

Immunological Effects of TBE Vaccination:

Increased Expression of Transcription factor T-bet Indicates

Activation of Th1-like Cellular Immunity

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Populärvetenskaplig sammanfattning.

TBE är en virussjukdom som sprids av fästingar. I takt med att fästingarna har blivit fler och spridit sig i landet har även fallen av TBE sjukdom ökat. Det finns ingen behandling mot TBE när en person väl smittats. I de flesta fall självläker infektionen men kvarstående problem efter sjukdom så som olika typer av neurologiska skador förekommer och även enstaka dödsfall. För att förebygga TBE infektion kan man använda ett så kallat inaktiverat vaccin. Det betyder att vaccinet består av hela viruspartiklar som med hjälp av formalin har avdödats. Detta gör att viruset inte kan dela sig inuti celler, vilket är det normala sättet för viruset att föröka sig.

Kroppens immunförsvar brukar traditionellt delas upp i två grenar, det cellulära försvaret och det humorala försvaret. Förenklat kan man säga att det cellulära försvaret är nödvändigt för att döda celler som infekterats av smittämnen som förökar sig inuti celler, t.ex. virus. Det humorala försvaret ska å andra sidan aktiveras och ta hand om smittämnen som förökar sig utanför celler t.ex. många typer av bakterier. Det som avgör vilken del av immunförsvaret som aktiveras vid en infektion är en komplicerad process som ännu ej är helt utredd. Man vet dock att de så kallade T-hjälparcellerna har en central betydelse. T-hjälparcellerna är innan de aktiveras i en så kallad naive eller 0 fas. Beroende på vilka signalämnen som finns i omgivningen när dessa celler upptäcker ett smittämne kan de bilda antingen T-hjälparceller av typ 1 eller typ 2. Bildas typ 1 T-hjälparceller kommer immunförsvaret aktivera det cellulära försvaret och bildas typ 2 T-hjälparceller aktiveras det humorala försvaret.

Eftersom inaktiverat TBE vaccin inte delar sig inuti celler, som levande virus gör, finns det anledning att tro att vaccinet enbart aktiverar det humorala försvaret. Eftersom det allmänt anses att det cellulära försvaret är nödvändigt för att skydda mot vissa typer av virusinfektion är det viktigt att klarlägga vaccinationens effekt. Till exempel skulle ett felaktigt immunförsvar kunna förklara varför vissa personer blir sjuka i TBE trots vaccination. Vår studie undersökte immunförsvaret vid vaccination genom att mäta användningen av två olika gener hos T-hjälparceller, T-bet och GATA-3. Våra resultat visade att vaccination ökar användningen av T-bet genen men inte GATA-3 genen hos T-hjälparcellerna. Eftersom T-bet genen används mest i typ 1 T-hjälparceller drar vi slutsatsen att det inaktiverade vaccinet förmodligen, trots allt, ger ett immunförsvar av cellulär typ. Detta fynd stärker uppfattningen att vaccination ger ett gott skydd mot TBE.

Abstract

Tick-borne encephalitis virus is the cause of much morbidity and sometimes a fatal infection. A vaccine based on formaldehyde inactivated virus is currently the only available way of preventing disease. This vaccine gives a high rate of seroconversion but there are reports of vaccination breakthrough, even in people who have demonstrated a neutralizing antibody response. The T cell response to inactivated TBE vaccine is largely unknown, but could be of importance for the effect of the vaccine. This study characterizes aspects of the T cell response by investigating the expression of two transcription factors, T-bet and GATA-3 with RT-PCR. T-bet is expressed in CD4⁺ T cells of the Th1 type, while GATA-3 is expressed in CD4⁺ T cells of the Th2 type. Our data show that vaccination with inactivated TBE vaccine leads to increase in expression of the T-bet gene when cells of vaccinated subjects are cultured with TBE virus. In contrast, the expression of GATA-3 remains unaffected by vaccination. Thus, this study suggests that the inactivated TBE vaccine leads to a Th1-like immune response in humans.

Introduction

The tick-borne encephalitis virus

The tick-borne encephalitis virus (TBEV) belongs to the family Flaviviridae, genus flavivirus. The flavivirus genus consists of more than 70 viruses including the viruses causing Japanese encephalitis, dengue fever, yellow fever and TBE. The flaviviruses have been classified into clusters and species based on nucleotide sequence. The major clusters are tick-borne, mosquito-borne and non-vector-borne viruses.¹ The tick-borne cluster comprises Omsk hemorrhagic fever and three subtypes of TBE, the European TBEV (TBEV-Eu), Siberian TBEV (TBEV-Sib) and Far eastern TBEV (TBEV-FE).²

The TBE viruses are positive-stranded RNA viruses of approximately 11000 nucleotides. They have one single open reading frame, which is translated into a single polyprotein. This polyprotein is cleaved by viral and cellular proteases into the individual proteins.² The result of this process is three structural and seven non-structural proteins. The structural proteins are assembled in the endoplasmic reticulum of the cell into immature virions consisting of capsid protein (C), envelope protein (E) and pre-membrane protein (prM). The immature virion is transported through the cellular secretory pathway and just before release the prM protein is cleaved into functional M protein and the fully functioning virus is released.³

Infectious TBEV enters cells by receptor mediated endocytosis. The E protein of the virus is composed of three beta barrel shaped structures. Structure III is an immunoglobulin-like domain and thought to be the receptor binding part of the protein. The precise receptor for TBEV cell infection has not yet been elucidated.⁴ A recent study made of TBE infected humans in Lithuania implicated a role for the mutated CCR5δ32 allele, a chemokine receptor, but its exact role in disease is not known.⁵ After endocytosis has been completed, the acidic environment in the endosome changes the E proteins' dimeric conformation to a trimeric conformation, which enables it to initiate fusion of the viral envelope with the endosomal membrane and release of the viral RNA into the cell cytoplasm.⁴ The E protein is also the major target antigen of neutralizing antibodies when a protective immune response is induced. The mechanisms of action of the polyclonal antibodies are not known but they probably act by preventing virus attachment or fusion of the viral envelope to the endosome.⁶

