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"ANALYSIS OF γδ T LYMPHOCYTES IN RASMUSSEN'S ENCEPHALITIS"

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CENTER FOR BRAIN RESEARCH

This thesis has been based on my research stay at the Center for Brain Research (CBR), located at 4 Spitalgasse Street, 1090 Vienna, Austria. My internship was carried out as part of the Department of Neuroimmunology, specifically in the Brain Inflammation research group, under the direction of Prof. Dr. Jan Bauer.

The CBR is a non-profit organization, based on a national framework. It is promoted and financed mainly by Austrian Science Fund (FWF), Austria's central funding organization for basic research, but as well by the European Community Framework Programmes, Oesterreichische Nationalbank and WWTF Vienna Science and Technology Fund. The investigation "Inflammation and Viruses in Epilepsy" (project number: P 26936 Einzelprojekte) in which I was working on is fund by the FWF.

The CBR defines itself as a young institution dedicated to the advancement of translational neurosciences. It focuses on studying clinically relevant topics such as mechanisms of acute and chronic pain (Dept. of Neurophysiology), immune surveillance of the nervous system (Dept. of Neuroimmunology), peroxisome-dependent malfunction of the nervous system (Dept. Pathobiology of the Nervous System), synaptic mechanisms of psychiatric and neurological diseases (Dept. of Molecular Neurosciences), synaptic mechanisms of learning and memory (Dept. of Neuronal Cell Biology) and the neuronal basis of cognitive functions (Dept. of Cognitive Neurobiology).

Research within the Department of Neuroimmunology focuses on brain inflammation and inflammatory diseases of the nervous system. As part of this department Prof. Dr. Jan Bauer main interest is the role of cytotoxic T lymphocytes and their implication in various human inflammatory diseases, such as Rasmussen encephalitis; an epileptic disorder of unknown aetiology mostly found in children.

ABSTRACT

Rasmussen Encephalitis (RE) is a rare progressive epileptic disorder with unknown aetiology. It is characterized by unilateral hemispheric inflammation, drug-resistant focal epilepsy and progressive neurological and cognitive deterioration. One of the histopathological hallmarks of RE is T cell infiltration, however, little is known regarding their presence in active lesions as well as their phenotype and activation state at early stages of the disease. (Varadkar et al., 2014)

In the present study, we analysed in detail the phenotype and activation state of gamma delta (γδ) T lymphocytes in the different stages of RE. Here, specific attention was paid to cytotoxic T cells with the ability to attack neurons, leading to neuronal loss. These studies were performed by qualitative and quantitative analysis by light microscopy and confocal fluorescence microscopy with various markers for T cells. The selection of markers consisted of antibodies against all T cells (CD3) and against γδ T lymphocytes (γδTCR). To identify if γδ T cells have a potential regulatory function, we then analysed the expression of the cytokine gamma-interferon (IFN-γ).

IHC stainings showed that γδ T lymphocytes do not constitute a specific population in early stages of RE. Nevertheless, they have the ability of targeting neurons, leading to the conclusion that they are capable of generating neuronal loss. In addition, it has also been proved that they constitute a cell population capable of expressing IFN-γ, suggesting that γδ T lymphocytes might be partially responsible for microglial activation in RE due to the production of this cytokine.

INTRODUCTION

Epilepsy is a brain disorder characterized by a permanent predisposition to seizures and by emotional and cognitive dysfunction. This disease has established itself as one of the most common neurological disorders, affecting approximately 50 million people worldwide. There is currently a wide range of antiepileptic drugs (AEDs), yet about one-third of individuals with epilepsy still experience seizures that do not respond to medication. This is due to the fact that AEDs basically act at a symptomatic level, which means that they block seizures but do not remedy the underlying pathology or slow the progression of the disorder. Therefore, there is a growing demand for the development of effective therapies that modify the epileptic process. To achieve this goal, one of the fundamental tools is to understand the mechanisms that generate the disease. (Vezzani, French, Bartfai, & Baram, 2011)

In the last decade, research in this field has been aimed at highlighting the connection between inflammatory processes and the development of epilepsy. Chronic inflammation in the brain is composed of the activation of microglia, astrocytes, endothelial cells of the blood-brain barrier (BBB) and peripheral immune cells, as well as the synthesis of inflammatory mediators. All this was first seen in patients with Rasmussen's encephalitis, which led to the suggestion that immune and inflammatory mechanisms play a role in some forms of epilepsy. (Vezzani et al., 2011)

