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## **Activation and inhibition of the type I interferon pathway in tick-borne encephalitis virus infection**

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## Summary

Tick-borne encephalitis virus (TBEV) is a member of the genus *Flaviviruses*, family *Flaviviridae* and contains a single stranded (ss) RNA genome with positive polarity.

Innate immunity is important to recognize viral structures and control viral replication of RNA viruses. Mammalian cells provide pathogen recognition receptors (PRRs) to detect products of viral replication and to trigger signal cascades, which result in the establishment of an antiviral state by the induction of type I interferons (IFNs).

Here, we investigated the interaction of TBEV with the host innate immune system. In particular, we were interested in the role of type I interferons on TBEV infections in vitro and on mechanisms leading to IFN activation.

We showed that infection of cells with replicating virus or transfection of self replicating viral RNAs lead to the activation of type I IFN mRNA transcription. In addition, only full length RNA of TBEV was able to activate an IFN $\beta$  promoter driven reporter gene. Our results indicated that IFN $\alpha/\beta$  signalling is RNA replication dependent. Moreover, virus RNA replication is a central mechanism in the activation of type I IFN induction.

Furthermore, we showed that the interferon regulatory factor 3 plays (IRF3) an important role in the control of viral replication. IRF3 deficient cells infected with TBEV exhibited a strong enhancement of virus replication. These cells did not activate IFN $\alpha/\beta$ . On the contrary, wild-type cells strongly induced IFN production, which lead to the suppression of virus replication, indicating that IRF3 is a main regulator in type I IFN signalling and essential to combat viral replication by inducing IFN $\alpha/\beta$ .

Surprisingly, TBEV infection only lead to low IFN $\alpha/\beta$  activation and therefore we analysed if TBEV inhibits IFN expression. Finally, we identified TBEV as an inhibitor of the IFN $\alpha/\beta$  system. The virus interferes with components of the type I IFN signalling cascade at early stages by inhibiting IFN induction and on later periods in the establishment of an anti viral state by inhibition of STAT-1 phosphorylation.



## Zusammenfassung

Das Frühsommer-Meningoenzephalitis (FSME) Virus gehört zur Gattung der *Flaviviren*, Familie *Flaviviridae* und besteht aus einem einsträngigem RNA Genom mit positiver Polarität.

Angeborene Immunität ist wichtig, um Virusstrukturen und Virusreplikation von RNA Viren zu erkennen. Säugetierzellen verfügen über pathogene Erkennungsrezeptoren (PRR) um Produkte der Virusreplikation zu erkennen und Signalkaskaden zu aktivieren, die zur Etablierung eines antiviralen Zustandes durch die Induktion von Typ I Interferonen (IFN) führen.

In dieser Studie untersuchten wir die Interaktion des FSME Virus mit dem angeborenen Wirtsimmunsystem. Von besonderem Interesse war die Rolle von Typ I Interferonen in FSME Virus Infektionen *in vitro* und die Mechanismen die zur Interferon Aktivierung führen.

Wir zeigten, dass die Infektion von Zellen mit replizierendem Virus oder die Transfektion von selbst-replizierender viraler RNA zu der Aktivierung von Typ I IFN mRNA Transkription führte. Zusätzlich war nur "Ganzlängen" RNA fähig ein Reporter gen mit einem davor geschalteten IFN $\beta$  Promoter zu aktivieren. Unsere Ergebnisse bewiesen, dass IFN $\alpha/\beta$  Signalisierung von RNA Replikation abhängt. Weiters ist die virale RNA Replikation ein zentraler Mechanismus in der Aktivierung von Typ I IFN Induktion.

Außerdem zeigten wir dass IRF3 (*interferon regulatory factor 3*) eine wichtige Rolle in der Kontrolle von viraler Replikation spielt. Mauszellen, die über kein IRF3 mehr verfügten und die mit TBEV infiziert wurden, wiesen eine stark erhöhte Virusreplikation auf. Diese Zellen aktivierten kein IFN $\alpha/\beta$ . Im Gegensatz induzierten Wildtyp Zellen sehr stark die IFN Produktion, was zu einer Verminderung der Virusreplikation führte und zeigt, dass IRF3 ein Hauptregulator in der Typ I IFN Signalisierung ist. Der Transkriptionsfaktor ist wichtig, um Virusreplikation durch die Induktion von IFN $\alpha/\beta$  zu bekämpfen.

Überraschenderweise führte die Infektion durch das FSME Virus nur zu geringer IFN $\alpha/\beta$  Aktivierung und aus diesem Grund testeten wir, ob es die Entstehung von IFN hemmt. Schließlich identifizierten wir das FSME Virus als einen Inhibitor des IFN $\alpha/\beta$  Systems. Das Virus interagiert mit Komponenten der Typ I IFN Signalkaskade in frühen Stadien durch die Inhibierung von IFN Induktion und in später Phase in der

Etablierung eines antiviralen Zustandes durch die Inhibierung von STAT-1 Phosphorylierung.

# 1. Introduction

## 1.1. Flaviviruses

The genus *Flavivirus* consists of 53 virus species and among them 27 are mosquito-borne, 12 are tick-borne and 14 are zoonotic agents with an unknown vector. It belongs to the family of *Flaviviridae* that are divided into three groups of closely related viruses: Flavivirus, Pestivirus and Hepacivirus. Viral classification is based on virion morphology, genome organization, vector associations and virus ecology (Fauquet and Fargette 2005).

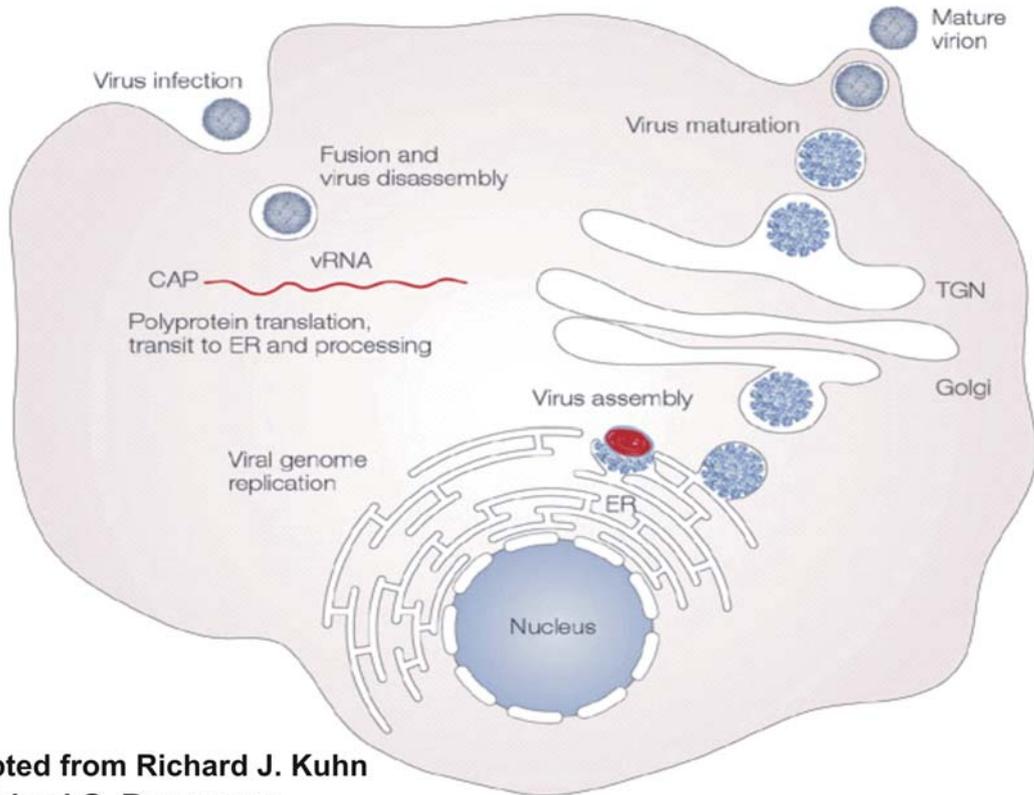
Flaviviruses are transmitted to vertebrates by blood sucking arthropods like mosquitoes and ticks. In very rare cases viruses can be transmitted vertically from vertebrate to vertebrate (Gaunt, Sall et al. 2001). Mosquito-borne flaviviruses include two major groups and can be distinguished by their clinical presentment in humans. On the one hand, Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV) present the encephalitic flaviviruses and on the other hand, yellow fever virus (YFV) and dengue virus (DENV) are viscerotropic and can cause hemorrhagic fever. The tick-borne Flaviviruses include tick-borne encephalitis virus (TBEV), Louping ill virus (LIV), Langat virus (LGTV), Powassan virus (POWV), Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), Kadam virus (KADV), Royal Farm virus (RFV) and its subtype Karshi virus and Gadgets Gully virus (GGYV) (Charrel, Zaki et al. 2001).

Flaviviruses have a genome that is single-stranded. The ~11-kb- long RNA molecule with positive-strand polarity carries a 5'-terminal Cap structure but no 3'-terminal poly(A) tail. It serves as the only viral mRNA that encodes all viral proteins in a single open reading frame (ORF). Virions are small round enveloped particles that consist of three structural proteins: capsid protein C, protein prM, which is a precursor to the small membrane protein M, and the large envelope protein E. The lipid envelope contains the two surface proteins, E and the membrane protein M. The capsid protein C and the viral genome can be found inside the viral envelope (Mandl 2005). The seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) have several functions in Virus replication. For example, they provide the RNA-dependent

RNA polymerase (NS5) and seem to have a role in modifying innate immune responses (Best, Morris et al. 2005).

## **1.2. Flavivirus life cycle**

Flavivirus binding and uptake includes receptor-mediated endocytosis and the virus is internalized via clathrin-coated pits. The low pH of the endosomal pathway triggers fusion of the virion envelope with host cell membranes to release the virus nucleocapsid (Chu and Ng 2004). Moreover, the RNA genome, which serves as messenger RNA (mRNA) for translation and template during RNA replication, is released into the cytoplasm. The RNA is translated into a single polyprotein, which is processed by host and viral proteases. Whereas replication occurs on intracellular membranes, viral assembly takes place on the surface of the endoplasmic reticulum (ER). The replication starts with the synthesis of a genome-length minus strand RNA that later serves as a template of a complementary plus strand RNA (Lindenbach and Rice 1997). This synthesis, where minus strands serve as templates for the production of plus strands is called asymmetric replication (Chu and Westaway 1985). Immature viral and subviral particles are transported through the trans-Golgi network (TGN) and cleaved by the host protease furin. Finally, they are released as mature and infectious particles by exocytosis (Fig. 1).



Adapted from Richard J. Kuhn  
& Michael G. Rossmann

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Fig. 1. Flavivirus life cycle

Virions attach to the surface of the host cell and enter the cell by receptor-mediated endocytosis. Acidification of the endosomal vesicle leads to conformational change in the virion and to the fusion of viral and cell host membrane followed by particle disassembly. When the genome is released in the cytoplasm, the RNA is translated into a polyprotein and replication occurs on intracellular membranes. Virus assembly arises in the endoplasmic reticulum (ER) and non-infectious, immature particles are transported through the trans-Golgi network (TGN). They are cleaved by the host protease furin and released as mature, infectious particles by exocytosis.

### 1.3. Tick-borne encephalitis virus (TBEV)

In 1931 TBEV was discovered in Europe and in 1937 the virus was isolated by Russian scientists. After *Ixodes* ticks were shown to be the vector of the disease, the virus was found to be spread in Eurasia (Lindquist and Vapalahti 2008). TBEV consists of the European (Eu), Far Eastern (FE) and Siberian subtype (Ecker, Allison et al. 1999). Whereas in Western Europe the principal vector is *Ix ricinus*, it is *Ix persulcatus* in eastern Eurasia (Mandl 2005). Cases of TBEV transmission to humans by the consumption of unpasteurized milk or by direct contact with infected sheep have also been documented (Komar 2003).

TBEV can infect all groups of people, but in general the symptoms are more severe in older people than in children. The incubation period lasts between 7 to 14 days after a tick bite (Lesnicar, Poljak et al. 2003). The clinical symptoms differ slightly between the three subtypes. Febrile illness occurs in more than 70 % of the people infected with the European subtype. The beginning febrile period lasts 2 to 7 days without syndromes of meningoencephalitis, while thrombocytopenia and leukopenia are often observed. This first acute phase of infection is followed by a symptom-free interval. 30 % of the patients develop a second phase of disease. The fever returns and CNS symptoms, such as meningitis occur. Clinical syndromes like ataxia, cognitive dysfunction, concentration difficulties, confusion and paralysis emerge in infected patients (Lindquist and Vapalahti 2008).

After the transmission to the host by the tick bite TBEV replicates in Langerhans cells and later in the macrophages, histiocytes and fibroblasts. When the virus enters the blood it multiplies in cells of the lymph nodes, in the spleen and in the liver. During the primary viremia, the virus is able to enter the central nervous system (CNS) via peripheral nerves, which leads to the second phase of viremia (Gelpi, Preusser et al. 2005). The neuropathogenesis of TBEV includes neuroinvasiveness and neurovirulence. Neuroinvasiveness shows the capacity of the virus to enter the CNS, whereas neurovirulence is the ability of the virus to replicate and cause damage in the neurons of the CNS (Mandl 2005).

There is no specific and established treatment for tick-borne encephalitis available, but the disease can be prevented by active immunization. Two vaccines based on nearly identical TBEV-Eu strains (strain Neudoerfl, FSME-IMUN by Baxter Vaccines,

Vienna Austria; strain K23, Encepur by Novartis, Basel, Switzerland) are approved and used successfully in Europe (Demicheli, Graves et al. 2000).

## **1.4. Pattern recognition receptors**

Innate immunity and antiviral immune programs of mammalian cells play an important role and are necessary for the control of virus replication and spread during infection by RNA viruses. The cell is able to use pathogen recognition receptors (PRR) to detect products of viral replication in the form of pathogen-associated molecular patterns (PAMPs) (Kawai and Akira 2006). Viral recognition through PRRs leads to the activation of signalling pathways and to the induction of various latent transcription factors (Saito and Gale 2007). These transcription factors trigger the reprogramming of the cell's gene expression and induce diverse genes to establish an antiviral state. Two latent and constitutively expressed transcription factors are interferon regulatory factor 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). They are essential for the establishment of the antiviral state by the induction of type I interferons (IFNs) and through the induction of proinflammatory cytokines.

Diverse classes of PRRs, Toll-like receptors (TLRs), the RIG-I-like helicase retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) have been demonstrated to take part in the activation of IRF3 and NF- $\kappa$ B signalling in response to Flaviviruses.

### **1.4.1. Toll like receptors (TLRs)**

TLRs belong to the interleukin-1 receptor (IL-1R)/TLR superfamily, which also contains IL-1, IL-18 and IL-33 receptors. These receptors have a great importance in host innate immunity and are highly conserved (Akira, Uematsu et al. 2006).

TLRs are either expressed on the cell surface or within endocytic vesicles (Saito and Gale 2007). Virus structures have been identified as target for TLR, including single-stranded (ss) RNA that is recognized by TLR7 and TLR8; double-stranded (ds) RNA sensed by TLR3 and viral DNA detected by TLR9. Numbers of special viral structural proteins have been demonstrated to induce signal transduction through TLR2 and TLR4 (Akira, Uematsu et al. 2006).

Protein kinase R (PKR) has been shown to protect against viral infection and to sense intracellular dsRNA. Therefore, it represents the first antiviral PRR. Nevertheless, it has been observed that mice lacking the PKR gene could still induce an antiviral response after stimulation with poly I-C (pIC), which is a synthetic analogue of dsRNA. Consequently, TLR3 was discovered as a PRR to recognize dsRNA (Alexopoulou, Holt et al. 2001). In general, TLR3 is located in the endosome and after detection of a viral pathogen the receptor recruits the TIR domain-containing adaptor protein-inducing IFN $\beta$  (TRIF). Moreover, TRIF initiates a signalling complex that activates the I $\kappa$ B kinases (IKKs) TBK1 and IKK- $\epsilon$ , which further phosphorylate IRF3 (Au and Pitha 2001). Consequently, IRF3 forms homodimers and translocates to the nucleus, where it interacts with the CBP/p300 coactivator to activate the expression of IRF3 target genes. This leads to the establishment of an antiviral state to combat viruses and avoid viral replication (Yoneyama, Suhara et al. 2002). The adaptor molecule TRIF also interacts with TRAF6 and RIP-1, which cause the induction of NF $\kappa$ B and the activation of proinflammatory cytokines (Cusson-Hermance, Khurana et al. 2005).

TLR7 and TLR8 are both expressed in mice and humans and are highly homologous (Du, Poltorak et al. 2000). Synthetic ssRNA was shown to induce the stimulation of IFN $\alpha$  and proinflammatory cytokines from dendritic cells (DCs) and macrophages via human TLR8 and murine TLR7 (Heil, Hemmi et al. 2004).

TLR9, which is located in the intracellular endosomal compartments, counteracts its PAMPs in endosomes like TLR3 (Ahmad-Nejad, Hacker et al. 2002). It has been known that TLR9 detects invading microorganisms by responding to bacterial DNA sequences that contain unmethylated CpG dinucleotides (Hemmi, Takeuchi et al. 2000). Large eukaryotic DNA viruses also contain a high number of these motifs. Therefore, infection of mice with members of the *Herpesviridae*, which are great DNA viruses, leads to the induction of type I interferons (IFNs) in plasmacytoid dendritic cells (pDC) via TLR9 (Krug, Luker et al. 2004).

Although all TLRs activate NK- $\kappa$ B via MyD88- or TRIF-dependent pathways, type I IFN induction is regulated in a different way. While TLR4 and TLR3 induce IRF3 through TRIF, TLR7 and TLR9 activate IRF7 through MyD88 (Ishii, Koyama et al. 2008).

### **1.4.2. Rig like receptors (RLR) - Rig-I and MDA-5**

The retinoic acid-inducible gene I (RIG-I) like RNA helicase (RLH) family of PRRs includes RIG-I and melanoma differentiation-associated gene 5 (MDA-5). They are expressed in the cytosol and exhibit an alternative defence mechanism as an essential component of host innate immunity to RNA viruses (Kawai and Akira 2007). RIG-I is involved in the recognition of flaviviruses, orthomyxoviruses, paramyxoviruses and rhabdoviruses (Kato, Takeuchi et al. 2006). The receptor detects the uncapped 5'-triphosphate end of the ssRNA that is produced by these viruses (Hornung, Ellegast et al. 2006). Contrary, MDA-5 senses picornaviruses, but the exact RNA structure has not been identified yet.

Further studies demonstrated that RIG-I is essential for recognizing HCV infection and especially binds to the non-translated regions (NTRs) within the virus genome (Saito, Hirai et al. 2007). Moreover, 5'ppp and dsRNA motifs were defined as RIG-I substrates (Hornung, Ellegast et al. 2006).

RIG-I and MDA-5 contain two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. RLRs possess an ATPase activity that allows them to unwind dsRNA. When RIG-I recruits its CARD-containing adaptor IPS-1 (also known as MAVS, VISA or Cardif), the adaptor relays the signal to the kinases TBK1 and IKK $\epsilon$ , which phosphorylate IRF3 transcription factor (Thompson and Locarnini 2007).

Diverse studies showed that RIG-I is necessary to activate expression of IFN $\beta$  in response to JEV infection (Kato, Takeuchi et al. 2006). RIG-I deficient mice showed a decrease in serum IFN $\alpha$  levels and an increased susceptibility to infection compared to wild-type mice, whereas MDA-5 knock out mice did not exhibit any phenotypic difference. Therefore, RIG-I plays an important role to initiate an antiviral response pathway to JEV.

Contrary, RIG-I knock out mouse embryonic fibroblasts (MEFs) still induced IRF3 target genes in response to DENV (Loo, Fornek et al. 2008). The same response occurred with MDA-5 null MEFs suggesting that the signalling pathway of DENV is more complicated and involves both RIG-I and MDA-5.

RIG-I null cells still detected WNV infection, but the innate antiviral response was delayed (Fredericksen and Gale 2006). Consequently, RIG-I is essential to mediate antiviral response; nevertheless, various secondary pathways are also involved.

## 1.5. The interferon system

Cytokines play a very important role in the host defence against viruses and the most prominent produced during viral infections are the interferons (IFNs). They contain three different classes: type I, II and III, according to the receptor they use (Randall and Goodbourn 2008). Type I IFNs consist of one to three IFN $\beta$  genes, multiple IFN $\alpha$  family members and other genes like IFN $\omega$ ,  $\epsilon$ ,  $\tau$ ,  $\delta$  and  $\kappa$ . The IFN $\alpha$  and  $\beta$  genes can be produced by all nucleated cells in response to virus infection.