Pathology of TBEV

Upon ingestion of virus from the ticks' saliva to the human host, the virus first invades and multiplies in the Langerhans cells of the skin and then invades macrophages, histiocytes and fibroblasts. The virus spreads to the regional lymph nodes, liver and spleen. During this primary viremia the virus also initiates its invasion of CNS.⁶ The mechanisms of transversion of the blood brain barrier are not yet elucidated. The possible ways of blood brain barrier crossing include passive diffusion, transcytosis, invasion of endothelial cells, or invasion of olfactory epithelium. The exact mechanisms of viral damage are not known but once inside the CNS the virus induces an inflammatory response which can be seen as perivascular infiltration of activated T cells and macrophages. Damage is spread through-out the CNS and affects primarily grey matter where neuronal degeneration, necrosis and neurophagia are seen.

The damage is most pronounced in the medulla oblongata, brainstem, cerebellum and the spinal cord.⁷

Clinical symptoms of disease

Serological studies indicates that 70 to 90 % of TBEV infections in human are sub-clinical or asymptomatic.⁸ In those who develop disease there is an incubation period of 4-28 days with a median of eight days. After this time there is an acute phase where the patient has uncharacteristic influenza-like illness with fever, joint and back pain, headache, nausea and vomiting.⁹ A second phase of disease characteristically seen after approximately eight days, develop in about 20-30% of patients experiencing a symptomatic acute phase of TBE-Eu infection.⁸ The symptoms seen in the second phase are meningeal signs (headache and neck stiffness), ataxia, altered consciousness, impaired concentration and memory, dysphasia, confusion, irritability, tremor, and paralysis of cranial nerves.¹⁰ Many patients have residual symptoms. In one study 80 % of TBE patients had symptoms of CNS dysfunction on follow-up 6 weeks after disease and 40 % still had symptoms at one -year follow-up.¹¹

Vectors and transmission

The vectors of the three subtypes of TBEV are all hematophagus ticks. The TBEV- Eu is primarily transmitted by the hard tick *Ixodes ricinus* while the two other subtypes primarily spread through another hard tick; *Ixodes persulcatus*. Transmission between ticks occurs both through feeding on viraemic hosts and through co-feeding, a process which involves an infected tick feeding in proximity to a naïve tick and the spread of virus in migratory skin cells of the host.¹⁰ Once infected by the TBEV the tick will stay infectious through-out its different stages of life and will also through vertical transmission pass the virus on to its offspring. The tick goes through three stages of development; larvae, nymph and adult stage. Development from one stage to another requires the tick to feed. The major targets for ticks are rodents, especially the *Apodemus* species, a genus of Eurasian field mice.⁶ These rodents are recognized as the major transmission host of the TBEV, while other targets of tick feeding such as humans and larger animals; goats, cows, deer, can become infected accidentally but play a minor role in transmission of virus between ticks.¹⁰

Epidemiology of TBEV

The occurrence of TBEV is determined by the distribution of the tick vectors. Thus, the TBEV is spread only during the active season of the tick which starts when the temperature rise above 5-7°C. In the central European region this means that the TBEV is spread from March-April until October-November when temperature declines below this level. Because of the longer duration of the active season of ticks in central Europe, there are two peaks of incidence of TBE infection reflecting the spring and autumn population of ticks. In Scandinavia only one peak in TBE infection is observed and it occurs in the summer. The geographical distribution of *Ixodes ricinus* is Europe, central Asia and North Africa and the TBEV- Eu has been found in all of these regions. *Ixodes persulcatus* is found from the Baltic countries in the west to the eastern parts of Russia and in these regions it acts as the major vector for the subtypes TBEV- FE and TBEV- Sib. The annual world wide incidence of clinical cases of TBEV is 10000- 12000. Most cases occur in Russia with 5000 to 9000 cases annually. The country with the highest incidence rate in the world is Latvia with 26.3 cases/100 000 population annually.¹² Sweden had 163 reported cases of TBE in 2006.¹³ This number increase in 2007 to 182 cases of TBE and although the majority of cases (75%) occur

in proximity of the Stockholm area, there are now reported cases also in Östergötland, around the lakes Vättern and Vänern and on the island Gotland.¹⁴

Vaccination

Currently there is no anti-viral treatment for TBEV once infection has taken place, instead only supportive treatment is given. There is also no way of interrupting the virus in the environment so the virus will remain endemic in areas inhabited by its vectors. Prevention by vaccination is therefore the main option to lessen the burden of disease. In Austria a massive vaccination program started in 1981, resulting in a vaccination rate of 86 %. This in turn has been followed by a steady decline in TBE incidence from 700 cases annually to 54 cases in 2001.¹⁵ There are currently two vaccines available in Western Europe. FSME-IMMUN[®] (Baxter Vaccine AG, Vienna, Austria) is prepared using formalin inactivated TBEV of the Neudörfl strain found in Austria. Encepur (Chiron.Behring, Marburg, Germany) is prepared from TBEV of the German strain K23. Trials with FSME-IMMUN[®] have shown a seroconversion rate after three vaccinations between 96 and 100 %.¹⁶ The administration scheme for both vaccines is three doses given intramuscularly, the first two given with a gap of three week to 3 month apart and the third one given nine to twelve months after the second. Thereafter, a booster dose every fifth year is recommended.¹⁵

The principles of immunity against viral infection

In the defence against viral infection, the adaptive immune system primarily utilizes two different responses. Antibodies produced against viral surface antigens and antigens on infected cells act to prevent the virus from infecting more cells. CD8+ T cell response against internal viral antigens shown on the cell surface by MHC I act to eliminate infected cells and hence to eradicate infection. In some viral infections, such as rabies, antibodies are enough to control the replication and spread of infection.¹⁷ In other viral infections such as lymphocytic choriomeningitis virus, protection can be mediated through a CD8+ T cell response alone.¹⁸ A combination of both is sometimes necessary for protection. Passive immunization with antibodies for arena virus infection protects against viral challenge when T cell response is fully functioning, but not in mice lacking CD8+ T cells.¹⁹ These examples illustrate that both of the two arms of adaptive immunity are important for viral immunity and that the relative importance of the humoral and cellular response depends on the infecting virus.