Inflammation in the Central Nervous System

Inflammation can be defined as the body's immune system response to various stimuli, involving the cascade of reactions and signals, which lead to activation of innate immune cells and adaptive immune response. The innate immunity is a non-specific defence mechanism activated either immediately or within hours after antigen invasion. The adaptive or acquired immunity, on the other hand, is a specialized process involving antigen presentation that aims to eliminate or prevent pathogen growth. Both types of inflammation manifest with recruitment of neutrophils, macrophages and production of pro-inflammatory cytokines and chemokines. (Shandra, Moshé, & Galanopoulou, 2017)
Neuroinflammation is the innate immunological response within the nervous system, involving microglia and astrocytes. At present, brain inflammation or

neuroinflammation has been associated with many central nervous system (CNS) pathologies. Such processes may have beneficial effects, like protecting against exogenous insults or promoting healing, but under certain situations they can be pathogenic. For example, previous studies associated elevated levels of pro-inflammatory cytokines with seizures, the pathogenesis of epilepsy and pathologies manifesting epilepsy. (Shandra et al., 2017)

Traditionally the brain has been labelled as an immune-privileged organ thanks to the presence of the BBB, the lack of a conventional lymphatic system and the limited traffic of peripheral immune cells. Under physiological conditions, the BBB limits the movement of cells and macromolecules between blood and CNS tissue. However, during systemic inflammation the BBB becomes more permeable, which means that, antibodies, peripheral immune cells and inflammatory molecules can migrate into the CNS, contributing to the pathology (Figure 1). During neuroinflammation, microglial cells resident in the brain are of great importance since they are able to synthesize inflammatory cytokines and chemokines which promote astrocyte activation and induce increased recruitment of peripheral immune cells. (Waisman, Liblau, & Becher, 2015)

However, prolonged and uncontrolled inflammation can lead to neuronal damage or death. This occurs in the case of chronic inflammation, which is characterized by the persistence of active inflammation and the infiltration of mononuclear immune cells such as macrophages, monocytes, lymphocytes and plasma cells. These cells, or their secreted products such as cytokines and cytotoxic products can contribute to tissue destruction and fibrosis under pathological condition. (Waisman et al., 2015)

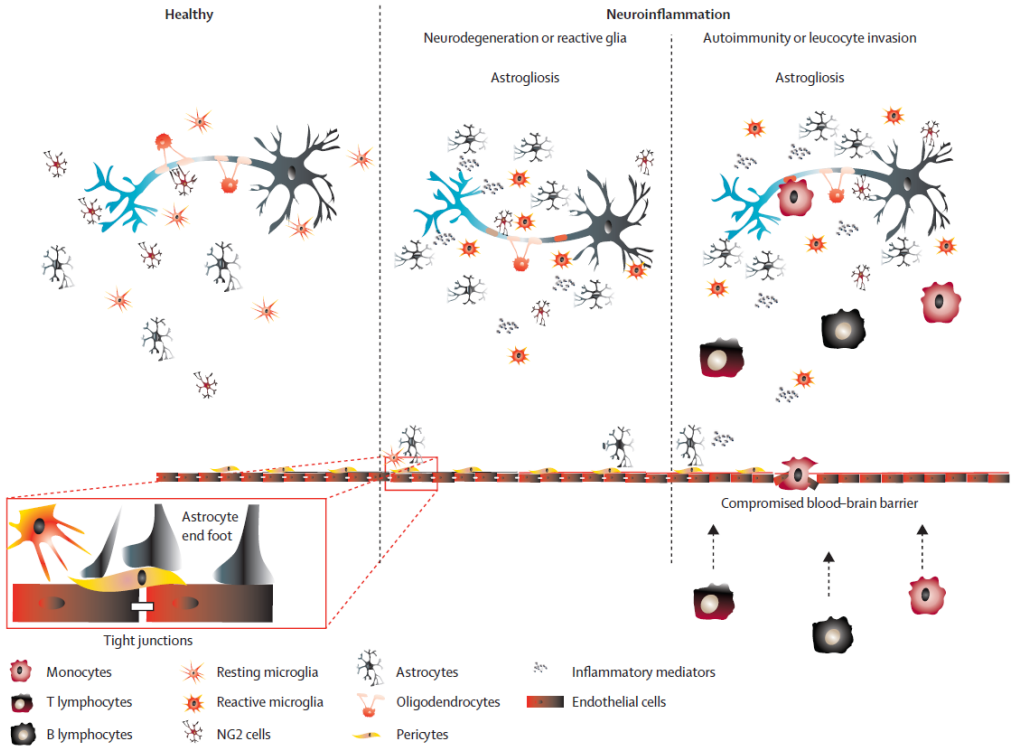

Figure 1| *Involvement of various cell types in CNS inflammation. In almost every non-physiological disorder, the CNS-resident astroglia and microglia become activated, proliferate, and change to have an inflammatory expression signature. In some inflammatory diseases of the CNS, myeloid cells cross the neurovascular unit and invade the CNS. Lymphocytes with recombined antigen receptors (T cells and B cells) can also penetrate the CNS and target CNS-resident antigens in many inflammatory diseases. (Waisman et al., 2015)*