Treatment of cells with type I IFNs leads to the upregulation and activation of several hundred genes, which promote the antiviral state. Some of the upregulated genes encode enzymes that are responsible to limit viral replication. For example, protein kinase R (PKR) and 2'5'-oligoadenylate synthetase (OAS) depend on viral co-factors like dsRNA and trigger huge changes in cellular function (e.g. translational arrest). Other IFN-inducible genes promote the upregulation of the major histocompatibility complex (MHC) class I complex and the presentation of viral antigens to the adaptive immune response (class II MHC transactivator (CIITA) and transporter of antigen presenting 1/low molecular protein 2 (Tap1/LMP2)). Moreover, IFN $\alpha/\beta$  has immunomodulatory functions by triggering the maturation of dendritic cells (DCs), inducing natural killer (NK) cells and CD8<sup>+</sup> T cells and promoting the synthesis of Interleukin-15 (IL-15), which is responsible for the division of memory CD8<sup>+</sup> T cells (Randall and Goodbourn 2008).

Type II IFNs contain only one member, IFN $\gamma$  and they are predominantly made by T lymphocytes and NK cells. The last group, type III IFNs, consist of IFN $\lambda$ 1,  $\lambda$ 2 and  $\lambda$ 3, which are also induced in direct response to viral infection and use the same pathway like IFN $\alpha/\beta$  to detect viral infection (Onoguchi, Yoneyama et al. 2007).

## 1.6. Activation of type I IFNs

IFN $\alpha/\beta$  production is tightly regulated at the transcriptional level. The IFN $\beta$  promoter has binding sites for several transcription factors, which cooperate for maximal induction. These are the IFN regulatory factor 3 and 7 (IRF3/7), NF $\kappa$ B and Ap-1 (Honda and Taniguchi 2006).

IRF3 and IRF7 are expressed in many cell types. Either of these factors, when expressed ectopically, can enhance IFN $\alpha/\beta$  mRNA induction levels (Sato, Hata et al. 1998; Sato, Tanaka et al. 1998). Transcriptional induction of IFN $\alpha/\beta$  genes during infection by different viruses, including vesicular stomatitis virus (VSV), herpes simplex virus (HSV), and encephalomyocarditis virus (EMCV), is commonly dependent on IRF family member (Sato, Suemori et al. 2000). However, IRF3 and IRF7 perform nonredundant and distinct roles from each other for the efficient induction of the IFN $\alpha/\beta$  gene, as well as for the diversity of the induction mechanisms within the IFN $\alpha$  gene family.

Whereas IRF3 is constitutively expressed in almost all cells, IRF7 is only primarily expressed in plasmacytoid dendritic cells. Type I IFNs have been proposed to produce feedbacks onto cells by synthesising IRF7, which further induces transcription of the “primary” genes (IFN $\beta$  and murine IFN $\alpha$ ) and activates transcription of the “secondary” genes (the remaining IFN $\alpha$  genes) (Sato, Hata et al. 1998). IRF7 is capable to bind the IFN $\beta$  promoter and can enhance transcription significantly.

IRF3 and IRF7 are activated through the phosphorylation of the C-terminus by the IKK related kinases IKK $\epsilon$  and TBK1, which leads to dimerization. IRF3-dimers are translocated to the nucleus and bind to consensus binding sites in the promoter regions of type I IFN genes. (Panne, McWhirter et al. 2007). NF- $\kappa$ B is located in the cytoplasm and retained there by an inhibitor molecule called inhibitor of NF- $\kappa$ B (I $\kappa$ B). Viral infection induces the phosphorylation and subsequent proteasomal degradation of I $\kappa$ B, which leads to the release and nuclear translocation of NF $\kappa$ B (Wullaert, Heyninck et al. 2006).

IFN $\beta$  gene transcription requires also the binding of a c-jun/AFT-2 heterodimer to the promoter. IRF3, NF $\kappa$ B and c-jun/AFT-2 form a complex on the promoter, which is called enhanceosome. They support in a cooperative way the recruitment of CREB-binding protein (CBP)/p300 that activates the congregation of the transcription machinery and RNA polymerase II (Merika and Thanos 2001).

The production of type I IFNs in viral infections activates the transcription factor IRF7 that seems to play a role in the induction process (Honda, Mizutani et al. 2004).

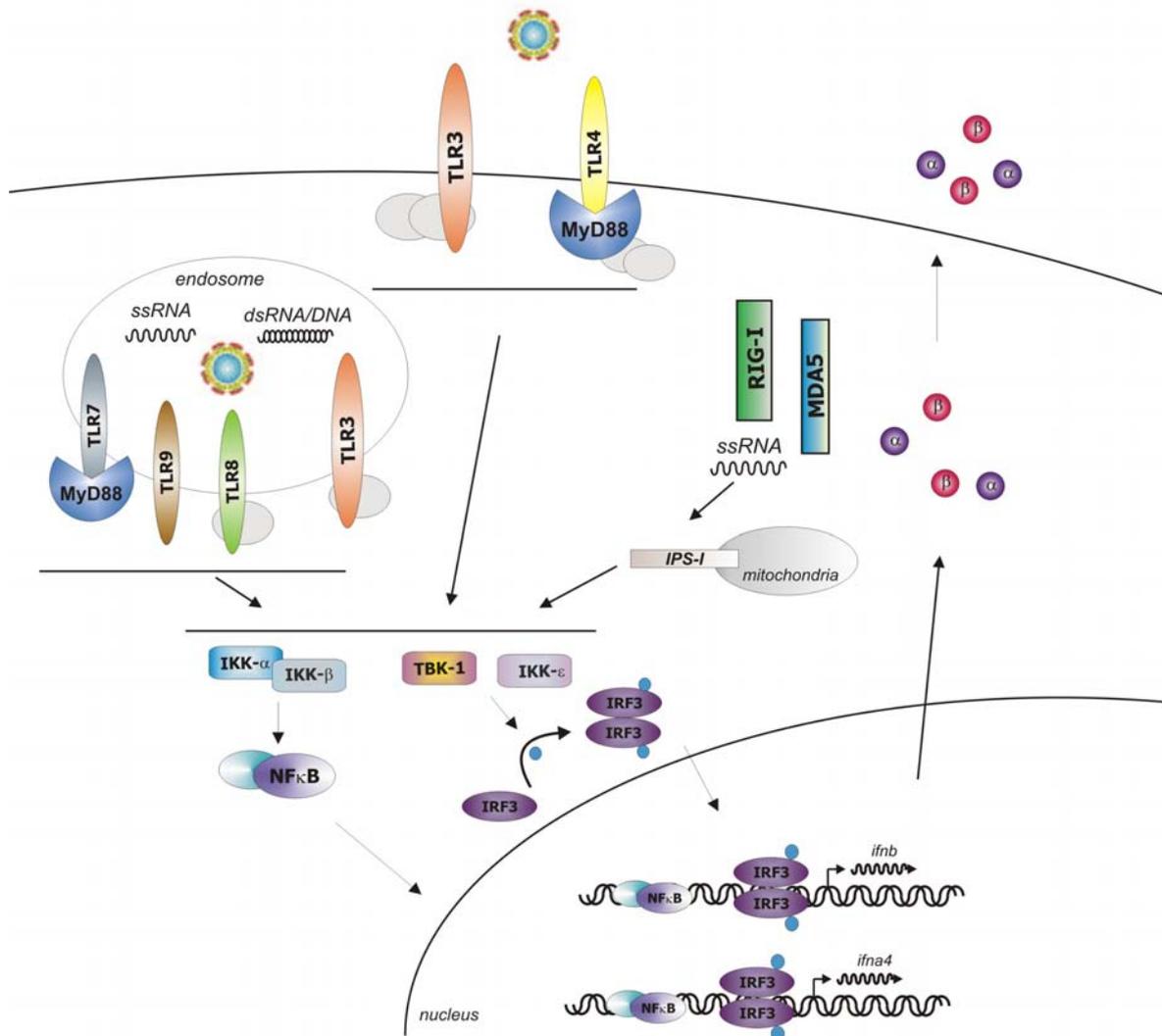


Fig. 2. Type I IFN pathway

Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I) like RNA helicase (RLH) are expressed on and in a number of cells in host tissues that recognize diverse virus structures. They cooperate together to recognize viral infection by sensing viral proteins (TLR4), ssRNA (TLR7, TLR8), dsRNA (TLR3) and DNA (TLR9) viruses. ssRNA, for example, can be recognized by TLR3 on the cell surface or in the endosome in a MyD88 independent manner. After pathogen detection TLR3 recruits the adaptor molecule TRIF and initiates a signalling complex that activates the I $\kappa$ B kinases (IKKs) TBK1 and IKK- $\epsilon$ , which further phosphorylate IRF3. Phosphorylated IRF3 dimerizes and translocates to the nucleus where it supports the transcription of IFN $\beta$  and IFN $\alpha$ 4 through the binding to their promoters. The kinases IKK $\alpha$  and IKK $\beta$  cause the induction of NF $\kappa$ B, which also leads to IRF3 phosphorylation. RIG-I and MDA-5 are located in the cytosol and present an alternative way of viral defence. RIG-I, for instance, senses viral ssRNA and activates TBK1 and IKK- $\epsilon$  via IPS-I.

## 1.7. JAK-STAT pathway

In general, all type I IFNs bind to a heterodimeric receptor, which is composed of IFNAR1 and IFNAR2. Whereas the cytoplasmic tail of IFNAR1 is associated with tyrosine kinase 2 (Tyk2), IFNAR2 is coupled to the tyrosine kinase JAK1 (Janus/just another kinase). IFN $\alpha/\beta$  binding leads to receptor oligomerization and activation of the receptor associated tyrosine kinases JAK1 and TYK2. The activated kinases phosphorylate IFN receptors and create recruitment sites for the cytoplasmic transcription factors signal transducers and activators of transcription 1 and 2 (STAT1/2). STAT1/2 proteins bind to the tyrosine phosphorylated receptors through their SH2 domains and become phosphorylated by JAK1 and TYK2 on tyrosine residues. This leads to heterodimerization of STAT1 and STAT2 and to the subsequent nuclear translocation. STAT1-STAT2 heterodimer further associate with IRF9 to form a stable complex called interferon-stimulated gene factor (ISGF3). This complex binds to the IFN-stimulated response element (ISRE), which is a consensus sequence in the promoter of IFN stimulated genes (ISG) and enhances transcription of these genes. (Tang, Gao et al. 2007) (Reich and Liu 2006).

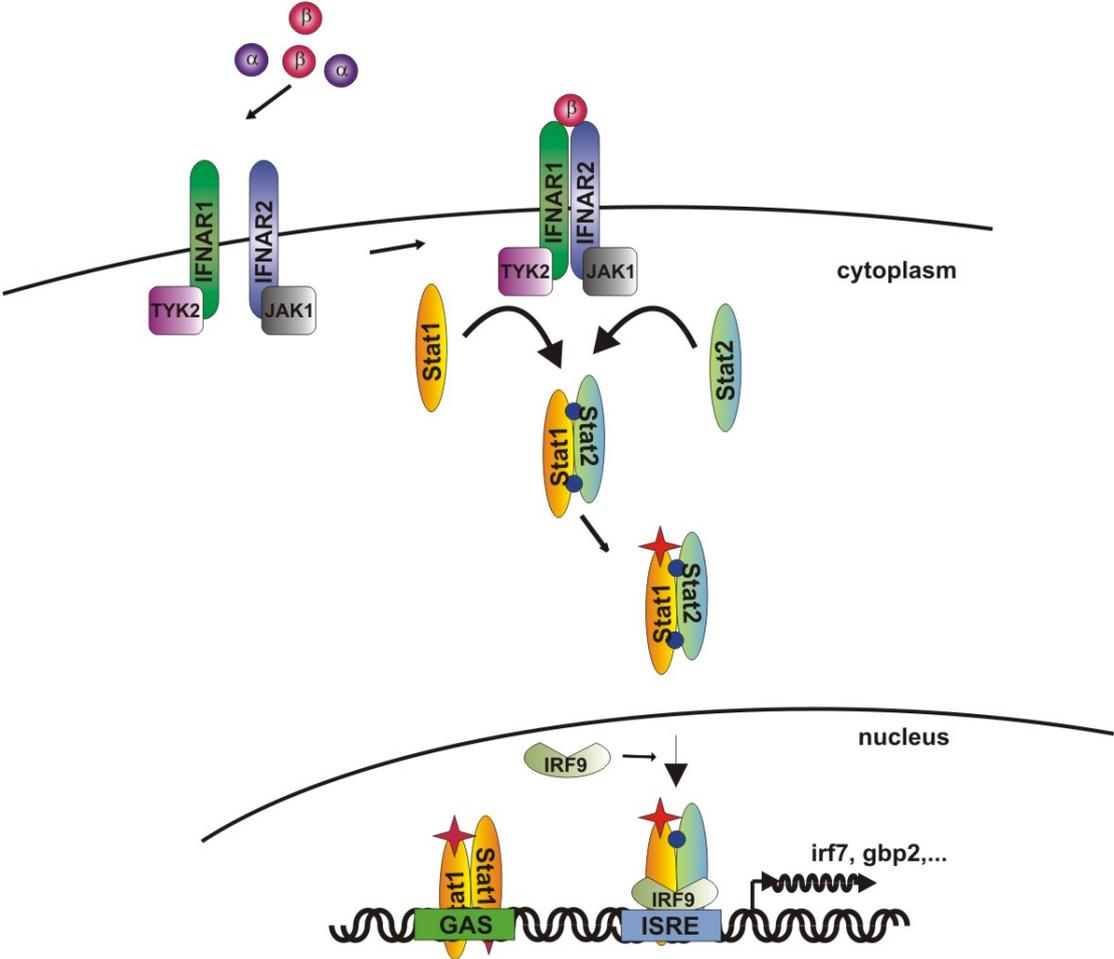


Fig. 3. JAK-STAT pathway  
Binding of type I IFN to their receptor leads to activation of the JAK-STAT pathway and subsequent activation of IFN target genes that play an important role in the antiviral immune response.

## 1.8. Viral evasion strategies

Viruses of the family *Flaviviridae* are capable to use multiple mechanisms to escape the antiviral effects of IFN signalling in various ways. For instance, the NS proteins of some members of the Flaviviruses have been shown to act as IFN-antagonist, inhibiting the JAK-STAT pathway (Samuel and Diamond 2006). The main function of this pathway is to support the antiviral action of type I IFNs through ISG expression. Viral disruption of JAK-STAT signalling results in rapid viral replication, elevated viral loads in tissue and blood and enhanced transmission between hosts (Samuel and Diamond 2005).

Pathogenic strains of DENV have been identified as very resistant viruses to the antiviral actions of IFNs. Viral NS2A, NS4A and NS4B have been suggested as possible IFN antagonists. Studies demonstrated that the mature NS4B protein in combination with NS4A blocks the nuclear import of STAT1 by inhibiting STAT phosphorylation (Munoz-Jordan, Sanchez-Burgos et al. 2003). Although the diverse virulence factors of the various DENV strains are still unknown, it might be obvious that they are able to interfere and suppress the JAK-STAT signalling cascade.

Analyses of the NS5 protein of JEV identified the protein as IFN antagonist that is able to inhibit the activation of Tyk2 and STAT1 (Lin, Chang et al. 2006). NS5 is the RNA polymerase of flaviviruses and essential for viral replication. Different studies showed that the NS5 protein of Langkat virus, which is a member of the tick-borne flaviviruses, also functions as an IFN antagonist. It was demonstrated that the virus blocked IFN-induced phosphorylation of Tyk2 and JAK1 and could resist IFN's antiviral effects after cells were already infected (Best, Morris et al. 2005).

WNV infection and replication has been shown to be connected with an inhibition of IFN-induced JAK1 and Tyk2 phosphorylation. It has been reported that NS2A, NS2B3, NS4A and NS4B inhibit IFN signalling through interference of STAT activation (Liu, Wang et al. 2005).

In general, chronic HCV infection is treated with IFN therapy, but in most cases HCV shows a low response rate to this therapy indicating that the virus is able to resist IFNs. Studies reported that HCV acts as an IFN-antagonist by inhibiting STAT1 through elevated levels of protein phosphatase 2A, which hypomethylates and inactivates the transcription factor (Heim, Moradpour et al. 1999; Blindenbacher, Duong et al. 2003). Additionally, the HCV core protein activates expression of

suppressor of cytokine signalling (SOCS)-3 that function as a negative feedback loop on IFN signalling by inhibiting signal transduction events of the JAK-STAT pathway (Bode, Ludwig et al. 2003). Different studies reported that HCV NS5A can inhibit IFN $\alpha$  indicating the protein as an IFN antagonist (Macdonald and Harris 2004).

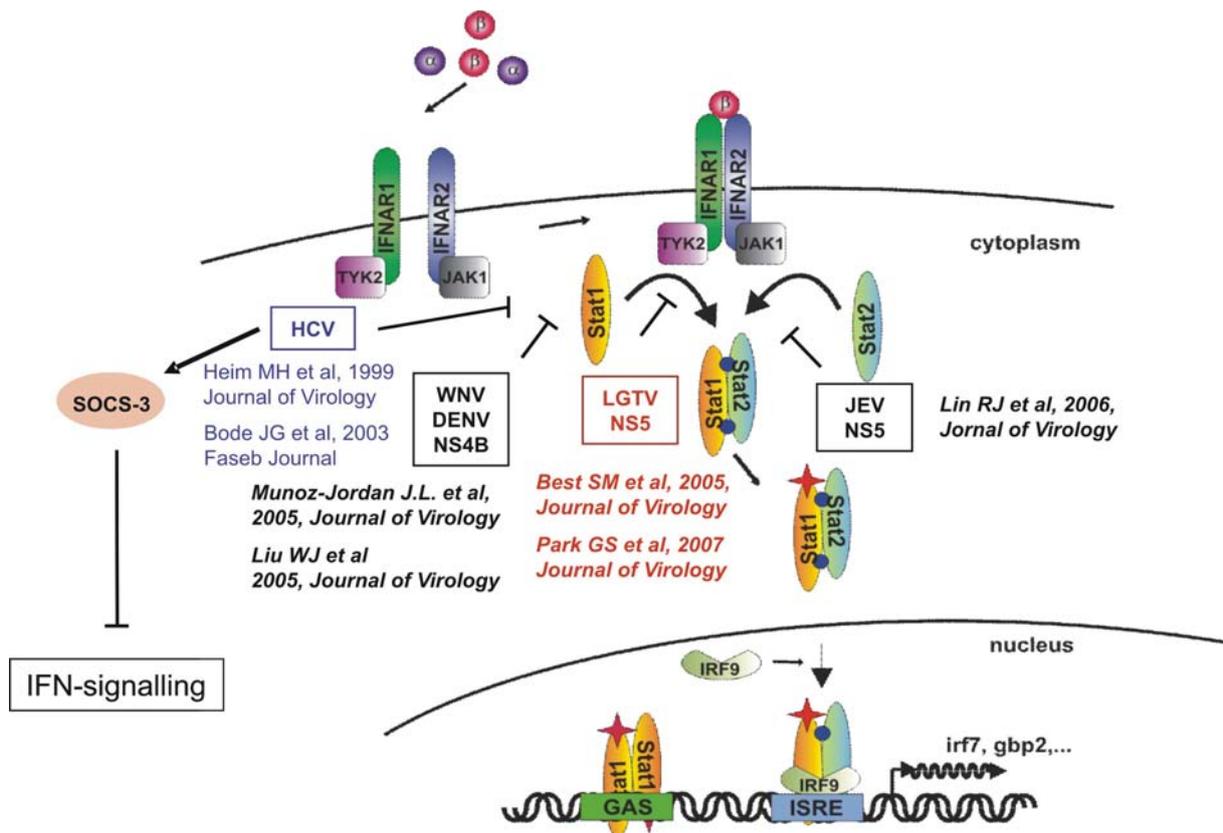


Fig. 4. Flaviviruses and HCV interfere with JAK-STAT signalling

HCV inhibits STAT1-phosphorylation and induces SOCS-3 mRNA, which is responsible for the negative regulation of IFN-signalling. WNV and DENV NS4B protein partially blocks STAT1 activation. NS5 protein of LGTV interferes with JAK-STAT pathway by inhibiting STAT1 phosphorylation, whereas NS5 protein of JEV blocks STAT1 translocation to the nucleus.

## **2. Aims**

Flavivirus infection leads to the activation of the host's innate immune system and to the establishment of an antiviral state by the induction of cytokines and interferons (IFNs). In our study we investigated the induction and role of type I IFNs in TBEV infection. It has not been analysed yet, how type I IFN pathways are activated in TBEV infection and how the virus interacts with the innate immune system. Therefore, we studied the interaction of TBEV with the host's interferon system. We were interested on the one hand which host cell factors contribute to the control of virus infection and on the other hand which parts of the viral replication cycle leads to the activation of host cell signalling pathways.