Antibodies are often directed towards the extra cellular domain of the surface antigen. On the viral protein there are often many different antigen sites that can be the target of antibodies. During a first exposure to the virus the immune system often only produces antibodies against some of these sites. During further exposure to viral antigen the immune response will evolve to involve more of the antigen sites. This is one explanation why it may take repeated immunizations to get a protective antibody response, as is the case with the TBE vaccines.²⁰

The CD8+ T cell response can be directed towards surface proteins as well as internal structural, regulatory and non-structural proteins. The internal structural and regulatory proteins are often more conserved, are produced in greater number and earlier in the virus life cycle than the surface proteins.²⁰ This make them important especially in infections with virus that undergo rapid mutation of surface antigens such as HIV where T cells towards the intracellular and non structural proteins Gag, Nef and Pol are present.²¹ On successive viral

challenges the CD8+ T cell response will expand to generate an increased number of antigen-specific T cells.²²

When a human is exposed to a virus the first line of defence is the mechanical barriers of the body; the skin and mucosa, also including cells and molecules of the innate immunity, which is followed by antibodies of the adaptive immune system. All of these mechanisms work to prevent the virus from infecting the cells of the body. Once infection has occurred a T cell response will be invoked. Viral infected cells can be destroyed by CD8+ T cell or by antibody and complement lysis, or by antibody mediated NK-cell activation. The ideal of an immunisation is to invoke an antibody response large enough to completely prevent virus from infecting cells, i.e. neutralizing the viral agents. Antibodies are most effective when the site of invasion is distant from the target organ, since the virus needs to reach the bloodstream to access the target and is thus exposed to the antibodies.²⁰ This is the case with TBE infection, which starts in the skin of the infected host but must pass through the lymph and blood stream to reach the CNS where the damage is done.

Inactivated vaccines

The currently used TBE vaccines are of the inactivated type.¹⁶ Inactivated vaccines are viruses that have been treated with formalin or other chemicals, making them unable to replicate. In comparison with the live attenuated vaccines they are generally less potent. This might be due to their lack of proliferatory (replicative) potential which leads to a lack of virus-encoded proteins in the cytosol of the cells, proteins that in a natural infection would be presented on MHC I leading to a CD8+ T cell response.²³ The advantages of inactivated vaccines are that they are more heat stable and that they lack the risk of reversal of virulence which is always a possibility in the case of live-attenuated vaccines.²⁴

The fact that inactivated vaccines only induce some parts of the immune system has been shown. In a study by Kreil et al., mice were first immunized with a three times course of FSME-immune and then challenged with a lethal dose of TBEV. As expected, high titers of antibodies against protein E were induced after the immunization, which resulted in survival rate of 100 %. However, even though viremia was not detected on challenge with live virus, a new kind of antibody developed towards non structural protein 1 that had not developed after vaccination. Also, TBEV specific CD8+ T cells were detected after but not before challenge with virus. The authors concluded that the vaccine protected against disease but not against infection.²⁵

Another study performed by Aberle et al. made on mice vaccinated with inactivated vaccine found that it induced a Th2 response measured as the amount IgG1 antibodies, which are Th2 specific in mice, compared to IgG2a, which are Th1 specific. This study also found that inactivated TBE vaccine was unable to induce a CD8+ T cell response in mice.²⁶

Adjuvants

To enhance the effect of vaccination, adjuvants are often added to the vaccination antigens. Adjuvants are defined as substances that enhance the immunogenicity of antigens.²³ In TBE vaccines the adjuvant is aluminium hydroxide (Al(OH)₃)¹⁶ In mice aluminium hydroxide favours a polarization towards a Th2 response.²⁴ Some adjuvants have been shown to induce a Th1 response when given with inactivated vaccines but it has been difficult to find any adjuvant that can induce a CD8+ T cell response in human.²⁷

Vaccination breakthrough

Measurement of neutralizing antibodies is used as a surrogate for protective efficacy in TBE vaccination.²⁸ After an immunization schedule of three vaccinations, the seroconversion has been found to be 99.6%.¹⁶ In Austria, where vaccinations have been extensive, 34 cases of TBE were reported in the vaccinated population between 1994 and 2004. Of these, a majority (20 of 34 cases) was over 60 years of age, indicating the waning of immune efficiency with age.²⁹ In a case study two patients were described having proven neutralizing antibodies of IgG type and still being infected by TBE.³⁰ A severe TBE infection was reported in a 54 year old patient who had taken the full 3 times schedule and 2 booster doses, the last one three years earlier. Although the patient survived, several residual symptoms were reported, such as neuropathy with stabbing pain in all limbs and persistent neuropsychological deficits.³¹

The adaptive immune system

As described above the immune response against viral infection depends on the adaptive immune systems' two major components; the humoral and cellular immune responses. The determination of which response that will be predominant in a viral challenge depends on the differentiation of the CD4+ T cell. The result of this differentiation is the creation of two kinds of CD4+ T cells; Th1 and Th2. The Th1 cell is characterized by the production of the cytokine interferon gamma (IFN- γ) and activation of macrophages. The Th2 cell, on the other hand, produces IL-4 and is specialized in activating B cells resulting in antibody production.³²

CD 4 T cell differentiation – transcriptions factors T-bet and GATA-3

The first requirement of CD4+ T cell differentiation is activation of the naïve T cell. This process begins when an antigen presenting cell (APC) such as a dendritic cell (DC) phagocytoses an antigen, gets activated and migrates to a peripheral lymphoid organ. In the lymphoid organ, e.g. a lymph node, the activated APC encounters naïve T cells. The CD4+ T cell binds the antigen presented on a MHC II molecule of the APC and at the same time its CD4+ protein binds the MHC II molecule. The T cell also needs a co-stimulatory signal which is the binding of its CD28 molecule to a CD80 or CD86 molecule which is highly expressed on APCs who have been activated.³²