Inflammation and epilepsy

Seizures and epilepsy manifest as changes in the CNS, although the mechanisms that induce them are not yet fully understood. It is speculated that the onset of seizures may be the result of increased neuronal excitation, lack of inhibition, or even both. These conditions, which lead to activation stages, are important for the generation of convulsive processes, and most likely for epileptogenesis. (Velasco-Ramirez, Rosales-Rivera, Ramirez-Anguiano, & Bitzer-Quintero, 2013)

In epilepsy, several pathogenic mechanisms leading to epileptogenesis and its progression have been associated with pro-inflammatory cytokines as predisposing factors. A few hours after the onset seizure activity, inflammatory processes have been observed in the brain, with the consequent release of proinflammatory cytokines, such as INF-γ, IL-1β, TNF-α and IL-6. These cytokines are expressed at basal levels under physiological conditions within the healthy brain, and after a wide variety of stimuli are rapidly induced and overexpressed. These findings suggest that there is an interaction between epilepsy and the immune system. (Velasco-Ramirez et al., 2013)

Microglia and astrocytes are the first cells to synthesize and release proinflammatory cytokines during convulsive process. As such, these cells represent the main source of proinflammatory molecules TNF-α and IL-1β, which are the most commonly expressed cytokines during seizures. During seizures, the receptors for these cytokines are rapidly overregulated in some groups of neurons, suggesting that the effect of cytokines is playing a mediating role in neuronal excitability. (Velasco-Ramirez et al., 2013)

When seizures are induced in experimental models, it is possible to see how the glia is activated, generating an epileptogenic focus, which, at the same time, induces the release of large amounts of TNF-α and IL-1β. The second one is considered an important mediator of changes in neuronal excitability. IL-1β released by microglia seems to increase the rate of neuronal excitability through an activating effect on astrocytes, altering the balance that controls glutamate homeostasis in synaptic junctions. Furthermore, it can also exhibit a direct effect on excitability and neuronal ion channels, as well as deteriorate neurotransmission systems dependent on GABA neurotransmitter. Besides TNF-α and IL-1β, other cytokines, such as IL-2, IL-10, fibroblast growth factor (FGF), IL-3, IL-8 and interferon gamma (IFN-γ), also are involved in seizure generation and epileptogenic processes. (Velasco-Ramirez et al., 2013)

Rasmussen's encephalitis

Rasmussen's encephalitis (RE) is a rare chronic neurological disorder, characterised by unilateral inflammation of the cerebral cortex, drug-resistant epilepsy, and progressive neurological and cognitive deterioration. Neuropathological and immunological studies support the notion that Rasmussen's encephalitis is probably driven by a T cell response to one or more antigenic epitopes, with potential additional contribution by autoantibodies. In addition, analysis of the association between histopathology and clinical presentation indicates that initial brain damage is mediated by T cells and microglia, suggesting a window for treatment if Rasmussen's encephalitis can be diagnosed early. (Varadkar et al., 2014)

Rasmussen's encephalitis is a rare disorder that mainly affects children or young adults. The acute stage is characterized by the presence of frequent seizures that arise from

one cerebral hemisphere. As the disease progresses, focal seizures appear as the main symptom, suggesting that there are inflamed areas in the brain. If children are not treated they may develop hemiparesis, hemianopia and cognitive impairment within one year of the onset of epilepsy. In addition, if the dominant hemisphere of language is affected they can suffer from dysphasia. Finally, children will undergo a residual stage in which severe neurological deficit, motor and cognitive problems, along with recurrent and difficult to treat epileptic episodes will predominate. (Varadkar et al., 2014)

Histopathologically, RE is characterized mainly by the presence of progressive cortical inflammation, neuronal loss and gliosis in one hemisphere of the brain. Frequently, microglial and lymphocytic nodules can be found, as well as severe neuronal death. All this leads to the belief that it may be an immune-mediated disease, associated with both an adaptive response, because of the presence of T lymphocytes, as well as innate immunity facilitated by microglia and astroglia. However, up to this day, the origin of RE remains unknown. (Varadkar et al., 2014)

