and live TBEV (MOI of 10) and stored at -80°C. The production of CXCL-10, MCP-1, and IL-8 was quantified by Cytometric Bead Assay (BD Biosciences, San Diego, CA, USA) according to manufacturer instructions.

Statistical Analysis

Statistically significant differences were determined using the unpaired Student’s t-test. Statistically 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

RNA-Seq identifies an I-TBEV-specific signature in human PBMCs

We previously showed the importance of type I IFN in the establishment of TBE vaccine-induced immune response [6]. Here, we further investigated the molecular factors and pathways involved in the innate response to the TBE vaccine by analyzing the transcriptional profile induced by its main component, whole inactivated TBEV (I-TBEV), in PBMCs from a healthy donor. The PBMCs, isolated and kept in liquid nitrogen until use, were thawed and stimulated for 24 hours – unless otherwise specified – with high quality (conforming) I-TBEV, non-conforming (heat-treated) I-TBEV (HT I-TBEV), matrix and live TBEV or left untreated. For each treatment group, 5 replicates were included (except for the non-stimulated control group, consisting of 4 replicates), their RNA was extracted and RNA sequencing (RNA-Seq) was performed. Gene expression was quantified with sequencing reads mapped to the human genome. Variance-stabilizing normalized gene read counts were used to identify differentially expressed genes (DEGs) following treatment (see Materials and Methods). Two RNA-Seq analyses were conducted: one examining the responses in cells treated with I-TBEV, HT I-TBEV, matrix or not stimulated (Analysis 1, Figure 1 and 2), and one comparing the transcription profiles of I-TBEV-treated, live TBEV-treated and untreated cells (Analysis 2, Figure 3 and 4). We will first focus on the results from Analysis 1.

Among the four treatment groups, 333 genes were considered differentially expressed based on the specified threshold level (fold change ≥ 2 and false discovery rate ≤ 5% for the average expression across the different groups). 264 genes (79.3%) were found to be upregulated upon I-TBEV treatment, and 69 (20.7%) were downregulated. Principal component analysis (PCA) of differentially expressed genes (Fig. 1A) showed a distinct profile for all I-TBEV samples, while samples in the matrix and HT I-TBEV groups clustered closer to the non-stimulated (control) group. Upon further selection of DEGs with a strong change in expression compared to the control group (minimal fold change of 2), 255 genes were found to be uniquely represented in the I-TBEV group; in the matrix and control groups, instead, only very few genes showed more than 2-fold changes in expression (Fig. 1B). A heatmap representation of the differences in expression of all 333 DEGs demonstrates that I-TBEV triggered stronger up- or downregulation of DEGs (compared to the non-stimulated cells) than treatments with HT I-TBEV or matrix (Fig. S1 for sample-specific responses, Fig. 1C for the average profile per treatment group). Interestingly, although HT I-TBEV induced a quantitatively weak response compared to treatment with I-TBEV, the profiles of up- and down-regulated genes were qualitatively similar for the two formulations. This was not the case for the matrix control.
Three main gene clusters were identified by hierarchical clustering (see below for detailed pathway analysis), and selected genes within each cluster were chosen for validation by RT-qPCR. Fold change in gene expression was assessed in 3 donors (including the donor used for RNA-Seq analysis, donor #1) (Fig. 1D). The RNA-Seq results were confirmed, as the RT-qPCR data for the three donors showed comparable I-TBEV-induced upregulation of the genes in the clusters A and B, and downregulation of genes in cluster C, despite the expected donor-dependent differences in the extent of responses. Thus, the responses identified in the NGS analysis were proven to be valid and not donor-specific.

We also assessed, in PBMCs from 4 additional donors, the cell responses at the protein level after 24 h treatment with I-TBEV, HT I-TBEV, matrix and in untreated cells (Fig. S2). While not statistically significant, a trend became apparent in which cell incubation with I-TBEV resulted in increased MCP-1, IL-8 and CXCL10 production compared to non-stimulated and matrix-stimulated cells. HT I-TBEV treatment resulted in responses mostly below those to I-TBEV, however with donor-dependent variations.

**I-TBEV induces an interferon-dominated immune profile**

To identify the biological pathways and processes associated with I-TBEV stimulation, we performed functional enrichment analysis of the identified gene clusters using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [17]. The top fifteen hits for each cluster are shown in Fig. 2.