The signal of the T cell receptor (TCR) starts with the release of intracellular calcium, which activates the calcium-dependent phosphatase calcineurin. This phosphatase dephosphorylates Nuclear Factor of Activated T cells (NFAT), which then translocates to the nucleus. NFAT activation results in chromatin remodelling which is necessary for further differentiation. NFAT may also be involved in the acute cytokine transcription of the activated T cell as it binds the *Ifng* promoter and the *Il4* promoter and transcription of both of these genes are increased on activation resulting in a slight increase of both IFN- γ and IL-4 secretion.³³ The *Il4* locus is hypermethylated in naïve T cells but during Th2 differentiation it is passively demethylated probably through some kind of interference with DNA methyltransferase 1 (Dnmt1). Dnmt1 is the enzyme which under normal conditions methylates the different alleles of the cell during replication. If this process is interrupted the *Il4* locus will lose more methylations for every replication, which means that every new generation of cells will produce more IL-4.³⁴ Demethylation is accompanied by increased acetylation of the *Il4* locus. This probably occurs due to the fact that deacetylation is normally mediated by acetylases incorporated in the multi-subunit complex which the methyl-CpG binding protein

(MBD2) assembles on methylated DNA. When the DNA is less methylated, less MBD2 is able to bind and thus, less deacetylation occurs.³⁵

All of these changes that occur on activation of the T cell increase the possibility of further change but are not enough for T cell differentiation. This process is dependent on the cytokine environment in which the T cell is activated and this in turn is believed to be largely dependent on the cytokines expressed by the DC that activated the T cell. There are two distinct subtypes of dendritic cells in human, one expressing IL-12 upon activation and inducing a Th1 response while the other does not express IL-12 and induces a Th2 response.³⁶

IL-12 act by binding the IL-12R receptor and this activates the second messenger signal transducers and activators of transcription 4 protein (STAT4). The effect of STAT4 signalling is increased survival of Th1 cells and increased transcription of the *Ifng* gene probably through acetylation mechanisms independent of the activation of transcription factor T-box expressed in T cells (T-Bet). Thus, IL-12 increases the IFN- γ expression and promotes a Th1 response. Th1 cells that have differentiated produce IFN- γ for approximately 72 hours and then the production decreases. The production is prolonged by addition of IL-12 and IL-18 indicating their role as IFN promoters.³⁷ IL-12 derived from activated DCs also increase IFN- γ production from NK cells and this effect is also enhanced by IL-18.³⁸

Th1 differentiation is also promoted by the cytokine IFN- γ . Through its second messenger STAT1 it activates T-bet.³⁹ This activation results in the transcription of *Ifng* as well as the downregulation of the *Il4* transcription.⁴⁰ STAT1 also induces the IL-12R β 2, which increases the sensibility of the T cell to IL-12 stimulation.³⁹

The differentiation of an activated T cell to a Th2 cell may be initiated by binding of IL-4 to the IL-4R. The second messenger of this receptor is STAT6. This protein associates with the *Il4* promoter and might thus have a direct effect on the IL-4 production. However, STAT6 also up-regulates the transcription of GATA-3. This transcription factor then binds the 3' enhancer of the *Il4* locus and this is associated with the binding of NFAT to the *Il4* promoter.⁴¹ Once activated this transcription factor can autoregulate its own transcription and it also leads to an intrinsic suppression of IFN- γ production independent of IL-4 production.⁴²

T-bet and GATA-3 thus determine the fate of the activated T cell. If T-bet is activated it up-regulates IFN- γ and down-regulates IL-4. GATA-3 does the opposite; it increases the transcription of IL-4 and down-regulates IFN- γ . The cytokines that are produced in this manner work in an autocrine way to encourage their own production as well as in a paracrine way to promote a Th1 or Th2 environment, respectively.

Aim

As here described, the TBE virus is the cause of much morbidity. Vaccination has been shown to be effective on a population basis but in cases of vaccination breakthrough, the course of the disease may be severe. The immune response induced by inactivated TBE vaccine in animals has been shown to be of a different kind than the one seen in natural infection. The TBE vaccination seems to induce a Th2 response instead of Th1 and also to lack induction of CD8+ T cells. The aim of this study is to characterize aspects of cellular immune response to inactivated TBE vaccine in humans. Blood samples were taken prior to and after full vaccination. The mRNA expression of the Th1 specific transcription factor T-bet and the Th2 specific transcription factor GATA-3 were measured after in vitro stimulation of T cells with inactivated TBE virus.

Materials

The blood samples used in this study came from seventeen people attending TBE vaccination at the Department of Infectious Medicine, University hospital of Linköping. Vaccinations took place from 2004 to 2007. The study group consisted of twelve women and five men in the age 29 to 77, median age 66. Out of these 17 subjects, seven were healthy, four had allergies, two had high blood pressure, one had pacemaker, one had asthma, one was under investigation for gastrointestinal problems and one had Hashimoto's disease. None of the subjects had been diagnosed with TBE or Dengue fever. Three had previously been diagnosed with Lyme disease i.e. infection of *Borrelia burgdorferi*. Prior to vaccination, serum samples were collected from all subjects. All subjects had given a serum sample before vaccination and this showed that 14 of subjects were negative for, two had borderline values and one was positive for TBE in the terms of antibodies as measured by ELISA. However, none of the subjects had neutralizing antibodies towards TBE before vaccination. Vaccinations took place according to the recommended immunization schedule.¹⁵ Blood samples were taken before the first vaccination and then again after the third vaccination. Although 17 subjects took part in the study, some samples had to be excluded. Out of the seventeen subjects, only seven patient-samples taken before vaccination could be used; in five subjects there were no stored cells available for RNA extraction, the remaining five samples had too low RNA concentration after RNA extraction to be used in RT-PCR. Also, in two of the subjects, one of the stimulations was excluded due to limited RNA content. As to samples taken after vaccination, two blood samples had to be excluded due to too low RNA concentration after extraction, leaving 15 useful blood samples in this group. The study was approved by the Regional Ethical Review Board in Linköping, Sweden. All subjects gave informed consent.