Rasmussen encephalitis is considered as a heterogeneous pathology. Any region of the brain can be damaged but currently, thanks to radiological studies, it has been shown that there is a predilection for the fronto-insular area. The occipital cortex is not commonly affected. When it is, patients tend to be younger and with a greater burden of disease. (Varadkar et al., 2014)

During the course of the disease, increased infiltration of T lymphocytes, astrogliosis and microglial activation reactions in the cortex are observed. According to this, Pardo et al. defined 5 stages based on pathological changes in RE (see Table 1). Stage 0 is considered a normal cerebral cortex, without neuronal loss and absence of reactive astrogliosis, microgliosis or lymphocyte infiltration. Stage 1, or early stage, is defined by focal infiltration of lymphocytes into the tissue, although no significant neuronal loss is observed. In stage 2 or intermediate stage, besides moderate to severe cortical neurodegeneration, there is a notable increase in the concentration of T lymphocytes. Furthermore, there is activation of microglia and astroglia, indicated by a notable increase in their cytoplasmic volume. In stage 3, also called late stage, there is a

significant decrease in the neuronal population. Moreover, as in the previous stage, the microglia and astroglia continue showing activation. However, remarkably, the presence of lymphocytes is lower than in the intermediate stage. The final stage or stage 4 is characterized by extensive destruction of the cerebral cortex and absence of T cells. (Pardo et al., 2004)

Table 1| *Staging of cortical pathology in Rasmussen encephalitis. (Pardo et al., 2004)*

Stages	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4
Definition	Normal cortex	Early stage	Intermediate stage	Late stage	End stage
Cerebral cortex	Normal	Mild focal inflammation and gliosis	Panlaminar cortical inflammation and gliosis	Panlaminar cortical degeneration and gliosis	Panlaminar cortical cavitation and/or gliosis
Neuronal loss	Absent	Minimal, focal	Moderate to severe, multifocal	Severe, panlaminar	Severe, rare neurons
Astrogliosis	Absent	Mild to moderate, focal	Marked, frequently panlaminar, gemistocytes	Marked, panlaminar, gemistocytes	Variable
Microglial activation	Absent	Mild to moderate, focal	Marked, panlaminar	Variable	Variable
T-cell infiltration	Absent	Mild to moderate, few T-lymphocyte clusters and perivascular cuffs	Marked, panlaminar or multifocal, frequent perivascular cuffs	Minimal	Rare

Until the cause of Rasmussen's encephalitis is known, it is difficult to anticipate how the treatments will improve. Currently, medications are aimed at reducing the severity and frequency of seizures and improving long-term functional outcome. To date, however, treatments have only relieved symptoms and have not addressed the underlying causes. Therefore, surgery is still the only cure for seizures despite having functional consequences, since it consists of completely disconnecting the affected hemisphere. For this reason, in the last decade there have been several attempts to generate new treatments using immunotherapy. Some of them slow the progression of the disease, but none has been able to cure or even stop its course. (Varadkar et al., 2014)

Innate immunity in Rasmussen's encephalitis

Innate immunity represents a nonspecific immediate host response against invading pathogens. The innate immune system components consist of cells such as; natural killer cells and granulocytes, macrophages, microglia and dendritic cells. These cells can produce a variety of inflammatory molecules and express specific receptors such as Toll-like receptors (TLRs), which represent the first line of defence against invading pathogens or virulence. (Vezzani et al., 2011)

Increasing evidence in epilepsy animal models has shown a prominent role of glial cells in the biosynthesis and release of the inflammatory molecules. These cells play the role

of intrinsic innate immunity cells of the brain in concert with extravasated macrophages and granulocytes. In particular, epileptogenic brain injuries or convulsive events rapidly activate microglia and astrocytes in the brain regions affected by the pathologic event. (Vezzani, Lang, & Aronica, 2016)