It is known that numbers of Flaviviruses suppress type I IFN signalling to replicate unchallenged in the host cell. Therefore, we investigated if TBEV also interferes with the type I IFN pathway and inhibits IFN induction.



### **3. Materials and Methods**

#### **3.1. Cells and viruses**

HEK 293T (293T cells): human kidney fibroblasts

MEFs: mouse embryonic fibroblasts

IRF3 -/- cells: mouse embryonic fibroblasts derived from IRF3 -/- mice

RAW 264.7 (RAW cells): mouse macrophages

L929 cells: mouse fibroblasts, established cell line, immortalised after 3T3 protocol (provided by Thomas Decker)

Tick cells (IRE-18/19): cells of *Ix ricinus*

BHK-21 cells: baby hamster kidney cells

TBEV: Tick borne encephalitis virus, strain Neudoerfl of Western subtype TBEV

VSV: Vesicular stomatitis virus, a member of the Rhabdovirus family, a (-)RNA stranded virus. Viral stocks were established from infected L929 murine fibroblasts

#### **3.2. Cell culture**

##### **3.2.1. Cultivation of MEFs, L929, 293T cells**

Standard conditions for cultivation of cells are 37°C, 5 % CO<sub>2</sub> and 95 % humidity. Medium has to be changed every 2-3 days. Whereas MEFs and 293T cells are split in the ratio 1:5, L929 have to be split 1:10.

Cells grow adherently in DMEM (GibCo Life Technologies), 10 % FCS and Pen/Strep (1x) in tissue culture treated dish. For passaging, medium is removed and cells are washed with 5 ml PBS. After PBS has been removed, cells are incubated in 1 ml 1x Trypsin/EDTA at 37°C for 1-2 min. Cells are resuspended and split in the appropriate ratio.

### **3.2.2. Cultivation of RAW cells**

Standard conditions for cultivation of cells are 37°C, 5 % CO<sub>2</sub> and 95 % humidity. Medium has to be changed every 2-3 days. Raw cells have to be split in the ratio 1:10.

Cells grow adherently in DMEM, 10 % FCS and Pen/Strep in tissue culture dish. For passaging, medium is removed and new one is added. Cells are scraped off the plat with a cell scraper, resuspended and split in the appropriate ratio.

### **3.2.3. Cultivation of BHKs**

Standard conditions for cultivation of cells are 37°C, 5 % CO<sub>2</sub> and 95 % humidity. Medium has to be changed every 3-4 days, when cells are split in the ratio 1:7.5. Cells grow adherently in medium (Minimal essential medium eagle, SIGMA #N1142), 5 % FCS, 1 % Glutamine, 0.5 % Neomycin in big cell culture bottles (175 cm<sup>2</sup>).

For passaging, medium is removed and cells are washed with 5 ml Trypsin. After discarding the Trypsin, another 5 ml are added. Trypsin is totally removed and cells are incubated at 37°C for 5 min. Cells are removed from the bottom by knocking against the cell culture bottle. They are resuspended with fresh medium and split in the appropriate ratio.

### **3.2.4. Cultivation of Tick cells**

Standard conditions for cultivation of cells are 28°C, without CO<sub>2</sub> or humidity in closed tubes (3 cm<sup>2</sup>). Medium has to be changed every week and cells are split in the ratio 1:2.

For passaging, medium is discarded and fresh one added. Cells are removed by pipetting the medium up and down for 10-20 times.

#### Medium for Ticks

<b>Total amount of medium</b>	<b>50ml</b>	<b>75ml</b>	<b>100ml</b>
L15	17ml	25.5ml	34ml
HBSS	18ml	27.5ml	36ml
Tryptose-PO4-broth	2.5ml	3.75ml	5ml
Lactalbumin hydrolysate 10%	1.25ml	1.8ml	2.5ml
FCS	10ml	15ml	20ml
L-Gln 200mM	0.5ml	0.75ml	1.0ml
PSA	0.5ml	0.75ml	1.0ml
<b>Added for infection</b>			
1M Hepes	1ml		
1N NaOH	300µl		

### **3.2.5. Freezing and thawing cells**

#### Freezing

- Cells are washed in a 10 cm confluent tissue culture dish with 5 ml PBS
- Remove PBS and add 1 ml Trypsin/EDTA
- Incubated at 37°C 1-2 min
- Resuspend cells in 9 ml medium and transfer to a Falcon tube
- Centrifuge for 5 min, at 1000 rpm at room temperature (RT)
- Discard supernatant and resuspend the pellet in 3 ml 90 % FCS / 10 % DMSO (Sigma)
- Transfer 1 ml aliquots into cryotubes and put them on ice for 10 min
- Put cryotubes at -80°C

#### Thawing

- Let cells thaw at RT and transfer them into Falcon tube
- Centrifuge for 5 min at 1000 rpm, RT
- Remove supernatant, resuspend cells in 1 ml medium and transfer them into a 10 cm culture dish with 9 ml medium

### 3.2.6. Coating of 24-well plates (293T cells)

#### Reagents

Borate Buffer      Dissolve 0.15 M sodium tetraborate decahydrate  
 ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) in ddH<sub>2</sub>O (pH 8.3)  
 Filter sterilize

293T cells are not very adherently and therefore 24 well plates have to be coated with Poly-D-Lysin before seeding cells.

- Dilute Poly-D-Lysin (1mg/ml) in Borate Buffer 1:4
- Add 200 µl per well
- Incubate 2-24 hours RT
- Remove Poly-D-Lysin and wash 2-3x with PBS
- Seed 293T cells

### 3.2.7. Cell seeding (transfection, elctroporation, immunofluorescence and western blotting)

	24 well	6 well	6cm plate
<b>MEFs</b>	$1 \times 10^5$	$2,5 \times 10^5$	$5 \times 10^5$
<b>293T</b>	$1 \times 10^5$	$2,5 \times 10^5$	$5 \times 10^5$
<b>L929</b>	$5 \times 10^4$	$1 \times 10^5$	$2 \times 10^5$
<b>RAW</b>	$5 \times 10^4$	$1 \times 10^5$	$2 \times 10^5$

### 3.2.8. Transfection of cells

#### 3.2.8.1. Transfection with Transmessenger Transfection Reagent (protocol Qiagen)

- Seed cells 1 day before transfection in 24 well plate
- Dilute 2 µl Enhancer-R in Buffer EC-R. Add 1 µg RNA/DNA and mix by vortexing for 10 seconds (s). The final volume should be 100 µl.
- Incubate at room temperature for 5 min, and then spin down the mixture.
- Add 4 µl Transmessenger Transfection Reagent. Mix by vortexing.
- Incubate 10 min RT

- While complex formation takes place, gently aspirate the growth medium from the plate and carefully wash cells 1-2 times with PBS using 1.5-2 times the volume of medium used for seeding
- Add 100  $\mu$ l medium without serum and antibiotics to the transfection complex. Mix by pipetting, then immediately drop it onto the cells.
- Incubate cells for 3 h 37°C.
- Remove complexes and add fresh medium containing 1 % FCS and P/S

#### 3.2.8.2. Transfection with Lipofectin (protocol Invitrogen)

- Seed cells 1 day before transfection in 24 well plate
- Dilute 1  $\mu$ g RNA/DNA with DMEM (without FCS and antibiotics) to a total volume of 50  $\mu$ l
- Dilute 2.5  $\mu$ l Lipofectin with 48  $\mu$ l DMEM (total volume 50  $\mu$ l)
- Incubate 30 min RT
- Mix DNA with Lipofectin and incubate for 5 min RT
- Wash cells with PBS
- Drop DNA-Lipofectin mix (100  $\mu$ l) on cells and incubate for 5 hours
- Remove complexes and add fresh medium containing 1 % FCS and P/S

#### 3.2.8.3. Transfection with Lipofectamin<sup>TM</sup> 2000 (protocol Invitrogen)

- Seed cells 1 day before transfection in 24 well plate
- Next day: dilute 0,8  $\mu$ g RNA/DNA in 50  $\mu$ l Opti-MEM (Gibco) without serum, mix gently
- Mix Lipofectamin gently before use, then dilute 2  $\mu$ l in 50  $\mu$ l of Opti-MEM
- Incubate for 5 min at room temperature
- Combine diluted DNA with diluted Lipofectamin
- Incubate for 20 min
- While complex formation takes place, gently aspirate the growth medium from the plate and carefully wash cells 1 time with PBS
- Add 100  $\mu$ l medium without serum and antibiotics
- Add the 100  $\mu$ l of complexes to each well containing cells and medium. Mix gently.
- Incubate cells at 37°C for 24 h.

- Remove complexes and add fresh medium containing 1 % FCS and P/S after 4-6 h

#### 3.2.8.4. Electroporation:

- Cells were incubated with 1xTrypsin/EDTA and resuspend in 5 ml medium (DMEM) with 10 % FCS
- Count cells and use  $2.5 \times 10^6$  for electroporation
- Centrifuge cells 5 min 1200 rpm
- Wash 2x with cold PBS (5 min 1200 rpm)
- Add 0.8 ml PBS, dissolve pellet
- Transfer cells in a cuvette and add 5  $\mu$ g RNA
- Electroporate with 2 pulses: 1.8kV; 200 $\Omega$ ; 25 $\mu$ F
- Add 5 ml medium
- Count cells
- Seed  $2-5 \times 10^5$  cells per well (for taking different time points)
- Use  $2 \times 10^5$  cells for cytoplasmic RNA isolation (see protocol page 44)

#### 3.2.9. Infection of cells

Infection Medium:

DMEM

1%FCS

15mM HEPES pH 7.4

- Wash cells 1 time with infection medium
- Infect cells with MOI 1 (TBEV:  $1 \times 10^8$  FFU/200  $\mu$ l virus  $\rightarrow$  0.2  $\mu$ l virus for  $1 \times 10^5$  cells) and incubate for 3-4 hours
- Remove medium and wash cells 1x with 2 ml infection medium
- Add fresh infection medium (0.5 ml for 6 cm plate)

#### 3.2.10. Immunofluorescence

- Put sterile small glass plates into 24 well plates and seed cells

Fixating of cells

- Remove medium and add 500  $\mu$ l PBS (ice cold)
- Remove PBS and add 500  $\mu$ l ice cold Methanol/Acetone (1:1)
- Put plate for 10 min at  $-20^{\circ}\text{C}$
- Remove MeOH/Ac and let plates dry on RT

Staining of cells

- Cover small plates with a drop of 1x PBS
- Remove the drop and add 25  $\mu$ l of the 1<sup>st</sup> antibody dilution: Kpm-2 (rabbit  $\alpha$  prM/E NS-1) diluted 1:50 in 1x PBS
- Incubate 1 h/ $37^{\circ}\text{C}$  in a wet chamber
- Wash 2 times with PBS, dry with ventilator
- Cover small plates with a drop PBS
- Remove the drop and add 25  $\mu$ l of the 2<sup>nd</sup> antibody dilution: FITC (goat  $\alpha$  rabbit IgG Fitc) diluted 1:25 in naphthalene black
- Incubate 1 h/ $37^{\circ}\text{C}$
- Wash 2 times with 1x PBS, dry with ventilator
- Fix small plates with mounting medium (Depex, Serva electrophoresis) on microscope slides
- Observe under the microscope

### 3.3. In vitro transcription

To obtain a linear DNA fragment for the in vitro transcription, the C17 and dBSSH plasmids (derivates of pTNd/c, an infectious cDNA clone of TBEV strain Neudoerfl (GenBank accession number U27495)) (Mandl, Ecker et al. 1997) were cut with NheI. Furthermore, the ends were blunted with Klenow. After the cleanup with Qiaex II System, the DNA was loaded on an agarose gel (Fig. 5).

#### 3.3.1. NheI digest and Klenow fill up

6 µg DNA Mega Prep

5 µl Tango Buffer

4 µl NheI

X µl ddH<sub>2</sub>O

50 µl total

→ 1 h 15 min 37°C

3 µl CTP

3 µl TTP

10 µl Klenow Buffer

33 µl H<sub>2</sub>O

1 µl Klenow fragment

50 µl NheI digest

100 µl total

→ 15 min 25°C

#### 3.3.2. Cleanup of DNA with Qiaex II System (according to Qiagen)

- Add 3 volumes of Buffer QX1 to 1 volume of sample
- Check that the color is yellow
- Resuspend QIAEX II by vortexing 30 s

- Add 10  $\mu$ l of QIAEX per 5  $\mu$ g of DNA and mix. Incubate at RT 10 min, mix every 2 min
- Centrifuge sample for 30 s and remove supernatant
- Wash the pellet twice with 500  $\mu$ l Buffer PE
- Air dry pellet for 10-15 min until it becomes white
- Elute the DNA in 32  $\mu$ l H<sub>2</sub>O

### 3.3.3. Cleanup of DNA with Phenol-Chloroform

- Add 100  $\mu$ l nuclease free (NF) water to 100  $\mu$ l sample and 200  $\mu$ l Phenol (1xVol), vortex sample
- Centrifuge 1-2 min full speed
- Put the water phase into new Eppendorf tube
- Add 200  $\mu$ l Chloroform-Isoamylalcohol (24:1), vortex
- Centrifuge 1 min 14000 rpm and put water phase into new tube
- Add NF water to the sample to a total volume of 200  $\mu$ l
- Add 1/10 (20  $\mu$ l) NaAc (3 M, pH 5-6)
- Add 3xVol 96 % EtOH (-20°C) and vortex
- Centrifuge 15 min 13200 rpm
- Discard supernatant (SN) and wash pellet in 1 ml 70 % EtOH
- Centrifuge 15 min 13200 rpm
- Discard SN
- Dissolve pellet in 32  $\mu$ l RNase free (RF) water

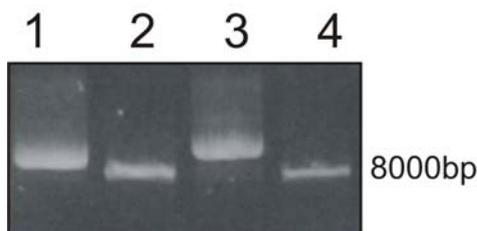


Fig. 5. C17 plasmid DNA (1) and dBSSH plasmid DNA (3) were digested with NheI (2, 4).

### 3.3.4. In vitro transcription with T7 megascript (Ambion)

After the DNA was in vitro transcribed into RNA a DNase digestion was performed. Moreover, the RNA was cleaned up with RNeasy Mini Kit or phenol chloroform extraction and then RNA quality was checked on a RNA gel (Fig. 6). The exact amount of RNA was determined via photometric measurement.

2 µl ATP  
2 µl CTP  
2 µl UTP  
0.4 µl GTP  
0.5 µl Cap  
2 µl buffer  
3 µl ddH<sub>2</sub>O  
12 µl total

→ 3-4 h 37°C

#### DNase digest

20 µl RNA  
1 µl DNase

→ 15 min 37°C

### 3.3.5. Cleanup of the RNA with RNeasy Mini Kit (Qiagen)

- Adjust the sample to a volume of 100 µl with RF water
- Add 350 µl Buffer RLT and mix well
- Add 250 µl EtOH (96-100 %) and mix well by pipetting. Do not centrifuge.
- Transfer sample to a column and centrifuge for 15 s at 10000 rpm
- Add 500 µl Buffer RPE to the column, centrifuge 15 s at 10000 rpm
- Add 500 µl Buffer RPE, centrifuge 2 min 10000 rpm
- Place column in a new 2 ml collection tube, centrifuge 1 min 13000 rpm

- Place column in new 1.5 ml collection tube. Add 60  $\mu$ l RF water and centrifuge 1 min 10000 rpm to elute RNA

### 3.3.6. RNA gel

#### Reagents

10x MOPS	0.2 M MOPS
	50 mM NaOAc
	10 mM EDTA
	+ ddH <sub>2</sub> O → 1l (pH 7.0)

RNA-Gel	0.5 g GTG-Agarose
	37 ml Ambion-H <sub>2</sub> O (nuclease free water, Ambion)
	5 ml 10x MOPS
	Boil up, cool down → add 8ml formaldehyde (HCHO)
	→ pour the gel

The quality of the RNA can be checked on a formaldehyde gel (RNA gel). Firstly the gel runs at 35 mA for 5 min and then at 55 mA for 55 min.

5  $\mu$ l digest  
5  $\mu$ l ddH<sub>2</sub>O  
10  $\mu$ l RNA loading buffer (LB)  
1  $\mu$ l Radiant Red  
16  $\mu$ l total

2  $\mu$ l marker  
3  $\mu$ l ddH<sub>2</sub>O  
5  $\mu$ l RNA LB  
0.5  $\mu$ l Radiant Red  
10.5  $\mu$ l total

### 3.3.7. Photometric measurement

The RNA is diluted in 10 mM Tris Cl (pH 7.5) and concentration is determined by measuring ultraviolet absorbance at 260 nm and 280 nm with the spectrophotometer. The received RSLT value has to be multiplied with the factor of dilution and the obtained result is the amount of RNA in  $\mu\text{g/ml}$ .

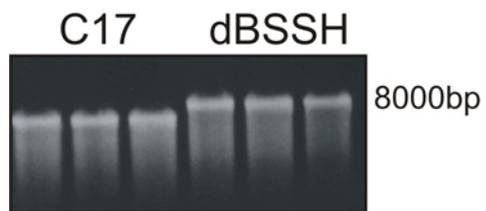


Fig. 6. C17 and dBSSH DNA was in vitro transcribed into RNA and a DNase digestion was performed. Moreover, the RNA was cleaned up with RNeasy Mini Kit and checked on a RNA gel.

### 3.4. Protein analysis

#### 3.4.1. Antibodies

- STAT 1 (Cell Signaling Technology)
- Phospho-STAT1 (Tyr701) Antibody (Cell Signaling Technology)
- ERK1 and ERK2 (pan ERK) (Cell Signaling Technology)
- Amersham ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked Species-Specific Whole Antibody (from sheep) (GE Healthcare)

#### 3.4.2. Cell extracts

##### Whole cell extracts using a detergent buffer

###### Reagents

Frackelton buffer (FB)	10 mM Tris base
	50 mM NaCl
	30 mM Na-pyrophosphat
	50 mM NaF
	1 % Triton X-100
	pH 7-7.5; store at 4°C
Add just before use:	
	1 mM PMSF
	1 mM DTT
	10x protease inhibitors (Roche)
	(dissolved in FB)
	10x phosphatase inhibitors (Roche)
	(dissolved in FB)
SDS sample buffer (24ml)	6 ml TRIS 0.5 M pH 6.8
	2 ml 2-β-mercaptoethanol
	2.5 ml glycerol

4 ml 10 % SDS  
9.5 ml H<sub>2</sub>O  
0.5 % (w/v) bromphenol blue  
Store at -20°C

The whole process has to be performed on ice and in the presence of protease and phosphatase inhibitors to prevent dephosphorylation by phosphatases.