This analysis revealed that, within the clusters A and B (comprising DEGs strongly and mildly upregulated upon I-TBEV treatment, respectively), the most over-represented functions and pathways were **Defense response to virus**, **Type I interferon signaling pathway**, **Interferon gamma-mediated signaling pathway** and **Innate immune response**. With a lower, but still significant, p-value we found activation of the KEGG pathway **RIG-I-like receptor signaling pathway** in cluster A, that comprised differentially expressed genes such as **RIG-I**, **MDA5**, **LGP2** and **IRF7**. Of note, genes such as **MxA**, **ISG56**, **CXCL10** and **Viperin** – identified in our earlier studies [7] as potential biomarkers for assessing I-TBEV responses – were found in cluster A as some of the most strongly upregulated DEGs upon I-TBEV treatment, thus confirming their important role in the response to the TBE vaccine. The role of Viperin, ISG56 and CXCL10 in the context of TBEV infection has been previously highlighted, although not in primary cells [21,22].
Figure 1. Transcriptional analysis of PBMCs stimulated with conforming and non-conforming I-TBEV (Analysis 1). PBMCs from a healthy donor were stimulated for 24 h with inactivated TBEV (I-TBEV), heat-treated I-TBEV (HT I-TBEV) or sucrose matrix at a concentration of 0.24 µg/mL (or an equivalent volume for the matrix). After treatment, the cells were lysed and processed for RNA sequencing. (A) Principal component analysis based on differentially expressed genes (DEGs) showing relative (dis)similarity for the samples. (B) Venn diagram showing DEGs with a fold change (FC) >2 in expression between the different treatment groups and the control group. Outside the circles, the number of DEGs with a FC <2. (C) Heatmap representing the fold change of DEGs in the three treatment groups, normalized to the control group (set as FC=1). (D) Validation of selected genes identified by RNA-Seq through RT-qPCR. The fold change in PBMCs from 3 donors was analyzed in duplicate for each gene and treatment. Results are shown as heatmap (normalized to the expression in the non-treated samples, set as FC=1), and compared to the RNA sequencing data.
In cluster B, the \textit{JAK-STAT cascade} was identified as upregulated, with DEGs such as \textit{JAK2}, \textit{STAT1}, \textit{STAT2} and \textit{SOCS}. The genes in cluster C, downregulated in PBMCs treated with I-TBEV, were enriched for processes associated with the \textit{Extracellular exosome}, \textit{Extracellular space} and \textit{Extracellular region}. Additionally, many DEGs in this cluster were enriched for functions related to lipid metabolism, with GO terms such as \textit{Long-chain fatty acid transport}, \textit{Fatty acid binding}, \textit{Lipid metabolic process} and \textit{Lipid catabolic process}. Interestingly, the function \textit{Inflammatory response} was found enriched for both cluster B and C: selected molecules involved with chemokine, cytokine and interleukin signaling were identified as upregulated (in cluster B) or downregulated (in cluster C) following treatment with I-TBEV. Thus, while activation of the IFN pathway was identified as an unequivocal signature induced by the main component of the vaccine, genes associated with the inflammatory response were regulated in a highly selective way.

\begin{figure}[h!]
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\caption{Top 15 GO terms and KEGG pathways identified in each cluster from Analysis 1. Hierarchical clusters depicted in Fig. 1C were analyzed for functional enrichment. The bar shows the $-\log_{10}$ of the raw p-value.}
\end{figure}
Additional analysis performed using other enrichment tools, such as Cytoscape, Ingenuity Pathway Analysis and Reactome, identified similar pathways associated with the stimulation of PBMCs with I-TBEV, in particular its induction of IFN responses, upregulation of RLRs and downregulation of genes associated with lipid metabolism and selected inflammatory responses (Fig. S3-5). Overall, functional enrichment of differentially expressed genes in PBMCs treated with I-TBEV demonstrated an interferon-dominated immune profile, and indicated a role for cytosolic pattern recognition receptors belonging to the RIG-I-like family.

**PBMCs treated with I-TBEV and live TBEV share similar transcriptional profiles**

To compare the innate immune signature of cells treated with the inactivated TBEV to that of cells incubated with the live virus, a second RNA-Seq analysis was performed on the data from the control, I-TBEV- and live TBEV-treated groups. The transcriptional profile for the live virus group was assessed with cells incubated at a multiplicity of infection of 10 for 48 h, since our previous experiments indicated that an infection of 24 h induced minimal changes in the expression of IFN stimulated genes (ISGs) such as *ISG56* (Fig. S6) and *MxA* (data not shown), earlier identified as potential biomarkers [7].