Microglia in Rasmussen's encephalitis

Microglia are the resident innate immune cells of the CNS and are responsible for normal maintenance of CNS tissue, they constitute the first cell population in responding to neuroinflammation and initiating an inflammatory response. Under homeostatic conditions, microglia plays a preventive role, monitoring the cellular microenvironment in order to detect lesions or infections. Consequently, if tissue homeostasis is altered, the functional phenotype of the microglia changes from "resting" to "activated". Once activated, microglia undergoes morphological changes, as well as alterations in gene expression. (Thompson & Tsirka, 2017) By using a broad array of immune receptors, they are able to recognize harmful stimuli and respond by producing inflammatory cytokines such as IL-1β, TNF-α and several other chemokines. (Cherry, Olschowka, & O'Banion, 2014) Microglia can act as an antigen presenting cell (APC), which is able to communicate with the adaptive immune system within the CNS. In RE, the presentation of the antigen by MHC, and the secretion of co-stimulatory molecules, by the microglia can promote the activation of T cells against CNS antigens, thus contributing to the generation of tissue damage. (Thompson & Tsirka, 2017)

Examining the role of the microglia in degenerative autoimmune disorders is of great interest since their pathogenic role is not yet fully understood, although reactive microgliosis is a well-founded hallmark of RE. In a study carried out by Wirenfeldt et al. immunohistochemical tests were performed on samples of RE, showing that there was extensive microglial activation in RE samples, with several different morphologies, indicating high reactivity. (Thompson & Tsirka, 2017)

The involvement of the microglia in epilepsy may differ depending on the aetiology of the disease. However, it could be speculated that microglia assumes a similar role in RE as in epilepsy, since epileptic seizures are characteristic of this disease. Despite the fact that pro-inflammatory mediators released by microglia are well known for being

beneficial in combating infection or tumour growth, they can also cause secondary neuronal damage. For example, Vezzani et al., by inducing seizures in rats, proved that this excitotoxic stimulation activated the microglia for the production of IL-1 β. IL-1 β is known for being a pro-inflammatory cytokine that accentuates induced seizures and, indirectly might contribute to neuronal loss. (Thompson & Tsirka, 2017)

Inflammasome in Rasmussen's encephalitis

Inflammation in the nervous system occurs in numerous neurological disorders with various pathological causes and phenotypes. Recently, the relationship between innate immunity and its contribution to neurological diseases has attracted strong interest, in particular the involvement of inflammasome activation. Inflammasomes form a molecular mechanism that is activated in case of cell infection or stress. As a result of their activation, they promote the maturation of pro-inflammatory cytokines, such as interleukin-1β, displaying an innate immune response. (Ramaswamy et al., 2013)

In the CNS, pattern-recognition receptors expressed by microglia, macrophages, and astrocytes are involved in the generation of the inflammasome. These receptors can be bounded to the cytoplasmic membrane and detect extracellular signals, known as Toll-like receptors (TLRs), or they can be located in the cytoplasm and therefore detect intracellular signals, in this case they are known as NOD-like receptors (NLRs). (Walsh, Muruve, & Power, 2014)

These NLR receptor cells are recruited to the site of injury or inflammation within the CNS. This represents one of the initial signature events during neuroinflammation and a hallmark of pathogenesis in neurodegenerative diseases.(Freeman & Ting, 2016) Thus, activation of the inflammasome, with the release of inflammatory mediators, can play a crucial role in the development of epilepsy after brain injury.

In RE, Ramaswamy et al. revealed a concurrent induction of inflammasome components in both the white matter as well as the cortex, revealing that microglia and macrophages are the main cells that exhibit the inflammasome cellular machinery.(Ramaswamy et al., 2013) As the principal role of the inflammasome is to mediate the release of proinflammatory cytokines, it represents a critical intermediary

in the generation of neuroinflammation and a potential therapeutic target for neurodegenerative diseases.(Freeman & Ting, 2016)

Adaptive immunity in Rasmussen's encephalitis

The adaptive or acquired immunity is the part of the immune system that prepares the body for current and future challenges from pathogens.(Vezzani et al., 2016) It is activated in response to innate immunity and enables the host to recognize and remember specific non-self-antigens to mount humoral or cell-mediated immune responses by B and T lymphocytes, respectively.(Vezzani et al., 2011)

Antigen-presenting cells or APCs, as dendritic cells, macrophages and B cells, and brain-resident microglia, stimulate naive T cells to become effector cells. When the antigen is presented, clonal selection and expansion of the lymphocytes occurs. However, adaptive immunity can also react against autoantigens when it deregulates and loses tolerance to self-antigens, leading to autoimmunity.(Vezzani et al., 2011)

The first epilepsy syndrome in which the autoimmune system was thought to play a significant pathological role was RE. Autoantibodies have been observed in the affected tissue of RE suggesting activation of the adaptive immune system, which may be a side effect after cytotoxic T lymphocytes intervened in the brain. (Vezzani et al., 2016)

Role of T cells in Rasmussen's encephalitis

It is believed that autoimmunity may be the cause of a high number of neurological disorders, such as RE. Although the mechanism that triggers autoimmunity is not yet known, it is clear that deregulation in the T cell population may be a key component because of its constitutive role in immune-vigilance. (Pilli, Zou, Tea, Dale, & Brilot, 2017) That is why in this section the main populations of T cells that are believed to play an important role in Rasmussen's encephalitis will be discussed.