- Remove medium from the cell
- Transfer plate on ice
- Wash cells with cold PBS
- Remove PBS well
- Add 80 µl ice-cold Frackelton Buffer to cells on a 6 cm dish
- Scrape cells with a cell scraper and transfer them to a micro centrifuge tube
- Centrifuge 5 min at 14000 rpm at 4°C
- Transfer supernatant into a new micro centrifuge tube
- Add 40 µl SDS-sample buffer
- Boil for 10 min at 95°C
- Store samples at -20°C or use directly

### 3.4.3. SDS polyacryamide gel electrophoresis

#### Reagents

10x running buffer for SDS PAGE	0.25 M Tris
	1.92 M glycin
	1 % (w/v) SDS

#### 10 % (w/v) SDS

Dissolve 10 g sodiumdodecylsulfate in H<sub>2</sub>O and add to 100 ml

4x separation gel buffer	1.5 M Tris-HCl, pH 8.8
--------------------------	------------------------

4x stacking gel buffer	0.5 M Tris-HCl, pH 6.8
------------------------	------------------------

	<b>Separation Gel (10%)</b>	<b>Stacking Gel (4%)</b>
<b>Acryl amid 40 %</b>	1 ml	0.2 ml
<b>Tris</b>	1 ml (pH 8.8)	0.6 ml (pH 6.8)
<b>H<sub>2</sub>O</b>	2 ml	1.3 ml
<b>SDS (10%)</b>	40 µl	20 µl
<b>TEMED</b>	12 µl	6 µl
<b>APS (20%)</b>	12 µl	6 µ

- Assemble the glass plates according to the manufacturer's instructions
- Prepare separation gel and pour it
- Cover separation gel with 70 % EtOH and wait until it is polymerized
- Remove EtOH and wash with dH<sub>2</sub>O
- Pour the stacking Gel and insert the comb
- Wait until gel is polymerized
- Put gel in the electrophoresis apparatus and add SDS running buffer
- Load 15 µl of the samples and 7 µl of a molecular weight marker (PageRuler™ Prestained Protein Ladder, Fermentas)
- Run the gel first 80 Volt until proteins enter the separation gel, then switch to 110 V

#### 3.4.4. Western blotting

##### Reagents

Anode buffer I      0.3 M Tris  
                              20 % Methanol  
                              pH 10.4

Anode buffer II      2.5 mM Tris  
                              20 % Methanol  
                              pH 10.4

Cathode buffer      0.04 M amino-capronic acid  
                              20 % Methanol

0.01 % SDS

Ponceau S      0.2 % (w/v) Ponceau S  
                  3 % (w/v) trichloroacetic acid

- Put PVDF membrane in Methanol, nitrocellulose membrane in dH<sub>2</sub>O, then shortly in Anode II Buffer
- Gel is inverted on the plate
- Put membrane on gel
- Put 3 Whatmann papers into Anode II buffer and then on gel
- Soak 6 Whatmann papers in Anode I buffer and add them
- Turn around gel and papers
- Add 6 papers soaked in Cathode buffer
- The blotting is performed in a semy dry blotting apparatus at 0.8 mA per cm<sup>2</sup> (= 60 mA/minigel), constant voltage of 20 V for 120 min
- After the blotting is completed the membrane is rinsed with H<sub>2</sub>O and stained with Ponceau S to test the quality of the protein transfer. The stain is removed by washing with water for 5 min.

### 3.4.5. Immunostaining

- Block the membrane with 5 % milk in TBST or 2 % BSA in TBST for 60 min
- Rinse the membrane with TBST and wash 3 times for 10 min with the buffer
- Incubate the membrane with the antibody solution overnight at 4°C on a shaker
- Wash 3x with TBST for 10 min
- Add secondary antibody- anti mouse or anti rabbit coupled to HRP 1:5000 in TBST for 30 min
- Wash 3x with TBST 10 min
- Detection with ECL-system (purchased from Pierce) in the dark room
- Cover the membrane with 1:1 mixture of Super Signal ECL – detection solution 1 and 2 for 1-2 min
- Expose to a Fuji X-ray medical film

The following antibody solutions have been used

Anti-pY701, 1:2000, 1 % BSA, 0.05 % NaN<sub>3</sub> in TBST

Anti-S1-C, 1:2000, 1 % BSA, 0.05 % NaN<sub>3</sub> in TBST

Anti-panERK, 1:2000, 1 % BSA, 0.05 % NaN<sub>3</sub> in TBST

### **3.4.6. Stripping of membranes**

#### Reagents

Stripping buffer	200 mM Glycin
	150 mM NaCl
	0.5 % (v/v) Tween 20
	pH 2.5
	autoclave

- Rinse the membrane shortly in water
- Incubate for 10 min in stripping buffer at RT
- Rinse membrane again with water
- Repeat the immunostaining procedure or dry the membrane between Whatmann papers

### 3.5. Real-time PCR

#### 3.5.1. Oligonucleotides

Real-time PCR Primers were purchased by VBC-genomics.

TaqMan (Tqm) probes were labelled with the reporter dye 6-carboxy-fluorescein (FAM) on the 5' end and the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) on the 3' end.

gene	<b>FSME-NS5</b>	
forward	FSME NS5-1	5'-GAAGCGGAGGCTGAACAAC-3'
reverse	FSME NS5-2	5'-TTGTCACGTTCCGTCTCCAG-3'
probe	FSME-NS5-Tqm	5'-TGTGTACAGGCGCACCCGGCA-3'
gene	<b>Ubiquitin conjugating enzyme 2d2 (Ube2d2)</b>	
forward	Ube2d2-f	5'-AGGTCCTGTTGGAGATGATATGTT-3'
reverse	Ube2d2-r	5'-TTGGGAAATGAATTGTCAAGAAA-3'
probe	Ube2d2 FAM	5'-CCAAATGACAGCCCCTATCAGGGTGG-3'
gene	<b>Cxcl10 (IP-10)</b>	
forward	IP-10(44)-f	5'-GTCTGAGTGGGACTCAAGGGATC-3'
reverse	IP-10(120)-r	5'-CACTGGCCCGTCATCGATAT-3'
probe	IP-10(88)FAM	5'-CTCTCGCAAGGACGGTCCGCTG-3'
gene	<b>Interferon alpha 4</b>	
forward	IFNa4-f	5'-CCTGTGTGATGCAGGAACC-3'
reverse	IFNa4-r	5'-TCACCTCCCAGGCACTGA-3'
probe	IFNa4 FAM	5'-AGACTCCCCTGCTGGCTGTGAGGACA-3'
gene	<b>pan Interferon alpha</b>	
forward	panIFNa(453)-f 18ATdeg	5'-CCACAGGATCACTGTGT(AT)CCTGAGA-3'
reverse	panIFNa(513)-r	5'-CTGATCACCTCCCAGGCACAG-3'
probe	panIFNa(479) FAM LNA	5'-AG+AA+GAA+A+C+AC+AG+CC-3'
gene	<b>GADPH</b>	
forward	GAPDH-f	5'-TGCACCACCAACTGCTTAGC-3'
reverse	GAPDH-r	5'-GGCATGGACTGTGGTCATGAG-3'
gene	<b>IFN<math>\beta</math></b>	
forward	IFN $\beta$ -f	5'-TCAGAATGAGTGGTGGTTGC-3'
reverse	IFN- $\beta$ -r	5'-GACCTTTCAAATGCAGTAGATTCA-3'

### 3.5.2. RNA isolation

#### Reagents

Na <sub>2</sub> PO <sub>4</sub> -gel	100 ml 1x RNA buffer (0.01 M Na <sub>2</sub> HPO <sub>4</sub> , 0.01 M NaH <sub>2</sub> PO <sub>4</sub> ) 2 agarose tablets 5 µl ethidium bromide Run gel for 1h 50 volt
RLN buffer (100 ml)	50 mM TrisHCl (1 M) 5 ml 140 mM NaCl (5 M) 2.8 ml 1.5 mM MgCl <sub>2</sub> (1 M) 250 µl 0.5 % (v/v) Igepal 500 µl 91.55 ml H <sub>2</sub> O
1M DTT	3.1 g dithiothreitol in 20 ml of 0.01 M sodium acetate (pH 5.2) Filter sterile and store at -20°C

#### 3.5.2.1. Purification of total RNA from animal cells using spin technology (according to Quiagen)

- Wash the cells with 1x PBS
- Add 350 µl Buffer RLT and scrape the cells off the plate
- Add 1 volume of 70 % EtOH and mix well by pipetting
- Transfer the sample to an RNeasy spin column and centrifuge for 15 s 10000 rpm

#### DNase digestion:

1. add 350 µl Buffer RW1, centrifuge 15 s 100000 rpm
  2. add 10 µl DNase stock solution (Quiagen) to 70 µl Buffer RDD, mix gently
  3. add the mix (80 µl) to the spin column membrane and place on the bench top for 15 min
- add 350 µl Buffer RW1, centrifuge 15 s 10000 rpm
  - Add 500 µl Buffer RPE, centrifuge 15 s 10000 rpm
  - Add 500 µl Buffer RPE, centrifuge 2 min 10000 rpm
  - place column in a new collection tube, centrifuge 1 min full speed

- Place column in a new centrifuge tube. Add 40 µl RNase-free water and centrifuge 1 min 10000 rpm
- Add another 40 µl RNase-free water and centrifuge 1 min 10000 rpm

#### 3.5.2.2. Isolation of cytoplasmic RNA (according to Quiagen)

- Prepare Buffer RLN (always add 1 mM DTT fresh)
- Add 175 µl cold RLN and resuspend cells
- Incubate on ice 5 min
- Centrifuge at 4°C at 1200 rpm 5 min
- Transfer supernatant into a new tube
- Add 600 µl Buffer RLT
- Add 430 µl EtOH, mix by pipetting, do not centrifuge
- Apply 700 µl to a column, centrifuge 15 s 10000 rpm. Discard the flow-through. Repeat with remaining sample
- Add 700 µl Buffer RW1, centrifuge 15 s 10000rpm
- Transfer column into new tube. Add 500 µl Buffer RPE, centrifuge 15 s 10000 rpm
- Add another 500 µl RPE, centrifuge 2 min 10000 rpm
- Place column in a new tube, centrifuge 1 min full speed
- Transfer column to a new 1.5 collection tube. Add 60 µl RF water directly on membrane, centrifuge 1 min 10000 rpm to elute.

The quality of the RNA is checked with a Na<sub>2</sub>PO<sub>4</sub>-gel and the concentration is determined in the spectrophotometer by measuring ultraviolet absorbance at 260 nm and 280 nm with the spectrophotometer. The received RSLT value has to be multiplied with the dilution factor and the result indicates the concentration of the RNA in µg/ml.

#### **3.5.3. cDNA synthesis**

RNA samples are reverse transcribed in cDNA for real-time PCR according to Biorad iScript™ cDNA synthesis users manual.

10 µl RNA (~1 µg)  
5 µl H<sub>2</sub>O  
4 µl 5x iScript Reaction Mix  
1 µl Reverse Transcriptase  
20 µl total

PCR reaction:

5 min 25°C  
30 min 42°C  
5 min 85°C  
Hold at 4°C

### **3.5.4. Real-time PCR**

The real-time experiments are performed on the 7300 Real-Time PCR System (Applied Biosystems).

#### 3.5.4.1. Calculation of the molecular weight of TBEV RNA copies

Program used: <http://www.basic.northwestern.edu/index.html>

Wt TBEV:  $3.46 \times 10^{12}$  µg/mol.....a

Avogadro's number:  $6.06 \times 10^{23}$ /mol RNA.....b

$b/a = 1.66 \times 10^{11}$  molecules/µg RNA

#### 3.5.4.2. Determination of RNA copies of TBEV

Viral RNA (C17) dilutions from  $10^2$  to  $10^7$  are used as a standard and for quantitation the standard curve method is used:

[http://www.biotech.uiuc.edu/centers/Keck/Functional\\_genomics/taqman/Guide%20to%20relative%20quantitation.pdf](http://www.biotech.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf)

#### *FSME-NS5*

Master Mix

12.5 µl TaqMan® Universal PCR Master Mix

0.4 µl primer 1 (P1) (100pmol/µl)  
0.4 µl primer 2 (P2) (100pmol/µl)  
0.1 µl TaqMan probe (100pmol/µl)  
6.6 µl H<sub>2</sub>O  
5 µl template (cDNA)  
25 µl total volume

#### 3.5.4.3. Determination of gene inducibility

*Pan alpha 4, pan IFN alpha, IP-10*

Ubiquitin-conjugating enzyme E2D 2 (Ube2d2), a housekeeping gene, is chosen as an endogenous control for normalization of the RNA load

Master Mix

12.5 µl TaqMan® Universal PCR Master Mix  
0.75 µl P1 (10 pmol/µl)  
0.75 µl P2 (10 pmol/µl)  
0.25 µl TaqMan probe (10 pmol/µl)  
6 µl H<sub>2</sub>O  
5 µl template (cDNA diluted 1:5)  
25 µl total volume

*IFNβ*

Glyceraldehyde-3-phosphate dehydrogenase (GADPH), a housekeeping gene, is chosen as an endogenous control for normalization of the RNA load.

Master Mix (IFNβ)

12.5 µl TaqMan® Universal PCR Master Mix  
0.5 µl P1 (100 pmol/µl)  
0.5 µl P2 (100 pmol/µl)  
1 µl probe (syber green 1:1000)  
0.375 FITC

0.125 µl H<sub>2</sub>O  
10 µl template (cDNA diluted 1:5)  
25 µl total volume

Master Mix (GADPH)

12.5 µl TaqMan® Universal PCR Master Mix  
0.2 µl P1 (100 pmol/µl)  
0.2 µl P2 (100 pmol/µl)  
1 µl probe (syber green 1:1000)  
0.375 FITC  
5.725 µl H<sub>2</sub>O  
5 µl template  
25 µl total volume

For quantitation of gene expression normalized to an endogenous control the standard curve method is used:

[http://www.biotech.uiuc.edu/centers/Keck/Functional\\_genomics/taqman/Guide%20to%20relative%20quantitation.pdf](http://www.biotech.uiuc.edu/centers/Keck/Functional_genomics/taqman/Guide%20to%20relative%20quantitation.pdf)

3.5.4.4. Standard curve equation:

The resulting C<sub>T</sub> values (PCR cycles to reach a fixed threshold of DNA synthesis) of the diluted cDNA sample are plotted against the log input copy number: C<sub>T</sub> values = m\* (log input copy number) + b

The copy numbers of the samples are normalized for diverse amounts of cDNA added to the reaction, to copy numbers of the endogenous Ube 2d2/GADPH control. Finally, the normalized amount of target is divided by the target quantity of the calibrator, which is the unstimulated control and has the lowest expression level of the target. Therefore, the stimulated samples are expressed as n-fold increase (inducibility) to the calibrator.

### 3.6. Luciferase assay

#### Plasmids

- *Renilla* pRL-SV40 Vector (Promega)
- *Renilla* pRL-null Vector (Promega)
- *Renilla* pRL-CMV Vector (Promega)
- p125-Luc: Firefly luciferase plasmid under the control of an IFN $\beta$  promoter (provided by Takashi Fujita)

#### 1<sup>st</sup> day:

##### Seeding of cells (293T)

1x10<sup>5</sup> cells per well are seeded in coated 24 well plates.

#### 2<sup>nd</sup> day:

##### Transfection of plasmids (*Renilla* + firefly) with Lipofectin

A transfection of 0.1  $\mu$ g *Renilla* as control plasmid and cotransfection of 0.1  $\mu$ g *Renilla* and 0.9  $\mu$ g firefly are performed.

#### 3<sup>rd</sup> day:

##### Transfection of RNA (1 $\mu$ g/ml) with Transmessenger Reagent and infection of cells

#### 4<sup>th</sup> day:

##### Preparation of cells for luciferase assay

- Wash cells with PBS 1 time
- Add 200  $\mu$ l Trypsin/EDTA for 1-2 min at 37°C
- Add 700  $\mu$ l Medium (EMEM)
- Resuspend the cells the plate and put them into a microcentrifuge tube
- Centrifuge 2-3 min with a table top centrifuge
- Remove supernatant
- Add 180  $\mu$ l RLN+DTT
- Put tubes 5 min on ice
- Centrifuge 5 min 1200 rpm 4°C
- Put the supernatant into a new tube
- Store samples at -80°C or use (measure luciferase) them directly

Measurement of relative light units (RLUs)

- Take 75  $\mu$ l of the sample for luciferase assay and add to 48 well plate
- Add 75  $\mu$ l firefly substrate (Dual-Glo™ Luciferase reagent, Promega)
- Measure the firefly luminescence with the luminometer (Victor Light Luminometer, Perkin Elmer)
- Add 75  $\mu$ l *Renilla* substrate (Dual-Glo™ Stop & Glo Luciferase reagent, Promega)
- Measure the *Renilla* luminescence with the luminometer

### **3.7. Mega Prep**

#### **3.7.1. Production of electro competent HB101 E.coli cells**

- Prepare 2x 5 ml over night culture of HB101 (37°C, 280 rpm)
- Next day: dilute the culture 1:100 and put it on the shaker until it reaches  $OD_{600}=0.5-1$
- Put it on ice 15-30 min
- Centrifuge 15 min 5000 rpm (Rotor F16, Sorvall)
- Discard the supernatant and resuspend pellet in 6x 165 ml  $H_2O_{dd}$ , 4°C
- Centrifuge 15 min 5000 rpm and discard supernatant
- Resuspend pellet in 3x 165 ml  $H_2O_{dd}$ , 4°C
- Centrifuge 15 min 5000 rpm and discard supernatant
- Resuspend pellet in 2 ml 10 % glycerin, 4°C
- Make 50  $\mu$ l aliquots on dry ice/EtOH
- Store aliquots at -80°C

#### **3.7.2. Transformation of plasmid in HB101 E.coli cells**

##### **1<sup>st</sup> day:**

- Dilute original plasmid Mega Prep 1:100
- Mix 45  $\mu$ l HB101 E.coli + 5  $\mu$ l plasmid (1:100 dilution)
- Incubate 1 min on ice
- Put in precooled 4 mm cuvette (Biozym)
- Electroporate: 1.8kV ; 200 $\Omega$  ; 25 $\mu$ F
- Add 500  $\mu$ l LB-Glu (0.02 M)
- Incubate 1 h 37°C on the shaker
- Centrifuge 3 min 3000 rpm in microcentrifuge tube
- Discard, plate 100  $\mu$ l on two plates LB-Amp (70  $\mu$ l + 30  $\mu$ l)
- Put plates upside down in the incubator over night

**2<sup>nd</sup> day:**

- Pick 1 colony and add to 400 ml LB-Amp (1 µl/ml Amp)
- Incubate bacterial cells in the shaker over night 37°C

**3.7.3. Mega Prep (according to Qiagen)**

**3<sup>rd</sup> day:**

- Harvest bacterial cells by centrifugation at 6000 rpm 10 min 4°C
- Screw the QIAfilter Cartridge onto a 45 mm neck glass bottle and connect it to a vacuum source
- Resuspend bacterial pellet in 50 ml P1 Buffer
- Add 50 ml P2, mix gently by inverting 4-6 times and incubate on RT 5 min
- Add 50 ml Buffer P3, mix immediately by inverting 4-6 times, mix well until white, fluffy material has formed
- Pour lysate into QIAfilter Cartridge and incubate at RT 10 min
- Switch on vacuum source; after all liquid has been pulled through, switch off vacuum source
- Add 50 ml Buffer FWB2, switch on vacuum source
- Equilibrate Quiagen tip by applying 35 ml Buffer QBT
- Apply the filtered lysate onto Quiagen tip, allow it to enter the resin by gravity flow
- Wash the tip with 200 ml Buffer QC
- Elute DNA with 35 ml with QF
- Precipitate DNA by adding 24.5 ml RT isopropanol, mix and centrifuge 4°C 4000 rpm 60 min
- Wash the pellet with 7 ml RT 70 % EtOH, centrifuge 15 min 4°C 4000 rpm, carefully decant supernatant
- Air dry pellet 10-20 min, redissolve DNA in 400-500 µl H<sub>2</sub>O

### 3.8. ELISA

#### Reagents

2x carbonate buffer	120 mM Na <sub>2</sub> CO <sub>3</sub> 80 mM NaH pH 9.6, aliquot and store at -20° C
Elisa buffer (EP)	1x PBS 2 % Tween 2 % Hammelserum (HS)
Washing buffer	1x PBS 0.05 % Tween pH 7.4
Elisa substrate	10 mg o-Phenylendiamin 10 ml phosphate buffer- citrate buffer pH 5.0 10 µl H <sub>2</sub> O <sub>2</sub> (Sigma)

- Coat the plates with antibody: γ GP2 (dilution 1:3000 in carbonate buffer pH 9.6) 50 µl/well and incubate 2 days 4°C or 24 hours at RT. (Carbonate buffer can only be used for 2 Weeks)
- Dilute supernatants 1:3 (50 µl +100 µl) in Elisa buffer (EP)
- Pipette 50 µl of the diluted sample on coated plate  
alignment on the plate: 1. row: Blanc, 2. -12. row samples (including positive control)
- Incubation 2 h 37°C in wet chamber
- Wash 4x with washing buffer
- Add 50 µl antigen-specific antibody (γ KP2) per well, diluted: 1:5000 in EP
- Incubation for 1 h 37°C in the wet chamber
- 4x washing with washing buffer
- add 50 µl anti-rabbit IgG-POX-conjugate (1:5000) (Amersham, NA 934, Batch)
- Incubation for 1 h 37°C in the wet chamber
- 4x washing with washing buffer

- Add 50 µl Elisa substrate per well
- Incubation 30 min protected from light at room temperature
- Stop the reaction by adding 100 µl 2N H<sub>2</sub>SO<sub>4</sub> per well
- Measurement in ELISA Reader ELX 808/KC Junior 490 nm/ reference filter 630 nm

### 3.9. Focus assay

#### Reagents

Blocking Solution            PBS 7.4  
   5 % Hammelserum (HS)

Antigen (Ag) buffer        PBS 7.4  
   0.2 % Tween  
   3 % HS

Conjugate buffer            TBS  
   0.2 % Tween  
   3 % HS

#### Substrate

= Sigma Fast® Fast Red TR/Naphtol AS-MX (#F -4648 = 1ml tablet)