After discarding unexpressed and non-differentially expressed genes, 337 DEGs were obtained from the analysis of the control group, I-TBEV- and TBEV-treated cells. PCA for differentially expressed genes shows distinctive clustering of the three treatment groups (Fig. 3A). Stimulation with I-TBEV and virus mostly induced qualitatively comparable expression signatures, as can be observed in Fig. 3B: the DEGs showing similar regulation in both treatment groups accounted for 71.5% of the total, while 28.5% of the genes showed opposite transcriptional signatures for I-TBEV- and live virus-treated cells. The heatmap representation in Fig. 3C displays the DEGs organized by hierarchical clustering, which identifies 5 major clusters: 3 include genes similarly up- (clusters C and E) or downregulated (cluster B) in both treatment groups, while 2 include DEGs that are downregulated in I-TBEV-treated cells and upregulated in live TBEV-treated cells (cluster A), or vice versa (cluster D).

To validate these findings, and confirm that the differential profile induced by the live virus was not resulting from the extended incubation time, the expression of selected genes within each cluster was validated by RT-qPCR in PBMCs (from the aforementioned 3 donors) treated with I-TBEV or live TBEV for 24 h. The changes in gene expression, averaged for the 3 donors, are shown and compared to the results from the RNA sequencing (Fig 3D). The expression signature induced by the virus was confirmed, as the genes in each cluster showed a similar up- or downregulation as observed in the NGS results.
Figure 3. Transcriptional analysis of PBMCs stimulated with I-TBEV and live TBEV (Analysis 2). PBMCs from a healthy donor were stimulated for 24 h with inactivated TBEV (I-TBEV) at a concentration of 0.24 µg/mL or 48 h with live TBEV at an MOI of 10. After treatment, the cells were lysed and processed for RNA sequencing. (A) Principal component analysis based on DEGs. (B) Representation of DEGs with distinct or overlapping change in expression in the I-TBEV and live TBEV treatment groups as compared to the control. In red the upregulated and in blue the downregulated genes. (C) Heatmap showing the FC of differentially expressed genes (DEGs) in the two treatment groups, normalized to the control group (set as FC=1) and hierarchically clustered. (D) Validation of RNA-Seq results by RT-qPCR. The fold change in PBMCs was analyzed in duplicate for each gene and treatment, and averaged for all 3 donors. Results are shown as heatmap and compared to the RNA sequencing data.

Again, we evaluated the cytokine production in PBMCs from 4 donors in response to I-TBEV and live TBEV stimulation (Fig. S2). On average, production of IL-8 and CXCL10 was increased with both treatments, while the protein level of MCP-1 appeared to be higher following incubation with the live virus (compared to inactivated TBEV) in all but one donor.
Functional enrichment analysis of the clusters was performed as previously described, and the top 7 hits for each cluster are shown in Fig. 4. Cluster A, that included genes downregulated following I-TBEV treatment and upregulated upon incubation with the live virus, comprises functions related to lipid metabolism, a process already identified in Analysis 1. Cluster B, including DEGs downregulated in both sample groups, shows enrichment of terms involved with RNA metabolism. Clusters C and E, both including genes upregulated in I-TBEV- and live virus-treated cells, show over-representation of pathways related to the antiviral and IFN response and RLR signaling. Cluster D, comprising DEGs upregulated upon treatment with I-TBEV and downregulated by the live virus incubation, includes functions involved with the immune response.

**Figure 4. Top 7 GO terms and KEGG pathways identified in each cluster from Analysis 2.** Hierarchical clusters depicted in Fig. 3C were analyzed for functional enrichment. The bar shows the -log10 of the raw p-value.
In summary, the analysis of DEGs identified in inactivated and live virus-treated PBMCs showed that the transcriptional profiles induced by the two treatments were mostly similar, with the exception of selected immune pathways (induced by the inactivated virus only) and of functions involved in the lipid metabolism (upregulated only upon incubation with the live virus). These differences may be ascribed to the intrinsic nature of the replicating virus, that acts to suppress certain immune responses [23] and to induce intracellular membrane rearrangements [24,25].