T lymphocytes, mainly cytotoxic CD8+ T cells, are found in areas of neuronal destruction, indicating an active role of these cells in the pathogenic process of RE. In a study carried out by Bien et al. an immunohistochemical evaluation of 11 RE brain samples was performed, it was found that CD8+ T cells may be responsible for triggering neuronal death. These active CD8+ T cells were found to be positioned on the

periphery of neurons and astrocytes. Not only that, but 7% of them presented granzyme B, a protease whose main function is to induce cell death, contained in cytotoxic granules. Therefore, CD8+ T lymphocytes can be found lying adjacent to neurons and astrocytes and polarizing these cytotoxic granules towards them.(Bauer, Bien, & Lassmann, 2002) (Pilli et al., 2017)

Related to this in an investigation carried out by Neumann et al. they studied the repertoire of T cell receptors (TCR) in the CNS in patients with RE. As a result it was revealed that there was presence of clonal expansion of CD8+ T cells, indicating that there is an antigen-specific T cell response, although the epitope of the disease has not been elucidated yet. (Neumann, Medana, Bauer, & Lassmann, 2002)(Pilli et al., 2017) Also in a study performed in 2016 by Schneider-Hohendorf et al. 23 brain, blood and cerebrospinal fluid (CSF) samples from patients with RE were evaluated. In this study, CD8+ T lymphocytes with the same dominant clone were found in the brain and peripheral blood of patients with RE. This finding has important implications, one of which is the confirmation that the effector lymphocytes responsible for brain destruction must migrate from the periphery to the parenchyma, where they cause the damage. Supporting the suggestion that, in RE, CD8+ T lymphocytes clonally expand in the periphery and constitute a specific antigen attack within the CNS. (Thompson & Tsirka, 2017)

Although immunopathology in RE appears to be driven by cytotoxic T cells, it is believed that CD4+ cells can play a crucial role in supporting CD8+ T cell activity. That is why this cell population requires special attention in RE, and in particular the IFN-γ producing lymphocytes.(Owens et al., 2013) These lymphocytes can be recognised by their expression of T-bet. T-bet is one of the Th1-lineage-specifying transcription factors and plays an important role in the production of IFN-γ. (Zhang et al., 2018)
According to Owens et al. IFN-γ can induce the expression of class I histocompatibility complex (MHC-I) molecules on the surface of neurons, making them more vulnerable to cytotoxic T cell attack. It has been reported that IFN-γ stimulates the production of various chemokines, such as CXCL9 by the microglia, CCL5 by the astrocytes and CXCL10 by both of them. Chemokine CCL5 produced by astrocytes can indirectly attract further

T cells, such as γδ T cells or NK lymphocytes at the areas of inflammation. (Owens et al., 2013)

γδ T cells in Rasmussen's encephalitis

Gamma-delta (γδ) T cells are the prototype of "unconventional" T cells and represent a small population of T cells, about 2-5% of peripheral lymphocytes. They are characterized by the expression of the heterodimeric T cell receptor (TCR) composed of the chains γ and δ (γδTCR). As a result, they are less represented compared to CD4+ helper T cells and CD8+ cytotoxic T cells, as they express the chains α and ß in their TCRs. (Hayday, n.d.) γδ T cells play an important role in innate and adaptive immunity, as well as being involved in various models of autoimmune diseases, bacteria, viral and fungi infections, and even ischemic brain injury. For instance, γδ T lymphocytes are overrepresented (up to 20%-30% of the total number of T cells) in T lymphocytes that infiltrate in early lesions in multiple sclerosis patients. (Hou, Wang, & Sun, 2015).