### Infection

#### **1<sup>st</sup> day:**

- Seed  $1 \times 10^5$  BHK-21 cells per well (24 well plate)

#### **2<sup>nd</sup> and 3<sup>rd</sup> day:**

- Wash with 0.5 ml infection medium
- Make dilutions of the virus (TBEV) with medium for infection ( $10^{-1}$ - $10^{-10}$ ) and add 200  $\mu$ l of every dilution (1<sup>st</sup> row of plate = cell control)
- Incubation: 3-4 h 37°C, 5 % CO<sub>2</sub>
- Remove virus suspension and cover cells with 1 ml 3 % CMC-Overlay in medium for infection. (Stock: 6 % CMC in medium for infection 1:1 diluted with fresh medium of infection)
- Incubation: BHK-21: 50-55 h, 37°C, 5 % CO<sub>2</sub> (attention: avoid abrasions)

**4<sup>th</sup> day:**

- Remove overlay and wash cells 2-3x with PBS (pH 7.4), 0.5 ml/well until overlay is totally washed off
- Fix cells with acetone methanol 1:1, 0.5 ml/well, 10 min -20°C
- Wait until cells are dry (dry plates can be stored at 4°C for 1-2 days)
- Blocking: 0.5 ml/well with Blocking Solution, 30 min room temperature
- Add 1<sup>st</sup> antibody:  $\gamma$  KP2 diluted 1:5000 in Ag-buffer, 200  $\mu$ l/well; incubation for 1 h 37°C
- Wash 2x with Ag-buffer, 0.5 ml/well; 3. washing step with conjugate buffer, 0.5 ml/well
- Add 2<sup>nd</sup> antibody: Goat anti-rabbit-IgG-AP (AP = alkaline phosphatase; Sigma # A-3812) diluted 1:400 in conjugate buffer and add 200  $\mu$ l/well; incubation for 45 min at RT
- Wash 2x with conjugate buffer: 0.5 ml/well
- Add substrate, 200  $\mu$ l/well; incubation for 5-10 min RT
- Stop of the reaction: remove substrate and wash with ddH<sub>2</sub>O 0.5 ml/well

### 3.10. Isolation of genomic DNA from cells

#### Reagent

Proteinase K lysis buffer	50 mM Tris/HCl pH 8.0 100 mM NaCl 100 mM EDTA pH 8.0 1 % SDS
Proteinase K	Stock solution (20 mg/ml) proteinase K in H <sub>2</sub> O
CIA	24 vol. chloroform 1 vol. isoamylalcohol
TE	10 mM Tris/HCl pH 8.0 1 mM EDTA pH 8.0

- Prepare cell pellets by centrifugation of cells, wash the pellet once with PBS
- Digest cell pellets in 500 µl Proteinase K lysis buffer supplemented with 3 µl Proteinase K at 60° C, 2 h – over night
- Add 500 µl CIA, invert the solution for 5-10 min and centrifuge for 10 min at 14000 rpm RT
- Transfer supernatant into a new tube. Precipitate DNA by adding 1 vol. isopropanol, invert solution for 3 times and centrifuge for 10 min at 14000 rpm
- Remove the supernatant and wash the DNA pellet once by adding 500 µl 70 % ethanol, centrifuge for 5 min at 14000 rpm RT
- Remove ethanol and air-dry the DNA pellet at RT
- Resuspend DNA in 70 µl TE

### 3.11. Common reagents and stock solutions

#### 20 % (w/v) APS

20 g ammoniumpersulfate

Add H<sub>2</sub>O to 100 ml

Store aliquots at -20°C

#### 10x phosphate buffered saline (PBS)

1.4 M NaCl

25 mM KCl

81 mM Na<sub>2</sub>HPO<sub>4</sub> x 2 H<sub>2</sub>O

15mM KH<sub>2</sub>PO<sub>4</sub>

pH 7.3

#### 100 mM PMSF

100 mM phenylmethylsulfonylflourid in 2-propanol

store at 4°C

#### 6x sample buffer for agarose gels

0.25 % (w/v) bromphenol blue

0.25 % (w/v) xylene cyanol FF

30 % (v/v) glycerol in H<sub>2</sub>O

store at 4°C

#### 10x TBE

890 mM Tris

890 mM boric acid

20 mM EDTA

add H<sub>2</sub>O to 1 litre

pH 8.0-8.3

autoclave

#### 1x TBST

10 mM Tris/HCl, pH 8

## Materials and Methods

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150 mM NaCl

0.05 % Tween 20

10x Trypsin/EDTA (SIGMA)

Working solution is diluted in sterile PBS and stored at 4°C

## **4. Results**

### **4.1. IFN $\beta$ mRNA expression is TBEV RNA replication dependent**

Innate immunity is important and necessary to detect viral spread and replication. The cell is able to recognize the presence of viruses by pathogen recognition receptors (PRRs). When an invading virus is detected, PRRs activate various signalling pathways, which lead to the induction of latent transcription factors and moreover to the production of type I interferons (IFNs) (Saito and Gale 2007).

To investigate if and when IFN $\beta$  mRNA induction takes place in the cell we infected mouse macrophages (RAW cells) with TBEV and isolated cytoplasmic RNA at multiple time points. Poly I-C (pIC), a synthetic double stranded RNA, is known as an efficient inducer of IFNs (Randall and Goodbourn 2008) and was therefore used as positive control in the experiment. Little IFN $\beta$  mRNA was detectable 12 hours post infection, whereas after 24 hours a high induction was measured (Fig. 7). To test whether viral surface glycoproteins play a role in the induction of type I IFNs or viral RNA replication, we treated the cells with formalin (FA) inactivated virus or recombinant subviral particles (RSPs), which both cannot replicate. After 24 hours no type I IFN mRNA was monitored in these cells, indicating that IFN $\beta$  expression depends on viral RNA replication.

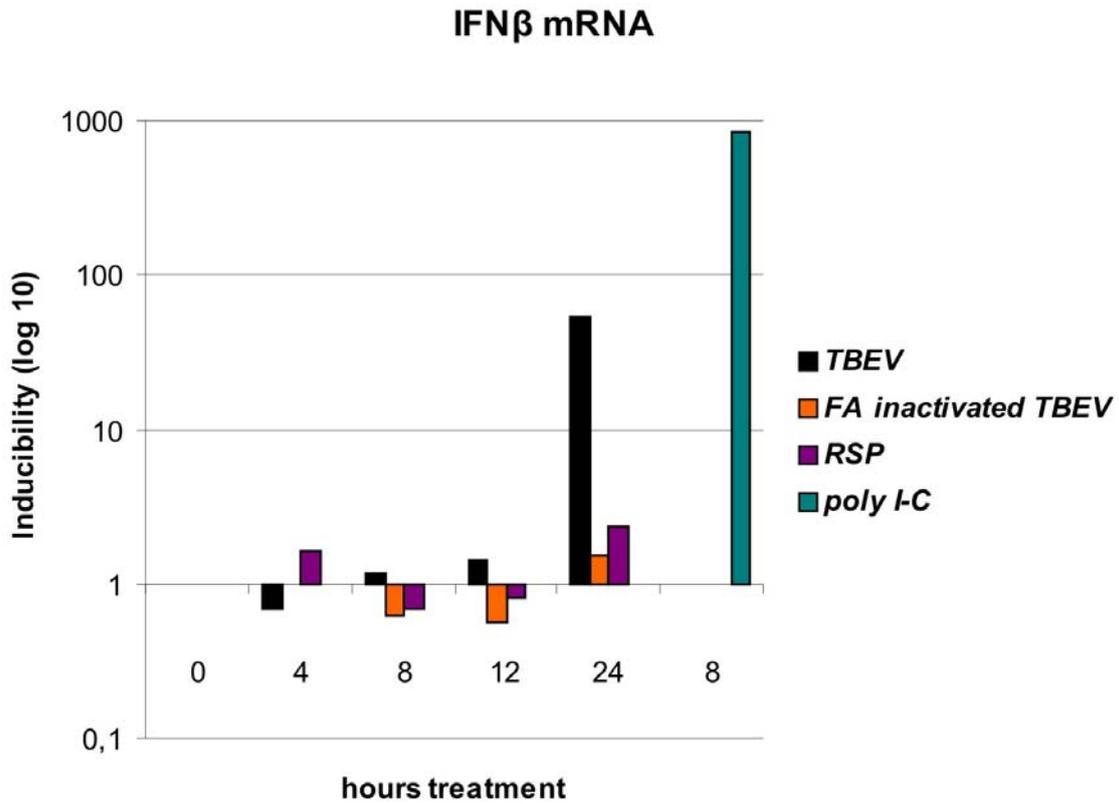


Fig. 7. TBEV infection induces IFN $\beta$  mRNA induction

RAW cells were infected with TBEV and treated with formalin (FA) inactivated virus and recombinant subviral particles (RSPs) for 0, 4, 8, 12 and 24 hours. Cytoplasmic RNA was isolated and the relative amount of IFN $\beta$  mRNA was determined by quantitative PCR and normalized to the house keeping gene *gapdh*. Poly I-C (pIC) was used as a positive control (1  $\mu$ g/ml).

## 4.2. TBEV RNA replication mediates type I IFN activation

The interferon regulatory factor 3 (IRF3) is a central transcription factor that is involved in the activation of type I IFNs. IRF3 was shown to play a crucial role in the activation of IFN $\alpha/\beta$  and subsequent control of virus replication by most RNA viruses like other members of the family *Falvivirusidae* (Daffis, Samuel et al. 2007). Here, we wanted to analyse the role of IRF3 in activation of type I IFN mRNA and control of virus replication.

We infected wild-type and IRF3 deficient mouse embryonic fibroblasts (MEFs) with TBEV and monitored virus replication by staining infected cells with a polyclonal antibody raised against TBEV (Fig. 8) and by measuring viral proteins by ELISA in the supernatant of infected cells (Fig. 9).

Cells that are deficient in IRF3 showed a strong increase in virus replication, which is shown by amplified viral antigen in the supernatant at MOI 1 and MOI 10 24 hours after infection. According to the export of virus to the supernatant, most IRF3 deficient cells were stained positive for TBEV already 24 hours after infection, while positive wild-type cells were only visible 48 hours post infection.

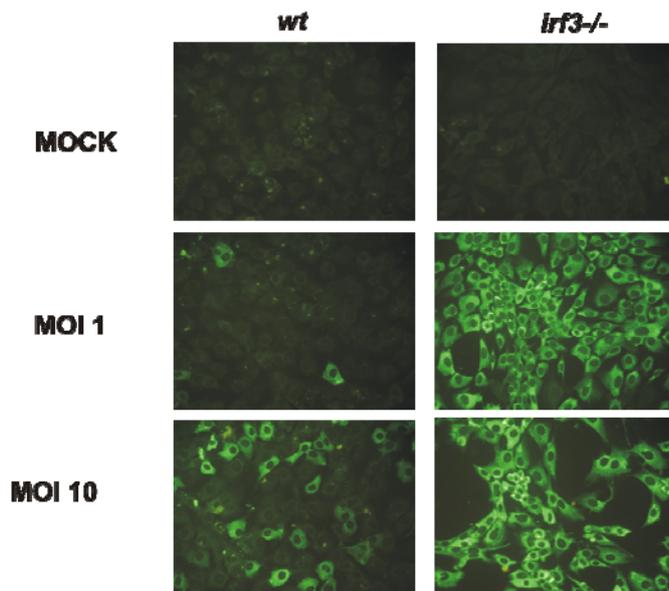


Fig. 8. TBEV replicates in IRF3<sup>-/-</sup> cells

Wild-type (wt) and interferon regulatory factor 3 (IRF3) deficient mouse embryonic fibroblasts (MEFs) were infected with TBEV multiplicity of infection 1 (MOI 1) and MOI 10 and left untreated (Mock). Cells were fixed with acetone methanol after 48 h and immunofluorescence staining with a polyclonal antiserum against TBEV was performed.

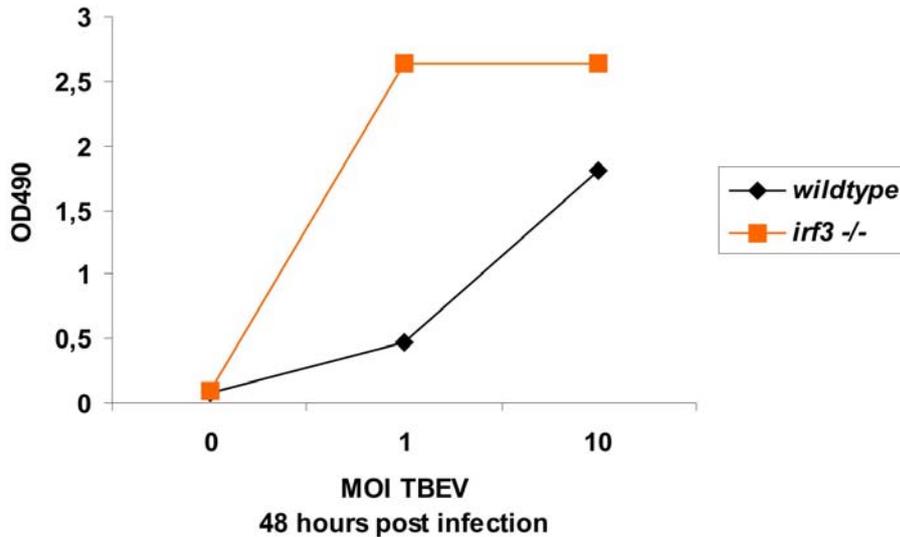


Fig. 9. Replication of TBEV is elevated in IRF3  $-/-$  MEFs

Supernatant from cells (wt/IRF3  $-/-$ ) infected with MOI 1 and MOI 10 was harvested after 48 h and subjected to ELISA measuring virus protein.

We further analysed the correlation between virus replication and type I IFN mRNA expression in these cells. We infected cells with TBEV MOI 1. At the indicated time points we isolated total RNA and measured the absolute amount of viral RNA copies (Fig. 10) and relative induction of IFN $\beta$  mRNA (Fig. 11) by quantitative real-time PCR. Again, virus replication was only measured in IRF3 deficient cells, in wild-type cells the input RNA measured at 2 hours post infection did not increase. The virus started to replicate between 8 and 24 hours. This is also the time frame where IFN $\beta$  mRNA expression is activated in wild-type cells. In these cells the virus did not replicate which was mediated by type I IFNs. This gives another indication that virus RNA replication but not the input RNA is a central mechanism in the activation of IFN $\alpha/\beta$  expression.

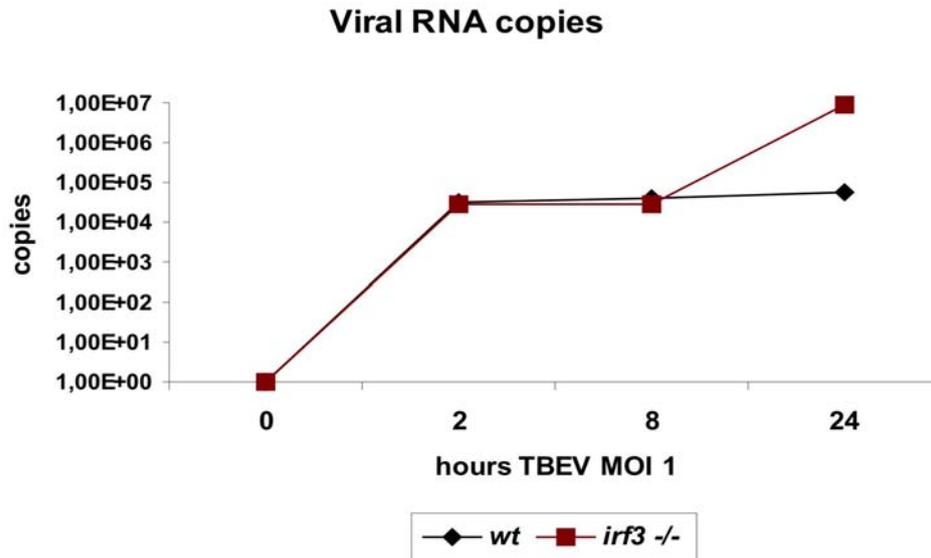


Fig. 10. TBEV replication occurs in IRF3 *-/-* cells

Wt MEFs and IRF3 *-/-* cells were infected with TBEV at MOI 1. The cytoplasmic RNA was isolated after 0, 2, 8 and 24 h of infection and reverse transcribed. The amount of RNA was measured by real-time PCR and was shown in the number of RNA copies post infection.

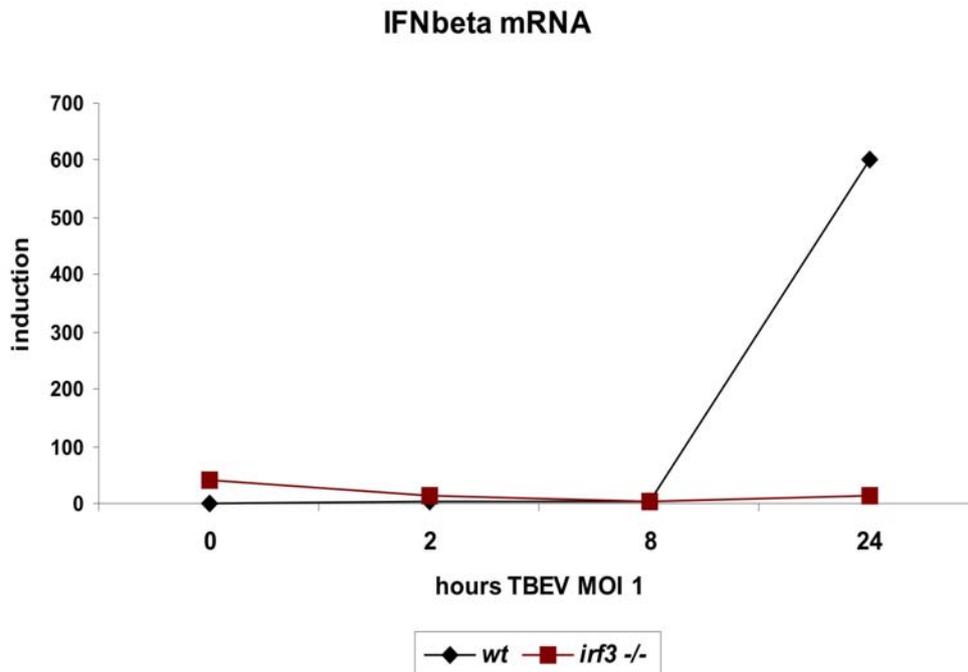


Fig. 11. TBEV infection induces IFN $\beta$  mRNA in wt MEFs

Wt MEFs and IRF3 *-/-* cells were infected with TBEV at MOI 1. The cytoplasmic RNA was isolated after 0, 2, 8 and 24 h of infection, reverse transcribed and subjected to quantitative real-time PCR measuring IFN $\beta$  expression normalized to the house keeping gene *gapdh*.

### 4.3. A TBEV replicon is able to replicate in cells deficient in IRF3

In the previous experiments we showed that replication of the RNA virus TBEV leads to type I IFN mRNA expression. To analyze which parts of the virus replication cycle contribute to IFN $\alpha/\beta$  induction, we used TBEV RNA constructs and electroporated them into wild-type mouse embryonic fibroblasts (MEFs). On the one hand we electroporated the replicon C17 (Fig. 12), which is a derivative of full-length infectious cDNA clone of TBEV strain Neudoerfl. In this mutant almost the complete structural protein coding region is removed, except the parts coding for the first 17 amino acid residues of protein C and the interior signal sequence at the carboxy terminus of protein E. This RNA is a so called replicon, a RNA molecule defined by its property to replicate in host cells; however, due to the lack of structural proteins, unable to infect neighbouring cells. The C17 replicon RNA can replicate and translate to wild-type levels. On the other hand we transfected a replication deficient mutant (dBSSH) (Fig. 13) into MEFs. dBSSH exhibits a partial deletion in NS5, the RNA polymerase, and therefore cannot replicate (Kofler, Hoenninger et al. 2006).