**Inhibition of RLRs, but not of TLRs, reduces I-TBEV-induced responses**

To get further insight into the molecular cascade engaged by the TBE vaccine, we assessed the I-TBEV-induced cellular responses in the presence of inhibitors of downstream molecules in selected signaling pathways. Given the suggestion from the RNA sequencing data of the involvement of RIG-I-like receptors, we first assessed the expression of biomarkers associated with I-TBEV stimulation upon treatment with Amlexanox (“ALX”) and BX795 (“BX”), two specific inhibitors of the noncanonical IkB kinases IKKε and TANK-binding kinase 1 (TBK1) [26,27] downstream of RLRs [28].

The two inhibitors were both capable of suppressing the expression of ISGs such as ISG56 and CXCL10 in PBMCs from a healthy donor upon treatment with the positive control PolyI:C (Fig. 5B, C), thus proving their functionality at the conditions used. Additionally, ALX and BX did not induce off-target effects on the inflammatory pathway (assessed as IL12-p40 expression, Fig. 5A). ISG56 and CXCL10 have been indicated as potential biomarkers for monitoring cell responses to I-TBEV [7]. The presence of the inhibitors during stimulation with inactivated TBEV resulted in decreased expression of ISG56 in PBMCs from 3 donors, indicating the involvement of the RIG-I pathway in the transduction of I-TBEV-associated signal (Fig. 5B, G). At the conditions used, ALX imposed the strongest reduction, and the reduction was enhanced when both inhibitors were used in combination (Fig. 5G). The impact of ALX and BX on the expression of CXCL10 was even greater, as it resulted in abolished changes in expression (Fig. 5C). We also assessed the effect of the two inhibitors on the live TBEV-associated signaling; both ALX and BX affected the expression of ISG56 and, more prominently, of CXCL10 (Fig. 5B, C).

Next, we sought to analyze the possible involvement of TLRs, as the whole-inactivated virus contains intact viral RNA reported to stimulate pDCs through TLR7/8 activation [6]. To do so, we used a MyD88 inhibitor peptide, Pepinh-MYD, interfering with the transduction of the respective TLR signaling cascade [29]. First, we monitored the expression of IL12-p40 following incubation of the cells with Pepinh-MYD or its aspecific control peptide. Expression of IL12-p40 was previously found to be upregulated in PBMCs upon treatment with the TLR7/8 agonist R848 [7,30–32].
RT-qPCR analysis confirmed the functionality of Pepinh-MYD, as the expression of *IL12-p40* was reduced in R848-stimulated cells that had been pre-incubated with the inhibitor, but not with the control peptide (Fig. 5D). We then focused on the inhibitor’s effect on I-TBEV-stimulated cells: the expression of *ISG56*, normally upregulated by I-TBEV treatment, was not affected by the pre-incubation of PBMCs from 3 healthy donors with Pepinh-MYD and its peptide control (Fig. 5G). Expression of *CXCL10* was affected by both Pepinh-MYD and the peptide control, pointing to an unspecific effect (Fig. 5F). The upregulation of *ISG56* and *CXCL10* upon incubation with the live virus was instead affected only by pre-treatment of the cells with Pepinh-MYD but not the peptide control pointing to involvement of the MyD88 pathway in signaling by live TBEV (Fig. 5E,F).
Figure 5 (in previous page). Gene expression levels in PBMCs stimulated with I-TBEV or live TBEV in the presence of inhibitors. (A-F) PBMCs from a healthy donor were pre-incubated with RLR inhibitors (ALX, BX) for 1 h (A-C) or with MyD88 inhibitor (Pepinh-MYD) and its peptide control (Control) for 6 h (D-F). Afterwards, the cells were treated for 24 h with Poly I:C (0.5 µg/mL), R848 (10 µg/mL), I-TBEV (0.24 µg/mL) or live virus (MOI 10). Following stimulation, the cells were lysed and changes in gene expression were analyzed by RT-qPCR. Results are from 3 replicates. (G) PBMCs from 3 healthy donors were pre-incubated with RLR inhibitors (ALX, BX) for 1 h or with MyD88 inhibitor (Pepinh-MYD) and its peptide control (Control) for 6 h. Afterwards, the cells were treated for 24 h with I-TBEV (0.24 µg/mL), or left untreated. Following stimulation, the cells were lysed and changes in gene expression were analyzed by RT-qPCR. Bars represent the average value and dots the individual value per donor. Levels of significance: ns: p > 0.05; *: p ≤ 0.05; **: p ≤ 0.01 and ***: p ≤ 0.001.