It is thought that γδ T cells are an important subset of T lymphocytes as they have the ability to recognize a broad range of antigens without the presence of major histocompatibility complex (MHC) molecules. They can attack target cells directly through their cytotoxic activity or indirectly through the activation of other immune cells. γδ T cell functional responses are induced upon the recognition of stress antigens, which promotes cytokine production and regulates pathogen clearance, inflammation, and tissue homeostasis in response to stress. (Lawand, Déchanet-Merville, & Dieu-Nosjean, 2017)

γδ T cells have been described as polyfunctional; which means that they can produce a wide array of cytokines, including IL-17A, IL-17F, IFN-γ, IL-10, IL-22, TNF-α... (Edwards, McGinley, McGuinness, & Mills, 2015) Over the years, they have been demonstrated to be the major early source of IL-17α. In contrast to CD4+ Th17 cells, γδ Th17 cells can immediately respond to a variety of stimuli including IL-23 and IL-1β. (Hou et al., 2015) Although γδ T cells do express a unique T cell receptor (TCR), engagement of this TCR with MHC-antigen complexes is not a prerequisite for their activation. Unlike conventional αβ T cells, cytokine stimulation alone is sufficient for activation of IL-17-

secreting γδ T cells, making these cells rapid and potent mediators of inflammation. (Edwards et al., 2015)

The cause of clinical symptomatology in Rasmussen's encephalitis appears to be an inflammatory response involving T cells and activated microglia. Yet it is not known what triggers this immune response. Several types of Herpesviridae have been detected in surgical brain samples from patients with RE; however, to date, there is no consistent evidence that it is a common pathogen in all cases of RE. Autoantibodies have also been described in cases of RE pointing to an autoimmune disease, but have not been found in all cases and, are not specific for RE. (Owens et al., 2015)

The presence of T cells in one brain hemisphere suggests that the initial inflammatory reaction may have been spatially restricted. CD8+ T cells positive for granzyme B have been observed in the brain parenchyma surrounding neurons and astrocytes. Thus, cytotoxic T cells are likely to react to foreign or self-antigens presented by neurons and astrocytes in the affected brain hemisphere. Consequently, brain-resident macrophages trigger a focused innate immune response, promoting the recruitment of non-resident immune cells unrestricted by MHCs, such as natural killer cells and γδ T cells. (Owens et al., 2015)

The presence of γδ T cells is a new finding and has implications for the immune response in RE. This cellular immune response involves both; classical αβ T cells and non-classical γδ T cells. The γδ T cells appear to be clonally restricted, and prevalent clones may recognize a common antigen, possibly a self-antigen associated with stressed cells. γδ T cells may facilitate the activation of autoreactive αβ T cells by releasing pro-inflammatory cytokines and promoting antigen presentation. Furthermore, γδ T cells may provide a link between inflammation in the brain and an adaptive immune response involving antigen-specific CD8+ T cells. On the other hand, they can act as an adaptive immune cell and bind a cognate antigen as well as produce IFN-γ. (Owens et al., 2015)

HYPOTHESIS

The neuropathological studies performed in Rasmussen's encephalitis have shown the variability and heterogeneity of the pathological changes in this disorder. The most characteristic signs of the pathology are microglia activation, early death of glia and neurons and infiltration of diverse populations of T lymphocytes (Varadkar et al., 2014).

It is believed that autoimmunity may be the cause of RE, because of its constitutive role in immune surveillance it is suggested that deregulation in the T cell population may be a key component of the pathogenesis of RE. (Pilli et al., 2017)

γδ T lymphocytes are a subtype of total peripheral T cells, regardless their low representation they constitute a very interesting population in the study of RE. γδ T cells recognize antigens without depending on antigen presentation by classical MHC molecules. Unlike conventional αβ T cells, cytokine stimulation it is sufficient for activation of γδ T cells, making these cells rapid and potent mediators of inflammation. (Edwards et al., 2015)

Since they are able to react to inflammation more quickly than αβ T lymphocytes, they can be expected to be overrepresented in the early stages of the disease. These facts led us to hypothesize that γδ T lymphocytes may have a special role in the first stages of RE.

In RE, γδ T cells can support the activation of autoreactive T cells by releasing pro-inflammatory cytokines and promoting antigen presentation. One such cytokine is IFN-γ, which can induce the expression MHC-I molecules on the surface of neurons, making them more sensitive to cytotoxic attack of T cells. Based on this, it is aimed to evaluate whether γδ T cells are capable of producing IFN-γ, thus attracting further cytotoxic T cells, γδ T cells and NK lymphocytes in the areas of inflammation. (Owens et al., 2013)

Therefore the hypotheses proposed in this thesis are the following:

- γδ T cells are a specific population of early stages in Rasmussen encephalitis; this population is antigen specific and capable of destruction of neurons.
- γδ T cells release interferon-γ, which plays a role in the innate immune response in Rasmussen encephalitis.