Methods

Blood samples were collected using phlebotomy. Peripheral blood mononuclear cells (PBMC) were extracted using gradient centrifugation on Lymphoprep (Medinor AB, Lidingö, Sweden). The cells were cultured in tissue culture medium consisting of Iscoves modification of Dulbeccos medium (Invitrogen) supplemented with NaHCO₃ 3,024g/l, L-glutamin(Sigma Aldrich, Sweden) 292mg/l, pencillin (In Vitro Sweden AB, Stockholm, Sweden) 50 IU/ml, streptomycin (In Vitro) 50 ug/ml, 100x non-essential amino acids (Invitrogen) 10 ml/l and 5% fetal bovine serum (Sigma Aldrich, Sweden) at a cell density of 1×10^6 /ml. From each subject four cultures were set up and stimulated with either Influenza antigen, formalin inactivated TBE virus (Chirion, Germany) or PHA (Sigma Aldrich, Stockholm, Sweden). In addition, one culture was left unstimulated to serve as negative control.

After stimulation, the cells were lysed by addition of buffer RLT (RNeasy 96 RNA extraction kit, Qiagen, Hilden, Germany), in accordance to the manufacturers protocol. The lysate were stored at -70°C until extraction of mRNA.

The resulting material of the process described above was used in the current study. Total RNA was extracted from the lysed cells by use of the protocol and kit RNeasy[®] Mini Kit (QIAGEN AB, Solna, Sweden). Briefly, the lysed cells were homogenized and then passed through an RNeasy Mini spin column. This column contains a membrane that the RNA binds to. Addition of water to the spin column released the pure RNA from the membrane. RNA quality and quantity was then assessed by spectrophotometry (ND 1000; NanoDrop technologies, Wilmington, USA).

The total-RNA extracted in this manner was then converted through reverse transcription to cDNA which is necessary for use in quantitative PCR. This conversion was done using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Stockholm, Sweden) and in accordance with manufacturers' instructions. In short, a mix containing nucleotides, primers and reverse transcriptase was put in a 96 well plate. The Total-RNA and water was then added in proportion to the RNA concentration so as to receive a sample of the same concentration as the sample with the least RNA content, in this study that RNA concentration was 22.69 ng/ μ L.

The cDNA samples were then used to in quantitative reverse transcription polymerase chain reaction (RT-PCR). This method was used to investigate the expression of three genes, 18S, GATA-3 and T- Bet. The 18S gene is a "house keeping"/ control gene and its product is part of the ribosome, this means that it should be expressed in equal numbers in all cells independent of the cells differentiation. The GATA-3 and T-bet genes were used as indicators of cell differentiation. All genes samples were run in duplicates and a standard curve were made for analyse of gene expression.

The analyse of data was made through the use of a standard curve. This curve was made with a cell lysate that had been diluted to a ratio of 1/1, 1/4, 1/16, 1/64, 1/256. Each of these samples had then been given an arbitrary value starting with 4096 for the 1/1 dilute to 16 for the 1/256 dilute. A threshold value of 0.05 was used as the point of measurement. A CV (coefficient of variance; Standard deviation / Mean x 100) of less than 15% was used as a limit of acceptable variation of duplicate values.

Statistics

Data were checked to determine if they were Gaussian distributed. The results indicated that data was not normally distributed and thus non-parametric Mann-Whitney U test was used. Statistical analysis was made using GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

Results

T-bet

The expression of the T-bet gene and the 18S gene was measured with quantitative RT-PCR in the samples taken before and after vaccination. The ratio of T-Bet to 18S was then measured for each sample and grouped in accordance to stimulation.

No significant difference was found before vaccination between the TBE stimulated and the un-stimulated samples when compared by Mann Whitney U test (Fig.1). Nor were there any significant difference between the PHA and Influenza when compared with the un-stimulated samples.

After vaccinations (Fig.2) a significant increase of the T-Bet/18S ratio was seen when the TBE samples were compared with un-stimulated ($p = 0.0005$). At this occasion there was also a significant increase in the other two groups of samples when compared with the un-stimulated samples (Influenza $p < 0.0001$ and PHA $p < 0.0001$). The TBE samples did not differ significantly when compared with the Influenza or the PHA samples (Influenza vs TBE $P=0.2455$ and PHA vs TBE $P=0.0745$).

Tbet/18S before and after vaccination

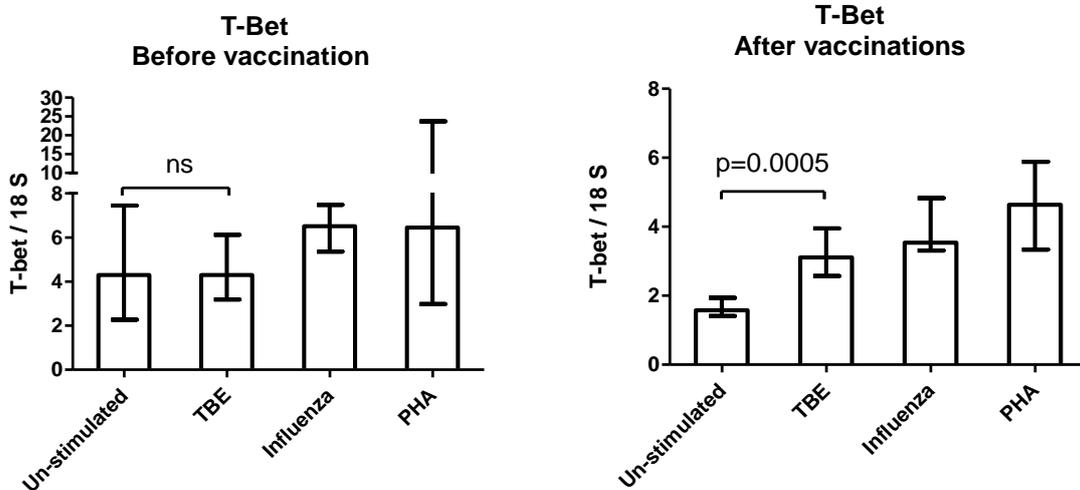


Fig 1. Graphs show the median and interquartile range before vaccination for the Tbet/18S expression ratio. TBE, influenza and PHA samples did not differ significantly from Un-stimulated samples.