TLR and NOD reporter cells do not respond to I-TBEV, but can be activated by live TBEV

To further assess the involvement of selected PRRs in the sensing of the TBE vaccine we turned to HEK Blue™ cells, human cells engineered to express a reporter construct upon ligand binding to various PRRs (individually expressed in each cell line). The cells were incubated for 48 h with increasing concentrations of I-TBEV or its matrix control, and their level of stimulation is reported as percentage of activation of the cells compared to the response to a fixed amount of TNF-α (set as 100% activation). While the different HEK Blue™ cell lines responded to their specific TLR and NOD2 ligands (data not shown), none of the receptors was triggered specifically by I-TBEV (Fig. 6A). The production of the reporter protein, found only at the highest concentration of I-TBEV used, was also induced by the same dose of sucrose-containing matrix solution alone, and, more importantly, the activation of the NF-κB pathway was also observed in the parental ‘Null’ cell line lacking all PRRs. Interestingly, when incubated with the replicating virus, TLR3, 7, 8 and NOD2-expressing cells did show the expression of the reporter protein in a dose-dependent manner (Fig. 6B). Thus, while some receptors could be activated by the live TBEV, the inactivated virus was unable to trigger PRR-specific responses in any of the reporter cells.
Figure 6. Activation of HEK Blue reporter cells by I-TBEV and live TBEV. (A) HEK Blue cells were stimulated with the indicated amounts of I-TBEV for 48 h at different concentrations. Subsequently, supernatants were added to the detection medium for assessment of NF-κB-induced production of the reporter protein. The stimulation of the cells is presented as percentage of activation relative to the activation achieved with 2.5 μg/mL of TNF-α (set as 100%). Filled bars represent the responses to I-TBEV, empty bars responses to the matrix. (B) HEK Blue cells were stimulated with live TBEV virus for 48 h at the indicated multiplicity of infection (MOI), and activation was assessed as described above. N=3.
Discussion

In this study, we assessed the cell responses and pathways induced by I-TBEV, the main component of the TBE vaccine, and by the replicating virus in human primary cells and reporter cell lines. Our first aim was to extensively characterize the innate immune signature induced by I-TBEV in our human PBMC platform – in comparison to untreated as well as to low-quality I-TBEV- and live virus-treated cells. The second aim was to identify the receptors responsible for (I-)TBEV sensing.

Addressing the first aim, we found that I-TBEV induced an interferon-dominated immune profile and upregulation of selected inflammatory genes. The low-quality formulation, HT I-TBEV, triggered a similar expression signature in quality, but with a much lower magnitude of responses. These findings are in line with previous studies on I-TBEV-induced responses [6,7]. Indeed, the induction of IFN responses has been identified as a common early signature of several vaccines [33]. Interestingly, selected genes involved in antigen presentation, interleukin signaling and interactions between lymphoid and non-lymphoid cells were underexpressed following treatment of PBMCs with I-TBEV. This downregulation of certain immune functions was for us unexpected, and in contrast with results from studies on whole inactivated influenza virus that found some of the same molecules upregulated in primary cells [15,34,35]. Thus, induction of selected inflammatory markers in vitro can be highly pathogen-specific. Nevertheless, the successful activation of an interferon cascade appears to be a general feature of promising vaccine candidates and was shown to correlate with favorable antibody titers [36,37] and T cell responses [38,39] in vivo.

Comparing the immune profiles in cells incubated with live or inactivated TBEV, we observed that the live virus induced a transcriptional signature overlapping in large parts with that induced by I-TBEV – with the exception of replication-related genes distinctly regulated in live virus-treated cells. Similarities in immune responses to live and whole inactivated viruses were previously observed in other studies [34,35], but the signature identified is of course cell- and pathogen-specific. TBEV, as many other (flavi)viruses, tries to evade the immune system during infection through replication in membrane vesicles (hindering the activation of PRRs), inhibition of signaling cascades by non-structural proteins, and impairment of antigen-presenting cell (APC) maturation [21,40–43]. Downregulation of IFN production is restricted to the early stages of infection, and – as also evident in this study – interferon signaling has recovered after 24 hours [25,44]. However, suppression of certain functions is still ongoing at this timepoint. Downregulation in the expression of adhesion molecules was previously reported in TBEV-infected cells [45,46]; our results extend this finding also to cells incubated with the inactivated virus. The induction of membrane rearrangements appears instead to be specific for the replicating virus, as only live TBEV induced upregulation of cellular pathways involved in lipid metabolism.
Overall, given the induction of interferon responses by the inactivated virus to similar or higher levels than those found in live TBEV-treated cells, I-TBEV stands out as a potent vaccine component.