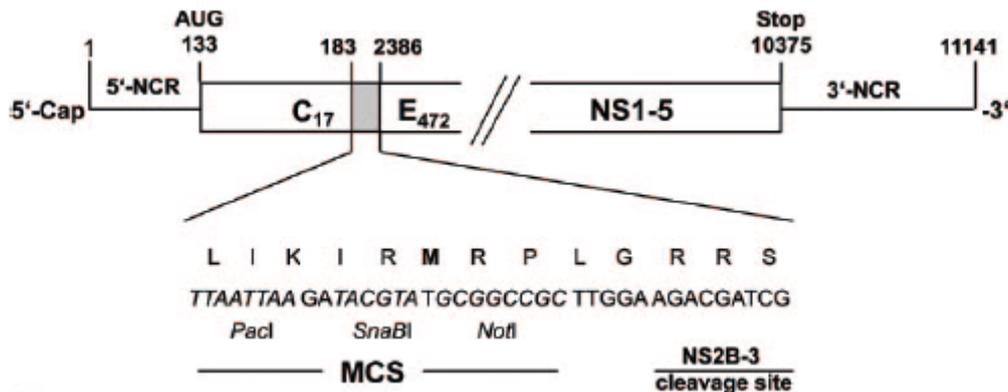


Fig. 12. Schematic drawing of C17

The nucleotide sequence between position 183 and 2386 (corresponding to amino acid residue 18 of protein C and 471 of protein E) of the wild-type TBEV genome was replaced by an artificial sequence that includes a multiple cloning site (MCS) (Kofler, Hoenninger et al. 2006).

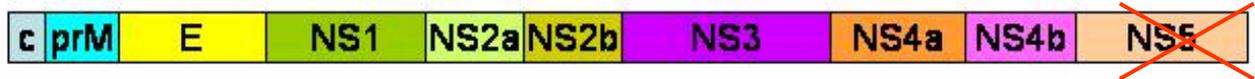


Fig. 13. dBSSH

The clone contains all structural (C, prM and E) and non-structural (NS) (NS1-NS5) proteins of the wild-type TBEV genome, but maintains a partial deletion in the NS5 protein, which provides the RNA-dependent RNA polymerase.



Fig. 14. NK4

NK4 is an infectious whole length clone of TBEV strain Neudoerfl.

In previous experiments we showed that TBEV can only replicate efficiently in cells that are unable to produce type I IFNs like IRF3 *-/-* cells (Fig. 8-11). Therefore, we electroporated the virus constructs in wild-type and IRF3 deficient cells and monitored for replication.

3, 8, 24 and 48 hours post electroporation the cytoplasmic RNA of the cells was isolated and the amount of virus RNA copies was determined by quantitative real-time PCR (Fig. 15A). After 24 hours the number of C17 RNA copies in IRF3 *-/-* cells was higher than in wt MEFs indicating that the construct replicated only in this cell line. As expected, the replication deficient dBSSH RNA was decreased in wt and IRF3 knock out (ko) cells. Moreover, we confirmed this result by immunofluorescence staining. After cells were electroporated with C17, we stained them with a polyclonal antiserum against TBEV. The pictures showed a few positive cells in both cell lines after 24 hours whereas after 48 hours the C17 clone was just detected in IRF3 ko cells (see Fig. 15B).

IFN $\alpha$ / $\beta$  signalling is important to defend the host from pathogens and we proved that the transcription factor IRF3 plays a key role in controlling viral replication. IRF3 *-/-* MEFs do not have the important transcription factor and therefore C17 can replicate in this cell line.

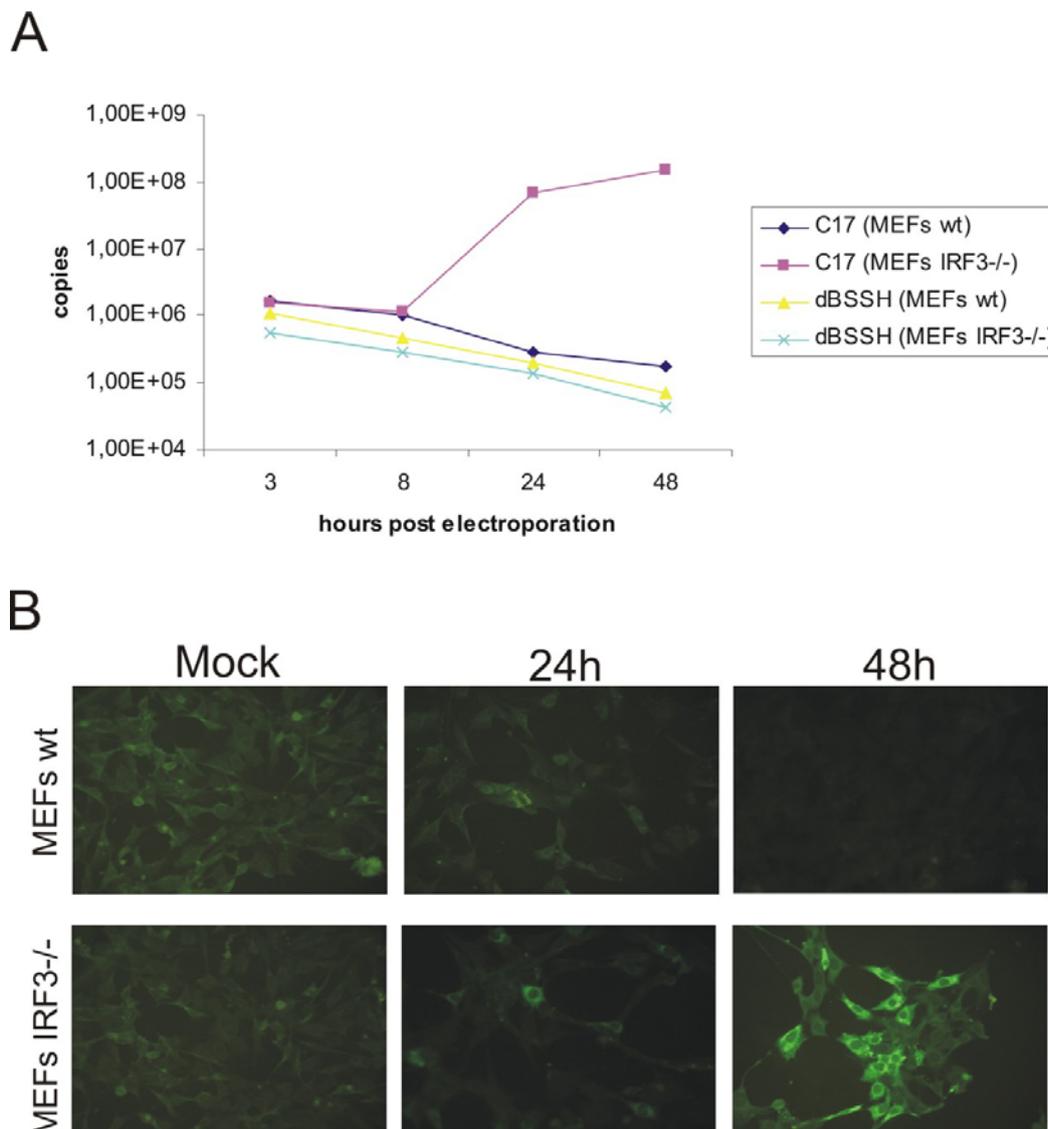


Fig. 15. C17 is able to replicate in IRF3 <sup>-/-</sup> MEFs

(A) C17 and dBSSH RNA were electroporated into wt MEFs and IRF3 <sup>-/-</sup> cells. The cytoplasmic RNA was isolated after 3, 8, 24 and 48 h. The amount of RNA was measured by real-time PCR and shown in the number of RNA copies post electroporation. (B) Replication of C17 in IRF3 deficient MEFs was also monitored by immunofluorescence. Cells were fixed with acetone methanol 48 h post electroporation and immunofluorescence staining with a polyclonal antiserum against TBEV was performed.

In principal, electroporation of the RNA constructs into the MEFs was possible. However, immunofluorescence staining showed only 30 % positive cells in IRF3 deficient cells 48 hours post electroporation (see Fig. 15B), indicating that the electroporation efficiency was very low. The electroporation method we used was a

standard method used in our laboratory, established to electroporate BHK-21 cells. Therefore, we also tried to transfect the RNA into cells with a lipid based transfection reagent, Transmessenger Transfection Reagent (Qiagen), which is optimized for RNA transfection. To determine the efficiency we electroporated and transfected C17 into IRF3  $-/-$  cells in parallel and compared the transfection efficiency of these two methods by immunofluorescence staining. After 48 hours 40 % positive cells were detected in cells that were electroporated, while with transfection only 10-20 % positives were monitored (Fig. 16). Consequently, we continued further work by electroporating viral RNA constructs.

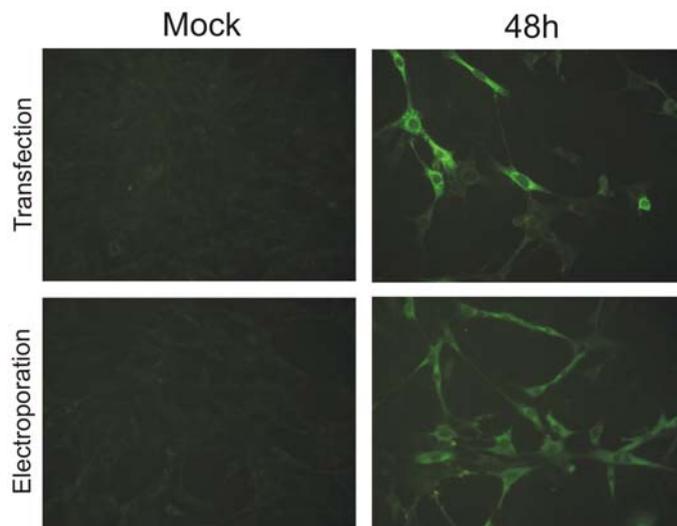


Fig. 16. Electroporation vs. transfection

C17 RNA was either electroporated or transfected with Transmessenger Transfection Reagent in IRF3  $-/-$  MEFs. Cells were fixed with acetone methanol after 48 h and immunofluorescence staining with a polyclonal antiserum against TBEV was performed.

To enhance electroporation efficiency we tested further electroporation conditions. On the one hand C17 replicon RNA was electroporated with 1.8 kV, 200  $\Omega$ , 25  $\mu$ F, 2 pulses (method 1) into wt MEFs. This condition is suggested and optimized for the electroporation of RNA into BHK-21 cells. On the other hand C17 was electroporated with 0.270 kV,  $\Omega$  none, 960  $\mu$ F (method 2), a method suggested for the electroporation of MEFs.

(Protocol: [http://www.bio-rad.at/LifeScience/pdf/Bulletin\\_3112\\_132.pdf](http://www.bio-rad.at/LifeScience/pdf/Bulletin_3112_132.pdf)).

After the indicated time points the cytoplasmic RNA was isolated, reverse transcribed and the amount of RNA copies was analyzed in quantitative real-time PCR (Fig. 17). Finally, no difference between these two methods was monitored and the established electroporation method (method 1) was used for following experiments.

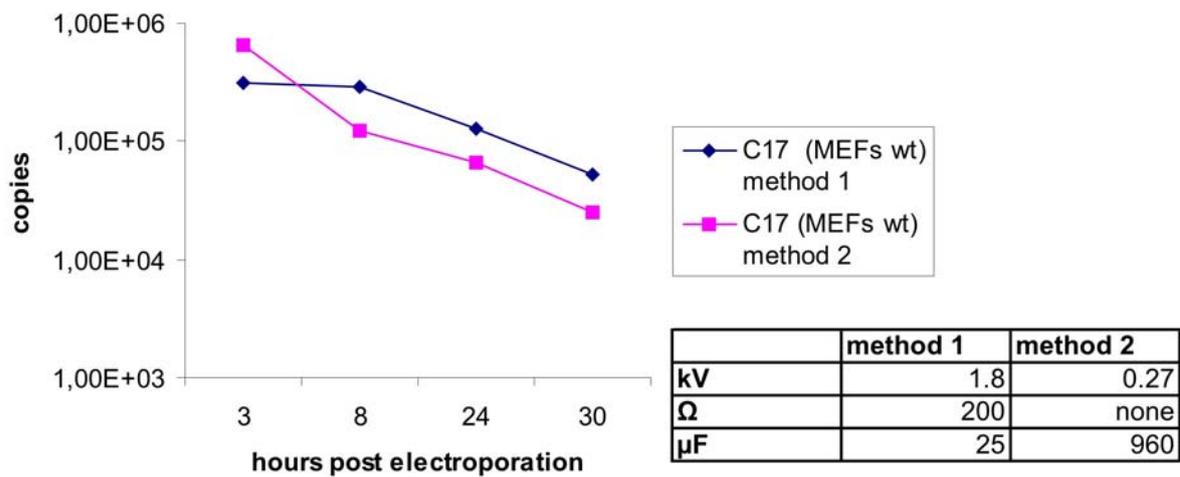


Fig. 17. Testing of two electroporation methods

C17 was electroporated into MEFs in two different ways, cytoplasmic RNA was isolated after 3, 8, 24 and 30 h. The amount of RNA was determined by quantitative PCR and shown in the number of RNA copies after electroporation.

#### **4.4. Endogenous type I IFN mRNA expression is not detectable after electroporation of viral RNA constructs**

We showed that TBEV infection leads to the activation of a signal-transduction pathway leading to the activation of type I IFNs (Fig. 11). In Figure 15 it was monitored that the replicon C17 is able to replicate in IRF3 deficient cells, but not in wild-type cells. This goes in line with infection of these cells (Fig. 9-10). Now we wanted to detect and compare the production of IFN $\alpha/\beta$  by electroporating the self replicating replicon C17 and the replication deficient dBSSH into wild-type MEFs. Cytoplasmic RNA was isolated at the indicated time points, the RNA was reverse transcribed and the relative induction of type I IFN mRNA (pan IFN alpha, alpha 4) was analyzed in quantitative real-time PCR. The genes of interest were normalized to an endogenous control, a housekeeping gene (Ube2d2). pIC, a synthetic double stranded RNA, which induces IFNs, was used as positive control. The cells were stimulated for 8 and 24 hours with 1  $\mu\text{g/ml}$  pIC.

After 8 hours an induction of IFNs was measured in cells treated with pIC, but after 24 hours numbers of cells had died and no mRNA was detected. In cells electroporated with C17 and dBSSH no induction of pan IFN alpha and alpha 4 mRNA was determined after 3, 8, 24 and 48 hours post electroporation (Fig. 18).

Since years it has been known that cells in culture respond to viruses by initiating a complicated signaling cascade, which leads to the production of type I IFNs (Decker, Stockinger et al. 2002). Therefore it was either a problem of electroporation efficiency or of detection limit, that no endogenous IFN mRNA could be determined. To address these problems we used a reporter assay system to increase the sensitivity of the assay.

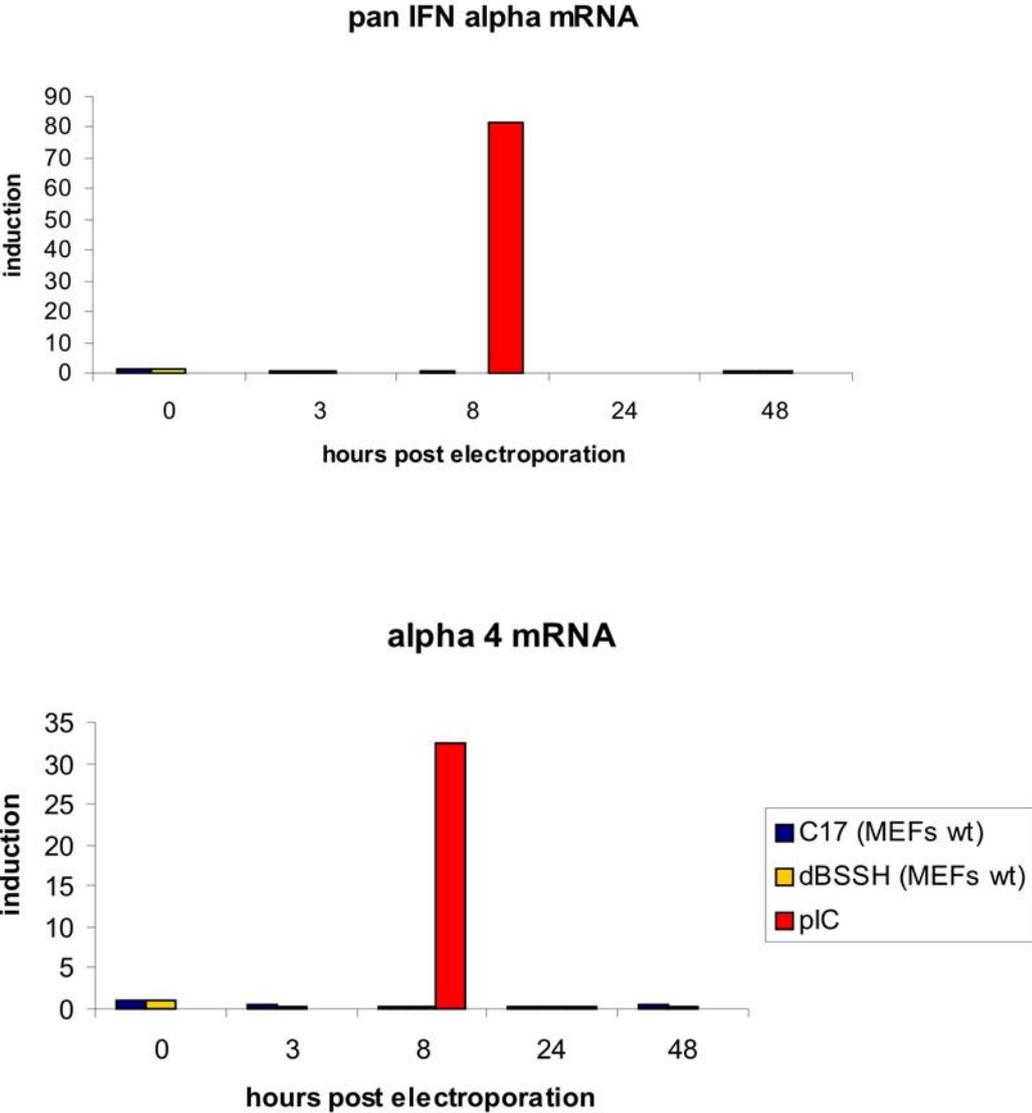


Fig. 18. C17 and dBSSH were electroporated into wild-type MEFs. The cytoplasmic RNA was isolated 3, 8, 24 and 48 h post electroporation. The amount of IFN alpha mRNA and alpha 4 mRNA was measured by real-time PCR; pIC (1 µg/ml) was used as a positive control.

#### **4.5. Activation of the IFN beta promoter by TBEV genome constructs**

To analyze mechanisms of type I IFN induction by replicating virus RNA we transfected self replicating RNAs (C17) or viral RNA deficient in replication (dBSSH) into mouse cells (Fig. 15A). Transfection of the individual constructs was successful; however, we were not able to detect endogenous type I IFN mRNA expression by the electroporation of replicating viral RNAs (Fig. 18). Therefore, we established a luciferase assay for the activation of the IFN beta promoter by TBEV in HEK 293T cells. We were kindly provided with a plasmid containing the firefly luciferase under control of the IFN beta promoter, named p125-Luc (provided by Takashi Fujita). For standardization we used a plasmid that constitutively expressed *Renilla* luciferase (pRLN-Null).

Before we started the experiment we optimized the transfection conditions for DNA and RNA and compared diverse transfection reagents. 293T cells were utilized for the establishment, because they are known to be easily transfected. Plasmid DNA expressing GFP (pIRES-EGFP) and RNA (Replicon C17) were transfected on the one hand with Transmessenger Reagent (Qiagen) and on the other hand with Lipofectin (Invitrogen) (see Fig. 19).

The pictures of GFP transfected living cells were taken 24 hours after transfection, whereas RNA transfected cells were fixed with acetone methanol and immunofluorescence was performed 48 hours post transfection. Finally, hardly a difference between these two methods was monitored. When GFP was transfected with Transmessenger (Qiagen) and Lipofectin (Invitrogen) approximately 10 % of the cells were positive, whereas the transfection of C17 with these two transfection reagents resulted in nearly 50 % positive cells. Transmessenger Reagent (Qiagen) is optimized for RNA according to the manufacturer's instructions. On the contrary, Lipofectin (Invitrogen) is suggested to be used for transfection of DNA. Consequently, we decided to continue our work referring to the manufacturer's instructions.