Fig 2. The Tbet/18S expression ratio of the TBE stimulated samples was significantly higher than the un-stimulated samples after vaccinations. The influenza and PHA samples also differed significantly from the un-stimulated samples but not from those stimulated with TBE.

GATA3

The GATA-3/18S ratio was calculated in the same manner as for T-Bet/18S (Fig.3 and 4). No significant differences were found before vaccination with any of the three stimulations when compared with the un-stimulated samples.

The TBE stimulated samples GATA-3 / 18S ratio after vaccination did not significantly differ from the un-stimulated samples. Neither did the influenza stimulated samples, whereas the PHA samples differed significantly from the un-stimulated (P=0.0035).

GATA-3/18S before and after vaccination

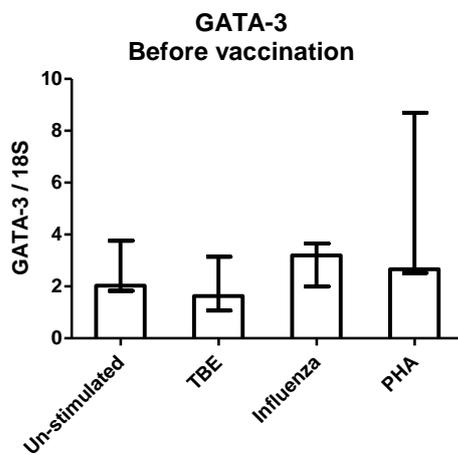


Fig 3. There were no significant difference in the GATA-3/18S ratio between the samples stimulated with TBE, Influenza or PHA and the un-stimulated samples before vaccination.

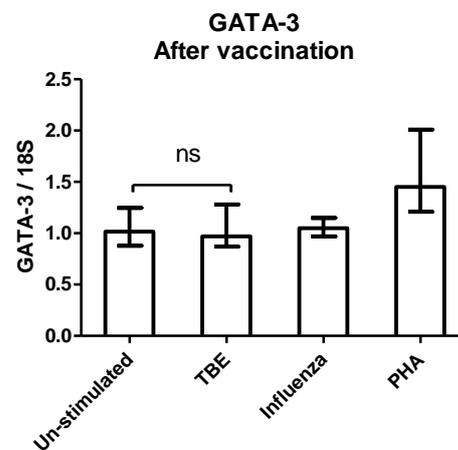


Fig 4. The TBE and the influenza stimulated samples GATA-3/18S ratio did not differ significantly from the un-stimulated samples after vaccination whereas the PHA stimulated samples did P=0.0035.

T-bet/ Gata3 ratio after vaccination

As shown above, there was a significant increase in the T-bet expression but no significant difference in the GATA-3 expression when comparing the TBE stimulated and the un-stimulated samples after vaccination. To directly compare the difference in expression of the two transcription factors after vaccination, and thus show which transcription factor that dominated after vaccinations, a calculation was done using the formula;

Subject X: TBE (T-bet/18S) / un-stimulated (T-bet/18).

This calculation was done for each of the 15 subjects and the median and inter-quartile range of these results was calculated and compared with the same calculations for the GATA-3 and 18S expression.

Sample X: TBE (GATA-3/18S) / un-stimulated (GATA-3/18S).

The result of these calculations makes it possible to directly compare the expression of the two transcription factors. The change in T-bet expression (median ratio of 1.89) was significantly higher ($p < 0.0001$) than the change in GATA-3 expression (median ratio of 1.09) after vaccination (Fig.5).

**GATA3 TBE/Un-stimulated & Tbet TBE/Un- stimula
After Vaccination**

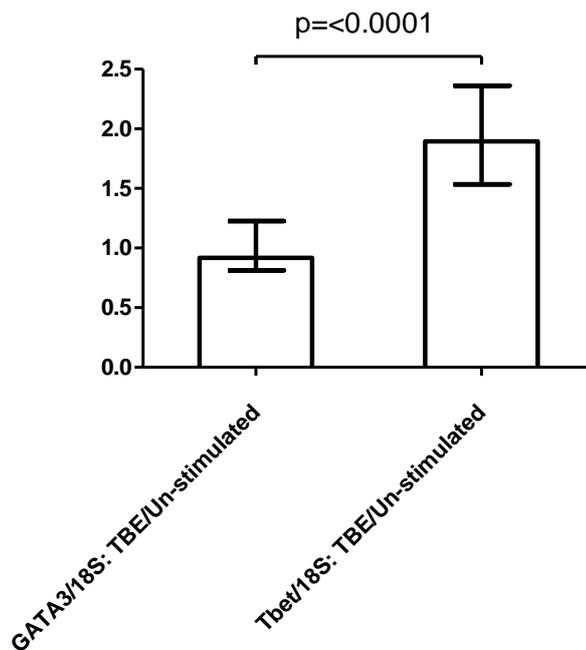


Fig.5. This graph shows the relative difference between the TBE stimulated and the un-stimulated samples for each of the two transcription factors. The median ratio was 1.89 for T- bet and 1.09 for GATA-3 with a P-value of < 0.0001 .

Discussion

The aim of this study was to characterize aspects of the cellular response to a three time vaccination schedule with inactivated TBE vaccine in humans. The results showed an increase in the expression of the T-bet gene when cells from vaccinated subjects were cultured with inactivated TBE virus compared with un-stimulated cells from the same subjects. In contrast, the expression of GATA-3 did not increase significantly when cells from the vaccinated subjects were stimulated with TBE. Finally, the difference in expression of the two transcription factors was compared to see which of the two that dominated, showing a significant predominance of T-bet over GATA-3. As T-bet is a Th1 specific transcription factor the interpretation of these results is that vaccination with inactivated TBE vaccine gives a T cell differentiation mainly of the Th1 type.