After having established the distinctive transcriptional profile of (I-)TBEV-stimulated cells, we sought to determine which pattern recognition receptors, once triggered, led to the identified responses. Using inhibitors of downstream factors of PRRs, we demonstrated the involvement of RLRs in I-TBEV sensing in cryopreserved PBMCs. Inhibition of MyD88 – an adapter protein downstream of TLRs – did not affect the expression of I-TBEV-induced genes such as ISG56 and CXCL10, while inhibition of TBK1/IKKε – factors downstream of RLRs – halted the signaling cascade. Activation of RIG-I-like receptors is a predominant mechanism for cellular recognition of flaviviruses [47,48]; expression of RIG-I and MDA5 is enriched in human neural cells following TBEV infection [49], and inhibition of RLR signaling was found to suppress TBEV-induced interferon production [24,41,50]. RIG-I detects uncapped single-stranded RNAs (ssRNAs) (with an exposed 5′-triphosphate group) and, together with MDA5, double stranded RNAs (dsRNAs) – both produced during viral replication [51]. As such, RLRs should not be triggered by the inactivated virus, since the viral genome is packaged only once mature and capped [52]. However, activation of RIG-I by panhandle RNA (a partially circularized structure) lacking a 5′-PPP moiety has been demonstrated for influenza A virus [53]. Given the presence of cyclization elements in TBEV (and other flaviviruses) RNA [54,55], we hypothesize that, after the uncoating of the (inactivated) virus, such structures are recognized by RIG-I in the cytosol.

The role of the viral genome in the immunogenicity of I-TBEV was previously assessed in plasmacytoid dendritic cells (pDCs) derived from freshly isolated PBMCs, which were found to sense I-TBEV through TLR7/8 [6]. The discrepancy with the results described here could be explained by the fact that we used cryopreserved PBMCs for our study. Cryopreservation can alter the relative proportions of APCs [56] and decrease the amount of pDCs [57], or affect cell responses to TLR agonists [58,59]. In pDCs, the RIG-I pathway is dispensable for IFN production, while it is of crucial importance in other DCs and in non-dendritic cells [44]. Comparison of responses to I-TBEV in fresh and frozen-thawed PBMCs and in the presence of RLR and TLR inhibitors could confirm that distinct pathways are predominant in different cell subtypes. Overall, the contribution of the viral ssRNA to the activation of APCs is undoubtful. For influenza, it has been shown that vaccines containing the viral genome – able to activate endosomal ssRNA receptors [60] – induce stronger immune responses than formulations lacking it [15,34,61], presumably through the activation of more diverse molecular pathways. These considerations should therefore be taken into account during vaccine development, as also for TBEV the presence of the viral genome is shown to provide self-adjuvanting properties.
Our study provides new insights into how I-TBEV activates the innate immune system in vitro. However, it has to be realized that the TBE vaccine contains aluminum hydroxide as adjuvant, which by itself also affects the innate immune system [62–65]. Unfortunately, the responses to the final vaccine formulation could not be analyzed in our platform since the alum adjuvant appeared not to be compatible with viability of PBMCs [7]. Therefore, while some conclusions can be drawn from an I-TBEV-based analysis of the immune signature in human PBMCs, a complete picture of the in vitro responses to the final TBE vaccine can be achieved only once a platform that tolerates the adjuvanted formulation is found.

As the scientific community strives to bring an effective SARS-CoV-2 vaccine to the market, the process of vaccine development and assessment receives now more than ever global attention. While vaccine potency and effectiveness can be assessed relatively easily, the knowledge of which host pathways should be activated for mounting a sufficient immune response is not yet solid. Especially in the case of ‘difficult’ vaccines (for rapidly mutating pathogens, as well as for viruses and bacteria with complex interactions with the host’s immune system), the traditional “isolate, inactivate and inject” strategy might be inappropriate for vaccine development. Next generation sequencing techniques have been proposed as tools eventually enabling rational and directed vaccine design [66–68]. As such, the present study contributes to the growing evidence of their applicability for assessing the batch-to-batch consistency of vaccine products and for understanding the mechanisms of action of vaccines and vaccine candidates.