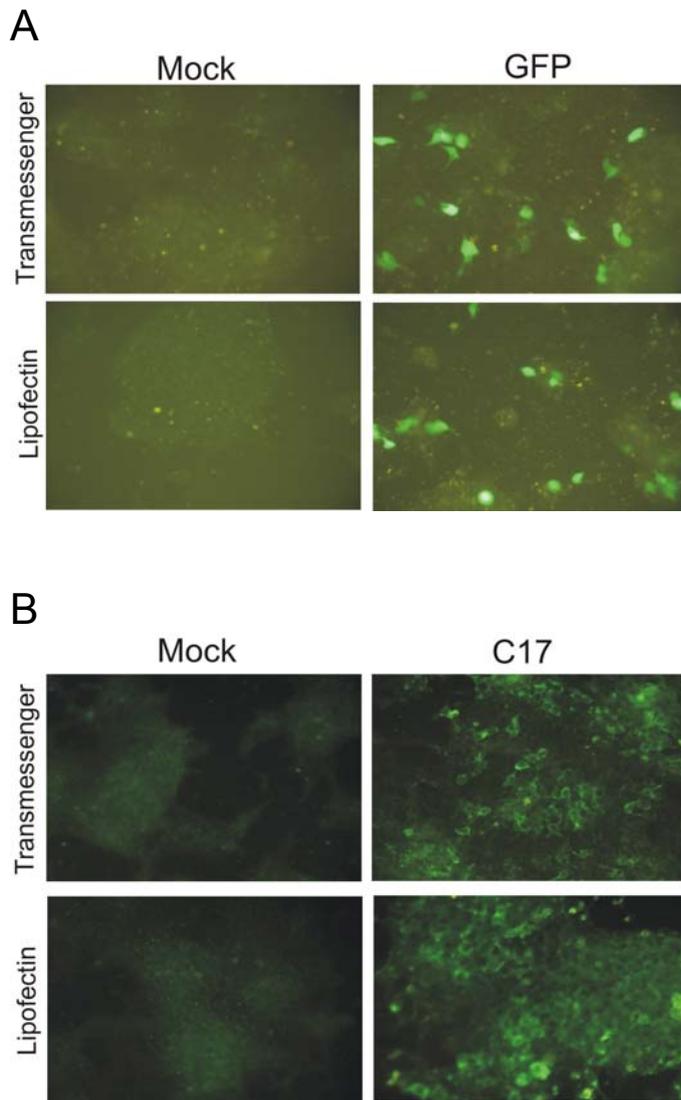


Fig. 19. Comparison between transfection methods.

GFP Plasmid (pIRES-EGFP) (A) and C17 RNA (1  $\mu\text{g}/\text{ml}$ ) (B) were transfected into 293T cells with Transmessenger Reagent and Lipofectin. Whereas GFP was detected 24 h post transfection, immunofluorescence of C17 was performed 48 h after transfection.

Firstly, an optimal *Renilla* control plasmid had to be found. The plasmid that was normally used in the lab had a SV 40 promoter, but the problem was that we received too high levels of relative light units (RLUs) for standardization with this promoter. Therefore, diverse *Renilla* plasmids (*Renilla* SV40 Vector, *Renilla* pRL-Null Vector *Renilla* pRL-CMV Vector (Promega)) were compared and tested (Tab. 1). With the plasmids containing SV40 and CMV promoters the RLUs were too high for standardization after 48 hours. Therefore, we decided to take the *Renilla* pRL-Null Vector as control plasmid, because the values for the RLUs were the lowest and had not increased dramatically 48 hours post transfection.

Tab. 1. Expression of *Renilla* luciferase under control of different promoters

Various *Renilla* plasmids were transfected into 293T cells with Lipofectin. After 24 h and after 48 hours *Renilla* luminescence was measured with the luminometer (Victor Light Luminometer, Perkin Elmer).

hours post transfection	24	48
Mock (RLUs)	32	46
<i>Renilla</i> SV40 (RLUs)	21210	542637
<i>Renilla</i> CMV (RLUs)	542637	202895
<i>Renilla</i> Null (RLUs)	956	8983

A firefly plasmid under the control of an IFN $\beta$  promoter, named p125-Luc (provided by Takashi Fujita), was used to detect IFN $\beta$  promoter activation. The two plasmids were co-transfected into 293T cells with Lipofectin. 24 hours later the cells, which included the plasmids, were transfected with various viral RNAs (C17, dBSSH, NK4) and infected with TBEV (MOI 1). NK4 is a whole length clone of TBEV that contains all structural and non-structural proteins of the virus (Fig. 14). Therefore, transfection of this RNA into cells leads to the production of viral particles that can spread to neighbouring cells. pIC was used as a positive control.

24 hours post transfection the activity of the firefly and the *Renilla* luciferase (control value) were measured with the luminometer. The values that we received from the firefly measurement were standardized to the control data of *Renilla*. When we transfected pIC, C17 and dBSSH into 293T cells a 3-fold induction of the IFN- $\beta$  promoter activity was monitored compared to mock transfected cells 24 hours post transfection. Transfection of NK4, the TBEV whole length clone, lead to a higher IFN $\beta$  induction compared to dBSSH (Fig. 20). dBSSH has a partial deletion in the non-structural 5 (NS5) protein, which provides the RNA polymerase and therefore cannot replicate. This viral RNA construct is a derivate of NK4 and it can be directly compared with the whole length clone. C17 induced also a lower induction of IFNs compared to NK4. In fact, C17 is able to replicate in cells, but replication occurs more slowly than in NK4 (see Fig. 23A).

The results indicted that a slower replicating RNA (C17) leads to lower IFN induction. Additionally, structural proteins could play a role in IFN induction; however we did not test this. Surprisingly, cells infected with TBEV MOI 1 only showed a 4-fold induction

of IFN $\beta$ . The promoter activity was also lower in infected cells compared to NK4 transfected cells, but the input RNA levels in cells infected and transfected are not comparable. 1  $\mu$ g transfected RNA contained  $1.66 \times 10^{11}$  molecules (see methods page 45: "Calculation of the molecular weight of TBEV RNA copies"), whereas with infection (MOI 1)  $1 \times 10^5$  RNA copies were used. Finally,  $1.66 \times 10^6$  more RNA molecules were utilized for transfection compared to infection.

Another explanation for the low levels of type I IFNs could be due to an inhibitory effect of viral proteins on the host antiviral response.

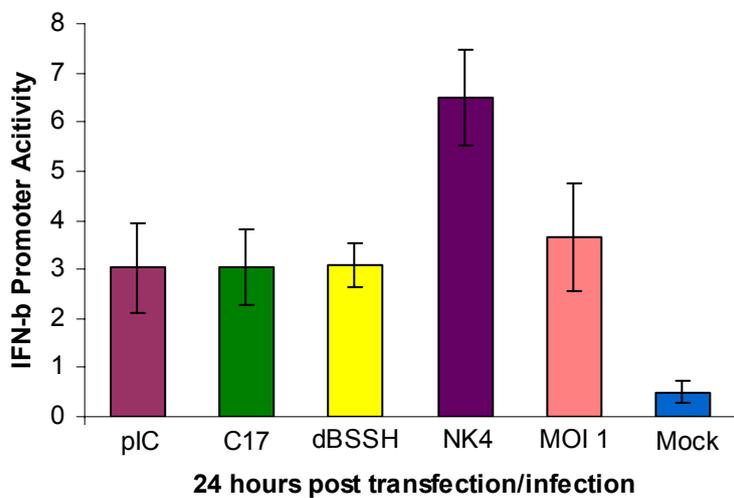


Fig. 20. Activation of the IFN beta promoter by TBEV genome constructs

293T cells were co-transfected with IFN $\beta$  promoter-luciferase and *Renilla* luciferase reporter plasmids. At 24 h post transfection, cells were either mock transfected or transfected with pIC (control), C17, dBSSH and NK4 RNA or infected with TBEV MOI 1. Cells were collected 24 h post transfection/infection for dual luciferase activity (expressed as relative IFN $\beta$  promoter activity). Error bars, standard deviations were calculated from three independent experiments.

#### **4.6. Activation of endogenous type I IFNs by efficient transfection of viral RNA constructs**

The luciferase assay showed that transfection of self replicating viral RNA constructs lead to the induction of type I IFNs. We still wanted to know the effect on the endogenous IFN $\alpha$ / $\beta$  activation, but as monitored in Figure 19 transfection efficiency was very low with Lipofectin (Invitrogen) and Transmessenger Reagent (Qiagen). When DNA was transfected only 10 % of the cells were positive, whereas transfection of RNA lead to approximately 50 % positive cells.

Consequently, a very promising transfection reagent, called Lipofectamin 2000 (Invitrogen), was tested. We compared the transfection efficiency of DNA expressing GFP in 293T cells by using various ratios of Lipofectamin 2000 and Lipofectin to DNA as it was proposed in the manufacturer's instructions (Fig. 21). A huge difference can be seen between these two methods 24 hours post transfection. With Lipofectamin DNA was transfected more efficiently than with Lipofectin. Approximately, 20 % of the cells expressed GFP after transfection with Lipofectin, whereas more than 80 % were positive after transfection with Lipofectamin 2000, when a ratio of 1:5 was used (GFP ( $\mu$ g):Transfection Reagent ( $\mu$ l)).

Finally, we tested if transfection with Lipofectamin 2000 of C17 into mouse cells (L929) is also more efficient than transfection with Lipofectin. L929 cells are an established cell line and a very useful tool to study innate immune answers. They express most of the receptors, which play an important role in innate immunity. When comparing the two transfection reagents, transfection of C17 with Lipofectin resulted in approximately 20 % positive mouse fibroblasts, while transfection with Lipofectamin 2000 lead to more than 60 % positive cells (Fig. 22). Therefore, Lipofectamin 2000 was used for further transfection experiments.

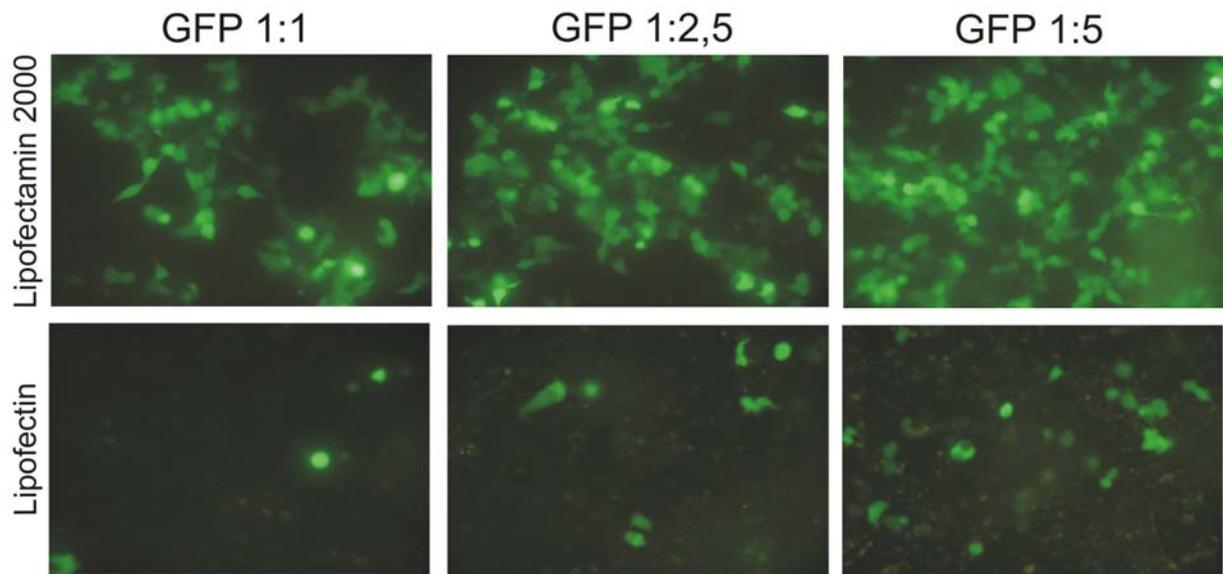


Fig. 21. Comparison of transfection methods by transfecting DNA  
 GFP was transfected into 293T by varying the ratios of the transfection reagents (Lipofectin and Lipofectamin 2000). Pictures of living cells were taken 24 h post transfection.

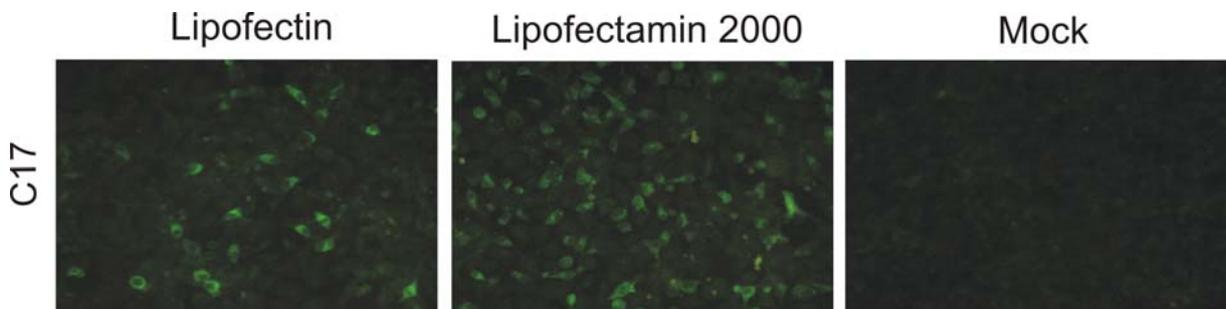


Fig. 22. Comparison of transfection methods by transfecting viral RNA constructs  
 C17 was transfected into L929 cells with the ratio 1:5 (C17 (µg):Transfection Reagent (µl)). Cells were fixed with acetone methanol after 48 h and immunofluorescence staining with a polyclonal antiserum against TBEV was performed.

#### **4.7. Transfection of viral RNA leads to the induction of IFN alpha 4**

Viral infection leads to type I IFN expression and to the activation of the host's innate immune system. In our study we were interested, which part and mechanism of the viral RNA induces IFN $\alpha/\beta$  and therefore transfected various viral constructs (C17, dBSSH, NK4). In previous experiments we electroporated RNA and tried to measure type I IFN production. We were not able to detect IFN mRNA by quantitative PCR because electroporation efficiency was low and IFN induction under detection limit. Therefore, we performed another experiment and transfected the viral RNA constructs in mouse fibroblasts (L929 cells) with Lipofectamin 2000, which transfected cells more efficiently. We also infected these cells with TBEV MOI 10. Total RNA of the transfected/infected L929 cells was isolated, reverse transcribed and the induction of IFN alpha 4 mRNA (Fig. 23B) compared to untreated cells was quantified by real-time PCR. By transfecting viral RNA with Lipofectamin 2000 an induction of IFN  $\alpha$ 4 mRNA could be shown. The transfection of NK4 lead to higher IFN  $\alpha$ 4 production compared to the transfection of C17 and dBSSH. dBSSH, which is a derivate of NK4 and therefore directly comparable with the whole length clone only showed a 20-fold IFN  $\alpha$ 4 induction, whereas NK4 transfection induced 6 times more mRNA. C17 transfection also caused IFN  $\alpha$ 4 mRNA production and lead to a 60-fold induction.

The transfection efficiency was monitored with immunofluorescence 32 hours post transfection (see Fig. 23A). With NK4 and C17 a few positive cells were visible, whereas with dBSSH no replication was detected with immunofluorescence staining. Interestingly, the cells that were infected with TBEV (MOI 10) nearly all appeared positive 32 hours post infection, while IFN  $\alpha$ 4 mRNA expression was very low. Surprisingly, infection of cells with TBEV did not show a high IFN expression compared to transfection with NK4, although nearly all cells were positive in immunofluorescence staining (see Fig. 23A).

The results suggested that RNA replication is important for viral recognition and for induction of IFNs in innate immunity. Moreover, it seems that the type I IFN pathway can be somehow suppressed by infection with TBEV.

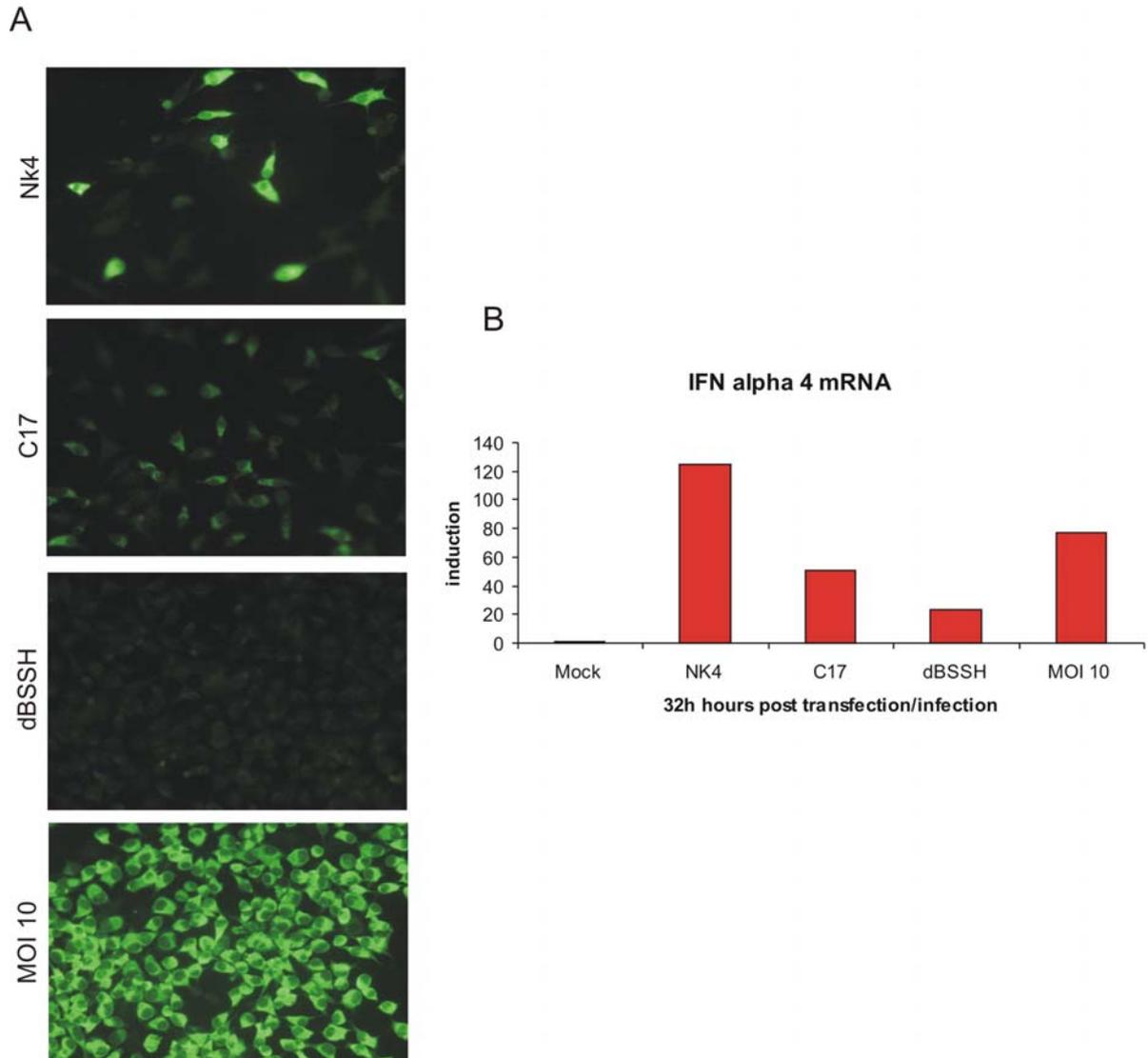


Fig. 23. Endogenous IFN  $\alpha$ 4 expression by transfection of TBEV RNA

L929 cells were transfected with NK4, C17, and dBSSH (Lipofectamin 2000) and infected with MOI 10. 32 h post transfection/infection cells were stained with immunofluorescence (A). Cytoplasmic RNA was isolated and the amount of alpha 4 mRNA was determined by real-time PCR 32 h post transfection/infection (B).

#### **4.8. TBEV interferes with the IRF3 pathway by inhibiting the induction of IFNs**

In general, flaviviruses have not been shown to cause a non-specific blockade on the IRF3 pathway and therefore inhibiting IFN production. However, WNV has developed a mechanism to avoid the transcriptional activity of IRF3. Human cells that are infected with WNV show a delayed activation of IRF3, 12 to 18 hours post infection. Consequently, the virus is able to replicate unchallenged by the host cell by evading detection at early times post infection (Fredericksen, Smith et al. 2004).

We supposed that TBEV has evolved similar strategies to escape the host immune system, because we could hardly detect an induction of type I IFNs in infected 293T (see Fig. 20) and L929 cells (Fig. 23B). Therefore, we analysed if TBEV somehow inhibits the production of IFN $\alpha/\beta$  and can replicate in the cell without being combated by the host's immune system. Firstly, L929 cells were infected with the virus (MOI 1, MOI 10) and mock infected. After 24 hours the total RNA was isolated and expression of IFN alpha 4 mRNA and pan IFN alpha mRNA were determined by real-time PCR. As shown in Fig. 22 an induction of IFN alpha 4 mRNA (Fig. 24A) and pan IFN alphas (Fig. 24B) could be monitored 24 hours post infection. However, infection with MOI 10 lead to 10 times higher levels of alpha 4 mRNA compared to infection with MOI 1 (Fig. 24B).