A weakness of this study was the inability to collect enough data before vaccination. Data was therefore unable to show a significant difference between the positive control (PHA stimulation) and the un-stimulated samples in the test subject before vaccination. This makes it difficult to exclude for certain whether the subjects of this study had a T cell response towards TBE antigen before the vaccinations took place. The reason for this is that although TBE stimulated cells did not differ from the un-stimulated cells in their expression of transcription factors before vaccination, neither did they differ from the positive control. Theoretically they could therefore have had a response towards the TBE antigen, which could not be detected in the test in line with the unexpectedly low PHA response. There is of course little reason to believe that the general population in Sweden would have a T cell response towards TBE before vaccination, and also all but one subject was negative for antibodies towards TBE before vaccination, indicating a lack of immune response. Still, this is a concern, which should be taken in to consideration when interpreting the result of this study.

Even so, the results show a significant difference between the expression of T-bet/18S between the TBE and un-stimulated groups after vaccination. Since it is unlikely that such a difference should exist in the un-vaccinated population in Sweden, our conclusion is that TBE vaccination most likely induces a T cell differentiation with an up-regulation of the T-bet gene. This notion is further supported by the absence of GATA-3 expression.

The reasons for a low RNA concentration in samples taken before vaccination are obscure. It should be possible to store cDNA at -70 °C. Still, it is possible that the longer storage time of the samples taken before vaccination was the reason for the low RNA concentration seen in some of those samples.

Another methodological consideration is the choice to look at transcription factors as an indicator of T cell differentiation. One must keep in mind that secondary modulations later in the signalling pathway may modify the final results of transcription factors and it could be argued that protein levels (IFN- γ or IL-4) or antibodies (IgG1/IgG3 for Th1 in humans ⁴³ or IgG4 for Th2 ⁴⁴) are better indicators of differentiation because they are the active immunological agents.

The result of this study, that an immunization with inactivated TBE vaccine gives a Th1-like immune response might seem unexpected, contradicting previous evidence of a Th2 response to inactivated TBE vaccine found in experiments made in mice. Aberle et al. investigated the nature of immune responses in mice immunized with different kinds of TBE vaccines. Of the

five tested vaccines, one was inactivated, two were live- attenuated in which parts of the viral genome had been removed, while the two remaining vaccines were of a novel kind consisting of the viral RNA genome with the exception of a sequence corresponding to 62 amino acid residues in protein C, thus producing non-infectious sub-viral particles lacking a functional capsid protein necessary for virulence. After immunization, the antibody response, which in mice distinctly mirrors the Th1/Th2 response, as well as the CD8+ T cell response, was measured. All types of immunizations resulted in 100 % seroconversion, i.e. all mice had TBEV specific antibodies. The IgG2a/IgG1 rate was used as a measure of the Th1/Th2 deviation of the response. Live attenuated and RNA vaccines both gave an IgG2a dominated, i.e. a Th1-like response. Inactivated vaccine, on the other hand, gave an IgG1 dominated Th2-like response. TBEV specific CD8+ T cells taken from the spleen of the immunized animals were measured. The result showed that live- attenuated and RNA vaccines gave a CD8+ T cell response while the inactivated vaccine did not.²⁶

Are these data totally contradicting the results of the present study or is there a possible explanation for the diverging results? One explanation of this difference could be an essential difference in the regulation of T cell differentiation in murine and human species, e.g. regarding the role of DCs.

DCs do play an important role in T cell differentiation. Not only do DCs perform the necessary steps for T cell activation by showing the antigen on their MHC molecules and express co-stimulatory proteins such as CD80/86, but they also produce cytokines that are responsible for the nature of the T cell differentiation. Studies have shown that DCs belong to different sub-classes as determined by their origin in humans and by their expression of certain proteins in mice. In humans, differences have been shown between the DCs generated from myeloid CD11c⁺ origin and those produced from plasmacytoid CD11c⁻ origin.⁴⁵ The myeloid DC, also called monocyte DC, or DC1 produces, after activation by the CD40L route, large amounts of IL-12, resulting in a Th1 response in naïve CD4⁺ T cells in vitro. The plasmacytoid DC, also called lymphoid DC or DC2, does not produce IL-12 after CD40L activation, and naïve T cells cultivated with DC2 differentiate into Th2 cells.³⁶ Plasmacytoid DCs do not produce IL-12 upon stimulation but may in some circumstances produce IFN- α when activated⁴⁶ These findings are comparable with the CD8 α ⁺ and CD8 α ⁻ types of DCs which can be found in mice. The CD8 α ⁺ is able to produce IL-12 leading to a Th1 response, while the CD8 α ⁻ is unable to produce IL-12 and therefore leads to a Th2 response.⁴⁷ Differences in which DC population that is activated upon stimulation with inactivated TBE vaccine could explain the different T cell responses in humans and mice. If the inactivated vaccine activated DCs of the monocyte/ DC1 origin in humans while activating CD8 α ⁻ DCs in mice then this would result in a Th1 response in humans and a Th2 response in mice. Factors that could influence the DC preference include route of administration, dose and interval between doses, number of doses and the adjuvant added to the vaccine.

Another factor of importance in T cell differentiation is differences in T cell signalling that result from the cytokines expressed by DCs. In the mouse, there is strong evidence that the CD8 α ⁺ DCs' ability to induce Th1 differentiation requires IL-12.⁴⁵ Since experiments indicates that IFN- α is unable to drive Th1 differentiation and that IFN- γ is merely a cofactor to IL-12.⁴⁸ In humans, on the other hand, the IL-12 is not the sole driver of Th1 differentiation and not the sole activator of STAT4, since STAT4 is also activated by IFN- α .⁴⁹ It has also been shown that IFN- α is able to induce a Th1 response in human naïve T cells in vitro, independent of IL-12 in contrast to the situation in mice.⁵⁰

Thus, these fundamental differences may affect the way T cells react on inactivated vaccines. For example, it is possible that DC macro-pinocytosis or receptor mediated phagocytosis, of the inactivated virus particles activated the plasmacytoid DCs to produce IFN- α that mediates a Th1 response in humans, while it does not in mice.