Acknowledgements

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References


Analysis of TBE vaccine-associated pathways in vitro


[38] Querec TD, Akondy RS, Lee EK, Cao W, Nakaya HI, Teuwen D, et al. Systems biology approach


[64] Ghimire TR, Benson RA, Garside P, Brewer JM. Alum increases antigen uptake, reduces antigen


# 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>
</tr>
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<tbody>
<tr>
<td>IL12-p40</td>
<td>CTGCCCAGAGCAAGATGTGTC</td>
<td>CATTCTCCAGGGGCATCCG</td>
<td>Own design</td>
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<tr>
<td>ISG56</td>
<td>CCTGGAGTACTATAGACGGG</td>
<td>TGGGTGCTAAGGACCTTGT</td>
<td>Holzinger et al., JVirol (2007)</td>
</tr>
<tr>
<td>CCL-8</td>
<td>GTTTCTGCAGCGCTCCTG</td>
<td>TGGCTGAGCAAGCTCCTGA</td>
<td>Ma et al., Exp Terap Med (2016)</td>
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<tr>
<td>CXCL-10</td>
<td>TGAAAATTATCTGCAAGC</td>
<td>CAGACATCTCTCTACCTTCTT</td>
<td>Ma et al., Exp Terap Med (2016)</td>
</tr>
<tr>
<td>STAT1</td>
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<td>TATGCACTGCACGGAAGC</td>
<td>Zhang et al., Immunol (2009)</td>
</tr>
<tr>
<td>IL4I1</td>
<td>GCTGAAGAAAGAAG.equals</td>
<td>CCTAACCAGCCACAGAAGGA</td>
<td>Erdman et al., MalariaJ (2014)</td>
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<tr>
<td>CHI3L1</td>
<td>TGCCCTTGACCCTCTGTACC</td>
<td>GAGCGTCACATATTCCACT</td>
<td>Warby et al., JVirol (2003)</td>
</tr>
<tr>
<td>JAK2</td>
<td>TCTGACAGGCAGCCTCTT</td>
<td>TCTGACAGGCAGGCAAGC</td>
<td>Ko et al., ThoracicCan (2014)</td>
</tr>
<tr>
<td>APOC1</td>
<td>TTCTGTGATGTCCTTGGAA</td>
<td>TCAGCTTATCCAAGCGACT</td>
<td>Okura et al., J Clin Immunol (2015)</td>
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<tr>
<td>CYBB</td>
<td>TAGGAGTAGACGACCTTCTT</td>
<td>ACATACACCACCTATACTGGA</td>
<td>Nowak et al., EBioMedicine (2019)</td>
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<td>EIF4B</td>
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<tr>
<td>GAPDH</td>
<td>AGGGCTGCTTTTAACCTCCTG</td>
<td>CCCCACTTGATTGGAGGA</td>
<td>Abubaker et al., PLOS One (2013)</td>
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</tbody>
</table>
Figure S1. Transcriptional analysis of I-TBEV-treated PBMCs. Heatmap showing the fold change (FC) of differentially expressed genes (DEGs) in each sample, normalized to the control group.

Figure S2. Cytokine production in PBMCs stimulated with conforming, non-conforming I-TBEV and live TBEV. PBMCs from 4 donors were stimulated with the indicated compounds for 24 h, after which the supernatants were collected and the cytokine concentration assessed by Cytometric Bead Assay. Bars represent the average value and dots the individual value per donor.
Figure S3. Functional analysis of DEGs identified by RNA-Seq on Cytoscape using the plug-in ClueGO. Functions of DEGs upregulated (A) and downregulated (B) by I-TBEV treatment.
Figure S4. Canonical pathways identified by functional analysis on IPA. Two canonical pathways associated with DEGs upregulated upon I-TBEV treatment are shown: "Role of RLRs in antiviral innate immunity" (A) and "Role of PKR in interferon induction and antiviral response" (B, next page). DEGs identified by the RNA-Seq are highlighted.
Figure S5. Functional analysis of DEGs identified by RNA-Seq on Reactome. Functions of DEGs upregulated (A) and downregulated (B) by I-TBEV treatment.
Figure S6. Upregulation of *ISG56* in PBMCs incubated with live TBEV. Changes in gene expression following 24 or 48 h incubation with different multiplicities of infection (MOIs) of live virus were analyzed by RT-qPCR in PBMCs from a healthy donor. Results are from 3 replicates.