To monitor the effect of the inhibition of type I IFN expression on downstream effectors functions of IFNs we analysed the expression of a classical IFN target gene. Interferon- $\gamma$  (IFN- $\gamma$ )-inducible protein-10 (IP-10), a member of the C-X-C sub-family of chemokines, is a highly inducible gene. IP-10 is known to stimulate monocytes, natural killer and T-cell migration in response to IFN- $\gamma$  (Singh, Venkataraman et al. 2007). We detected the induction of IP-10 mRNA with quantitative PCR to show the innate immune response of the host after infection with TBEV. 24 hours post infection, IP-10 mRNA levels were clearly elevated (Fig. 24C). Whereas with MOI 1 only a ~200-fold induction could be measured, with MOI 10 nearly a 2000-fold induction was monitored. Consequently, after infection with MOI 10, 10 times more IP-10 mRNA was produced compared to infection with MOI 1.

The transfection of pIC with Lipofectamin 2000 caused a strong induction of type I IFN and IP-10 mRNA levels. We wanted to analyse if TBEV is able to suppress the production of this mRNA. Therefore, L929 cells were infected with TBEV MOI 1, MOI

10 and mock infected. 8 hours post infection pIC was transfected into TBEV and Mock infected cells. After isolating total RNA of these cells, IFN alpha 4, pan IFN alpha and IP-10 mRNA levels were determined by quantitative PCR 24 hours after infection. When cells were infected with TBEV prior to the transfection of pIC, the induction of mRNA was dramatically reduced compared to non-infected cells treated with pIC (Fig. 24D, E and F). The inhibitory effect was even more dramatic in cells infected with MOI 10.

Taken together this clearly showed that TBEV was able to inhibit the production of IFNs and ISG (IP-10). Also, the multiplicity of infection (MOI) of the virus seems to play an important role in the suppression of the host's immunity. The higher the MOI the more TBEV was able to inhibit the production of type I IFNs that are necessary to combat replication of the virus.

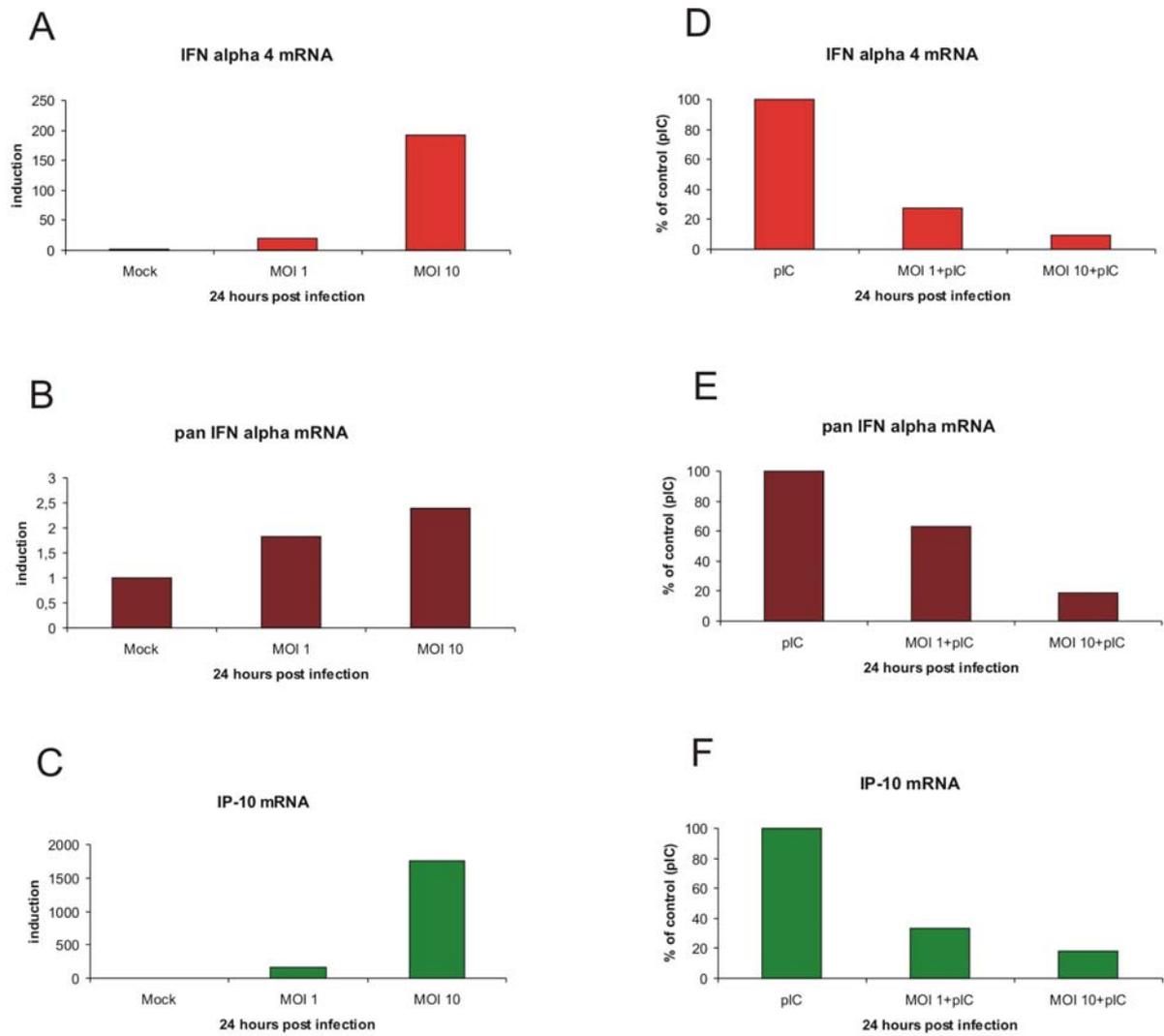


Fig. 24. TBEV can suppress the induction of type I IFNs

(A-C) L929 cells were infected with TBEV (MOI 1, MOI 10) and Mock infected. Total RNA was isolated after 24 h and the amount of alpha 4 mRNA, pan IFN alpha mRNA and IP-10 mRNA was determined by real-time PCR.

(D-F) L929 cells were infected with TBEV (MOI 1, MOI 10) and Mock infected. After 8 h pIC was transfected with Lipofectamin 2000. 24 h post infection total RNA was isolated and the amount of alpha 4 mRNA, pan IFN alpha mRNA and IP-10 mRNA was determined by real-time PCR.

#### **4.9. Inhibition of interferon-stimulated JAK-STAT signalling by TBEV**

The non-structural (NS) proteins of diverse members of the flaviviruses have been identified as IFN antagonists, whose action impaired signalling processes of the JAK-STAT pathway (Samuel and Diamond 2006). For example, the NS5 protein of Langat virus, a member of tick-borne flaviviruses and a close relative of the TBEV strain we were using in our studies, has been identified as an antagonist of IFN actions. Langat virus was found to resist IFN's antiviral effects when cells were treated with IFN $\beta$  after infection was already established (Best, Morris et al. 2005).

To further analyse the inhibition of the type I IFN pathway by TBEV we examined the interaction of the virus with JAK-STAT signal transduction pathways. In general, tyrosine phosphorylation of STAT2 and STAT1 occurs after IFN $\alpha/\beta$  ligation to the cell surface receptors. We analysed phosphorylation of STAT1 at Tyr701 by immunoblot analyses of infected L929 cell lysates. L929 cells were infected with TBEV MOI 1 and MOI 10 for various time points. To monitor the effect of TBEV infection on activation of STAT1 infected cells were treated with recombinant IFN $\beta$  (Fig. 25).

STAT1 Y701 phosphorylation was activated by the addition of recombinant IFN $\beta$  for 30 minutes in Mock treated cells. However, cells that were infected prior to IFN $\beta$  treatment showed a strong reduction after 24 and 48 hours of infection. The accumulation of Tyr701-phosphorylated STAT1 in response to IFN $\beta$  was inhibited in MOI 1 infected cells after 48 hours and already after 24 hours in cells infected with MOI 10.

In addition, cells infected with MOI 10 showed STAT1 phosphorylation after 24 and after 48 hours, indicating that the IFN signalling pathway is activated by infection. Interestingly we also observed STAT1 Y701 phosphorylation in cells that were infected only for 2 hours. At this time point we did not observe type I IFN production in previous experiments. Moreover, STAT1 levels had increased with time of infection.

The results clearly demonstrate that TBEV infection leads to the production of type I interferons and to activation of the JAK-STAT pathway. However, once TBEV established an infection in the host cell interferons cannot mediate their inhibitory functions anymore.

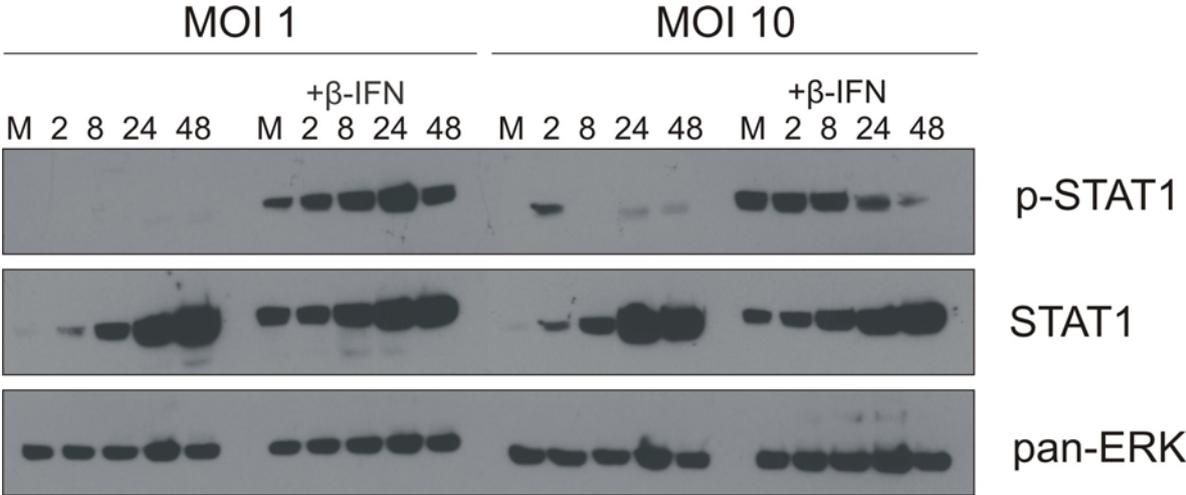


Fig. 25. Inhibition of tyrosine701-phosphorylation of STAT1 in response to IFNβ  
Uninfected L929 cells and cells infected with TBEV (MOI 1, MOI 10) for 2, 8, 24, 48 h were treated with IFNβ (500 U) for 30 min or left untreated. The cell lysates were examined by Western blotting with antibodies to Tyr701- phosphorylated STAT1 (p-STAT1), STAT1 and pan-ERK for loading control.



## 5. Discussion

### 5.1. Activation of IFNs by TBEV infection

In general, Flaviviruses interact with the host's immune system and viral infection leads to the activation of type I IFN signalling to limit viral replication and spread. The host antiviral response depends on the recognition of viral PAMPs by various pattern recognition receptors (PRRs) and results in the production of cytokines, which promote an antiviral state (Saito and Gale 2007).

In our study we investigated the induction and role of type I IFNs in TBEV infection. It has not been analysed yet, how type I IFN pathways are activated in TBEV infection and how the virus interacts with the innate immune system. Therefore, we studied the interaction of TBEV with the host's Interferon system. We were interested on the one hand which host cell factors contribute to the control of virus infection and on the other hand which parts of the viral replication cycle leads to the activation of host cell signalling pathways. We showed that infection of mouse macrophages with a replicating wild-type virus leads to a strong up regulation of IFN  $\alpha/\beta$  mRNA (Fig. 7) Treatment of the cells with subviral particles (RSP) or formalin inactivated virus did not lead to IFN production. This clearly indicated that only replicating intermediates of the virus can be recognized by the host cell.

IFN $\alpha/\beta$  gene induction takes place downstream of PRRs. For positive-stranded RNA viruses like flaviviruses, PKR, TLR3, RIG-I and MDA 5 recognize double-stranded RNA in the cytoplasm and endosome. Sensing of foreign molecules by PRRs leads to the activation of signalling pathways and subsequent activation of type I IFN and proinflammatory cytokines. The transcription factor IRF3 has been shown to play a central role in type I IFN activation by various viruses (Fredericksen, Keller et al. 2008). Therefore, we addressed the question which role IRF3 plays during TBEV infection *in vitro*. Our experiments demonstrated that infection of IRF3 deficient mouse embryonic fibroblasts (MEFs) with TBEV resulted in a strong enhancement of viral replication, whereas in wild-type cells the virus could not replicate (Fig. 9, 10). The virus started to replicate between 8 and 24 hours in IRF3 knock out cells. During this time IFN $\beta$  mRNA expression is also activated in wild-type cells, where no viral replication occurred.

Moreover, we analysed important parts of the virus replication cycle, which possible contribute to IFN $\alpha/\beta$  activation by electroporating and transfecting viral RNA constructs into different cell lines. Our study revealed that the TBEV replicon C17, which has almost all of the structural proteins deleted, was able to replicate efficiently in IRF3 deficient mouse cells, whereas the replicon was replication negative in wild-type cells. When we electroporated the replication deficient TBEV RNA dBSSH, the construct was not detected in wild-type and knock out cells, as expected (Fig. 15A). The results indicate that viral replication is a central activator of type I IFN expression. It was also shown that IRF3 is an indispensable factor in innate immunity and a key regulator in the defence against viral replication in TBEV infection. Our experiment revealed that IRF3 plays a key role in controlling TBEV spread and replication. Importantly, it plays a central role in the activation pathway of IFN $\beta$  gene transcription. In IRF3  $-/-$  cells TBEV and a TBEV replicon can replicate unchallenged, because type I IFN signalling is destroyed.

Similarly, it was shown that the Flavivirus West Nile Virus (WNV) is an effective trigger of IRF3 activation and its antiviral response combats viral spread. IRF3 deficient mice show a greater WNV burden in the periphery and extended tissue tropism compared to wild-type mice after infection with low doses of virus (Daffis, Samuel et al. 2007)

To analyze mechanisms of IFN $\alpha/\beta$  induction we transfected self replicating RNA or viral RNA defective in replication into HEK293 cells and monitored IFN $\beta$  promoter activation in a luciferase assay (Fig. 20). The whole length clone of TBEV NK4, lead to a robust IFN $\beta$  promoter activation compared to the replication defective dBSSH, which induced the promoter only weakly. Interestingly, transfection of the TBEV replicon C17 also lead to a very weak IFN $\beta$  promoter activation, although it is able to replicate. One possible explanation would be the delayed replication of C17 compared to the full length clone NK4 (see Fig 23A).

Also, other factors besides replication are important for type I IFN activation. Structural proteins could play a role in IFN production. To verify this hypothesis, one could transfect cells with various viral RNA constructs, in which different structural or non-structural proteins are knocked out and measure IFN $\alpha/\beta$  activation. We would expect different inductions of type I IFNs and this would allow to explore something about the importance of the multiple structural and non-structural proteins. For example, in Hepatitis C virus (HCV) the core protein, which is one out of three

structural proteins, was shown to stimulate the host's immune system and trigger cytokine production via TLR2 (Chang, Dolganiuc et al. 2007). Furthermore, a study reported that measles virus (MV) wild-type strains specifically activated cells via TLR2, and this was dependent on the expression of the envelope glycoprotein hemagglutinin (H). MV that expressed the wild-type H activated TLR-responsive genes in monocytes (Bieback, Lien et al. 2002).

Furthermore, we were able to show endogenous type I IFN expression by quantitative PCR after transfecting TBEV RNA constructs into a mouse cell line (L929 cells) (Fig. 23). The experiment demonstrated that transfection of a TBEV full length clone (NK4) lead to a higher IFN $\alpha$ 4 induction than transfection of its derivate, a replication deficient construct (dBSSH) did. The IFN $\alpha$ 4 mRNA expression of NK4 was even 6 times higher compared to that of dBSSH. The transfection of the replicon C17 caused IFN  $\alpha$ 4 mRNA activation, but it was two times lower than the IFN induction of the whole length clone NK4. These results revealed that viral replication is an important factor of type I IFN induction and necessary for the host cell to recognize and combat viral structures.

The exact mechanism how TBEV recognition in the host takes places and how the various PRRs are activated has not been investigated yet. As analysed in previous studies it seems that diverse RNA structures play an important role in viral recognition (Hornung, Ellegast et al. 2006). The group showed that 5'-triphosphate RNA directly binds to RIG-I and RIG-I is responsible for the recognition of 5'-triphosphate RNA. Generally, the mRNAs of flavivirus infecting cells contain 7-methyl-guanosine cap structures at their 5' ends. Nevertheless, RNA synthesis leads to cytosolic viral RNA intermediates with an uncapped 5'-triphosphate end. It is known that flaviviruses start replication with an uncapped 5'-triphosphate end and members of this genus were reported as being recognized by RIG-I (Sumpter, Loo et al. 2005; Kato, Takeuchi et al. 2006).

For future studies we are interested in the work with RIG-I and MDA-5 deficient cell lines. By infecting them with TBEV and measuring type I IFNs with quantitative real-time PCR we could prove the importance of the different PRRs for TBEV recognition and their influence on IFN $\alpha$ / $\beta$  activation.

## 5.2. Inhibition of IFNs by TBEV infection

The host antiviral response depends on the rapid recognition of viral PAMPs by innate immune cells to promote an antiviral state. Viruses are in the constant need to gain immune evading mechanism to escape the host's immune system. They have evolved diverse strategies not to be recognized and to replicate unchallenged by host cells.

Our findings showed only low IFN $\alpha/\beta$  activation in TBEV infection in mouse (L929) and human (HEK 293T) cell lines. Therefore, we investigated if TBEV inhibits IFN expression. It is known that pIC, a synthetic dsRNA, leads to type I IFN activation (Randall and Goodbourn 2008). We showed that in mouse cells (L929), where TBEV infection was already established, IFN $\alpha/\beta$  induction was lower after pIC treatment than in uninfected cells. These results indicate that TBEV interferes with type I signalling by inhibiting the activation of IFN $\alpha/\beta$  to allow replication.

Fikrig et al. (Arjona, Ledizet et al. 2007) reported in a study that WNV structural protein E, which is the first viral molecule to interact with the host, specifically inhibits the induction of antiviral cytokines induced by dsRNA. Further research is necessary to identify the protein responsible for IFN $\alpha/\beta$  inhibition in type I IFN signalling by TBEV infection.

In addition to the inhibitory effect of TBEV on type I IFN production we also analysed if the virus can interfere with effector functions of IFNs by interfering with the JAK-STAT pathway. We showed that once the virus established replication in the host cell STAT1 phosphorylation is inhibited (Fig. 25). STAT proteins are essential in mediating IFN $\alpha/\beta$  signalling in innate immune responses and are important for the host control of viral infections.

Tick- and mosquito-borne flaviviruses have developed numbers of strategies to avoid type I IFN response by their non-structural (NS) proteins, which downregulate individual signalling components of the JAK-STAT pathway (Werme, Wigerius et al. 2008). It was shown that the NS5 protein of Langkat virus (LGTV), a member of TBEV, interferes with the IFN response by blocking the phosphorylation of JAK1 and Tyk2 (Best, Morris et al. 2005). Similarly, the JEV NS5 protein was identified as an IFN antagonist that blocks type I IFN signalling of Tyk and STAT1, possibly via a PTP-dependent mechanism (Lin, Chang et al. 2006). Other flaviviruses proteins

analysed as IFN antagonists include WNV and DENV NS4B (Munoz-Jordan, Sanchez-Burgos et al. 2003).

In summary, we have identified TBEV as a type I IFN antagonist, which interferes with the JAK-STAT pathway by blocking STAT1 phosphorylation. Moreover, TBEV reduces IFN $\alpha/\beta$  induction by inhibiting an essential component of the pathway at the early beginning of receptor-mediated type I IFN signalling.

In future work it would be interesting to clarify the exact mechanism of IFN $\alpha/\beta$  signalling and its inhibition by TBEV. In particular, which parts of the virus lead to viral recognition and which component is responsible for the virus interference with the host's innate immunity.



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## 7. Appendix

### 7.1. Curriculum Vitae

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**Mar. 2008– Mar.2009:** Diploma Thesis at the Institute of Virology in the working group of Prof. DDr. Mandl  
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## 7.2. Lebenslauf

### PERSÖNLICHE DATEN

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