It should be noted that there is no consensus about the difference in IFN- α signalling in mouse and humans. There is indeed evidence of an IL-12 independent mechanism for Th1 induction in mice infected with certain viruses such as lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and mouse hepatitis virus (MHV).⁴⁹ Also, there are reports that IFN- α may be of importance for Th1 differentiation in mice being necessary for IFN- γ production in the viral infection LCMV. This IFN- γ induction was found to be STAT4 mediated and IL-12 independent.⁵¹

Differences in expression of Toll like receptors (TLRs) in humans and in mice are another possible explanation for differences in T cell differentiation. Toll-like receptors are of importance for activation of DCs and their expression of IL-12 to pathogens. Knock-out mice lacking the MyD88 protein, a second messenger in Toll-like receptor signalling, inhibited the Th1 response to *Toxoplasma gondii* which is normally produced in infection with this organism.⁵² The TLRs -3, -7 and -9 are all involved in IFN- α and - β production in DCs to viral infection.⁵³ Lundberg et al have found that TLR-3 signalling in response to dsRNA differ in mice and humans, inducing TNF- α and IL-6 in mice but not in humans. Differences in expression of IFN- α was unfortunately not investigated in this study.⁵⁴

These are some possible mechanisms, which could explain the species difference found between the result of this report and earlier investigations of T cell differentiation in mice. Further studies are needed to investigate the nature of the T cell differentiation. Important questions include; how does inactivated vaccine induce a Th1 response in humans? Is there a real difference in T cell response to cytokines in mouse and human and what are the causes of these differences? Do DCs respond differently to inactivated vaccine in mice and humans and what mechanisms do DCs use to recognize the inactivated virus, i.e. what type of TLR or other pattern recognition receptor is used? Does an inactivated vaccine give a CD8+ T cell response in humans and if so how is the antigen showed on the MHC I molecule?

With the results presented here, we are hopeful regarding the use of inactivated vaccines in immunization against infectious diseases requiring a Th1 response. Other data from this vaccination study show that TBE vaccination induces both CD4+ and CD8+ T cells (Jarefors, Ernerudh et al., unpublished data). Thus, data question the current paradigm that inactivated vaccines are unable to induce a sufficient CD8+ T cell response due to lack of intracellular replication. The findings put emphasis on the fact that T cell differentiation is not mediated by MHC I expression but by cytokines and DCs and that these cells may recognize and initiate a Th1 response to inactivated virus even in the absence of intracellular replication. Given the induction of Th1 differentiation by the inactivated TBE vaccine there is no reason that it could not also induce a CD8+ T cell response in humans as well. There is evidence that DCs may present extra-cellular antigens on MHC I molecules through a mechanism called cross presentation and that this triggers a CD8+ T cell response, a mechanism called cross-priming.⁵⁵ Whereas MHC I molecules present endogenous peptides on the surface of all nucleated cells, the capacity of cross presentation seems to be mostly attributed to professional antigen presenting cells (pAPCs) including B-cells, macrophages, perhaps endothelial cells and DCs, the last cell type being the major player.⁵⁶ The focus of this research has been mostly on experiments where viral antigens are produced in antigen donor

cells which enable the binding of heat-shock proteins and the transfection of these complexes to DCs, a scenario quite different from the one that takes place when inactivated whole vaccine is given as immunization. Experiments have never the less shown that different viral proteins can give a cross presentation response without involvement of de novo synthesis. This process is far from elucidated, but at least one endocytosis receptor, the mannose-receptor, has been shown to give a soluble protein access to the cross presentation pathway in vivo.⁵⁷ The TLRs have been shown to be of major importance in cross presentation and TLR 9, which recognizes bacterial DNA (CpG motifs) is able in combination with Gp120, a major HIV surface protein, to induce CD8+ T cell response towards Gp120.⁵⁸ Also TLR 3 ligation with dsRNA has been shown to induce cross presentation of ovalbumin (OVA) proteins in DCs.⁵⁹ These data indicates possible mechanisms by which viral antigens can induce a CD8+ T cell response in humans with-out intracellular replication. If such a response is induced by inactivated TBE vaccine can not be entirely proved by data from the current study, but additional data indicates that TBE vaccination does increase a TBE-specific CD8+ T cell response, as measured by increased proliferation after co-culture in vitro (Jarefors, Ernerudh et al., unpublished data). If so, then the exact mechanism would be of interest for further vaccine development. If further studies, on the other hand, shows that a CD8+ T cell response is not induced by inactivated vaccine, then the experiments accounted for above give clues of possible adjuvants that might trigger a cross presentation mechanism, for example addition of dsRNA or bacterial DNA to the vaccine.

If vaccination with TBE gives a Th1 response as this study suggests and maybe also a CD8+ T cell response as future studies will have to investigate, then the reasons for vaccination breakthroughs must be looked for elsewhere. One such reason could be strain differences in the antibody inducing antigens of the TBE strain used for vaccine production and the many strains present in the environment, such antigenically defective strains have been found in Russia.⁸ In addition, it must also be kept in mind that although a Th1-like T-bet response was recorded at the group level, there are a number of individuals that did not mount such a response. It remains to be elucidated whether this lack of response is due to genes, in particular HLA genes, or if there indeed might be a response that was not detected by the present method but could be detected for example at the protein level.

To conclude, the present study shows that TBE vaccination induces increased expression of the Th1-related transcription factor T-bet. In contrast, no increased expression was found for the Th2-related transcription factor GATA-3. These data suggest that TBE vaccination leads to Th1-like memory response. Further studies, e.g. at the protein level, are needed for confirmation.

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