OBJECTIVES

The objectives of this these thesis are outlined below:

- γδ T cells are present in early stages in Rasmussen encephalitis.
- γδ T cells are cytotoxic.
- There is a relation between γδ T cells and neurons.
- Interferon-γ producing cells are present in Rasmussen tissue, as well as in microglial nodules.
- γδ T cells are capable of producing interferon-γ in RE.

MATERIALS AND METHODS

Study Samples

In this study formalin-fixed, paraffin-embedded (FFPE) human biopsy brain tissue of patients suffering from Rasmussen encephalitis (RE) (n=18) were used, as well as FFPE human autopsy and biopsy brain tissue controls (C) (n=19) (see Table 2). Both of them were provided by the Bethel Epilepsy Center in Bielefeld-Bethel (Germany).

Patients were from both genders, male (m) and female (f). The age of surgery between the patients was variable. The stage of the disease was determined in each patient prior to start with any experiment. Three different groups were defined (stage 0, 1 and 2), according to Pardo et al., based on immunohistochemical (IHC)-staining method using the following markers: CD68 (microglia marker), CD3 (T cells marker) and NeuN (neuronal marker).

Table 2| *Samples of RE and control patients used in this study.*

CASE	DISEASE	STAGE	GENDER	AGE OF OPERATION	AFFECTED AREA
395-99-f	Control	-	M	4.85	Temporal cortex
826-06-b	Control	-	M	40.21	Temporal cortex
873-04-I2	Control	-	F	-	-
1241-07-IIa2	Control	-	M	9.14	Temporal cortex
1251-98-I1	Control	-	F	4.04	-
1641-05-IIa2a	Control	-	M	8.97	Temporal cortex
1737-01-b	Control	-	F	-	-
221-02-f	RE	0	M	4,45	Temporal cortex
57-03-I3	RE	0	F	15,9	Cortex hemispherectomy
834-03-5a	RE	0	M	4,45	Temporal cortex
247-05-I2	RE	0	M	6,89	Temporal cortex
143-12-III4	RE	0	F	8,1	Cortex hemispherectomy
1307-12-I4	RE	0	M	9,3	Temporal cortex
578-97-I5a	RE	1	M	18,05	Frontal cortex
1762-00-I3	RE	1	F	24,58	Frontal cortex
247-05-I7	RE	1	M	6,89	Temporal cortex
876-12-I10	RE	1	M	9,13	Cortex hemispherectomy
128-14-I3	RE	1	F	8,3	-
144-14	RE	1	M	10,6	Cortex hemispherectomy
1186-90-4a	RE	2	M	4,48	Cortex hemispherectomy
1385-92-I2a	RE	2	F	5,62	Cortex hemispherectomy
1470-95-I	RE	2	F	29,3	Extratemporal/MST
1394-02-2a	RE	2	M	33,75	Frontal cortex
1892-10-7	RE	2	M	29,3	Cortex hemispherectomy
899-13-I2	RE	2	F	5,19	Temporal cortex

Immunohistochemistry - ABC System with peroxidase activity for detection

Materials

- 3-5 µm thick FFPE sections mounted on normal glass slides.
- Xylene (Avator Materials; Ref. 3410)
- Ethanol (EtOH) 96%, 70% and 50% (Brenntag CEE GmbH, Ref. # 442269)
- TBS-buffer:
    - TBS-stock solution pH 7.5 (1 L): 60.57g of 25mM Tris buffer, 180 g of 150 mM NaCl and 400 ml of 1N HCl were dissolved in deionized water (a.d). The pH was adjusted to 7.5 by adding 1 N HCl.
    - TBS working solution: the stock solution was diluted 1:20. 50 ml of stock buffer were diluted in 950 ml of a.d.
- PBS-buffer:
    - PBS-stock solution pH 7.4 (2.5 L): 13.8 g of 0.04 M NaH_2PO_4, 71.2 g of 0.16 M Na_2HPO_4 and 90 g of NaCl were dissolved in a.d. The pH was adjusted to 7.4.
    - PBS working solution: the stock solution was diluted 1: 4. 250ml of stock solution were diluted in 750ml of a.d.
- H_2O_2 /Methanol: 150 mL of Methanol and 1 ml H_2O_2 (30%) were mixed.
- 10% FCS/DAKO-Buffer: the commercial DAKO-buffer solution from Dako corporation 10x (# S3006) was diluted 1:10 with deionized water. For dilution of primary, secondary antibodies and Avidin Peroxidase a solution with 10% FCS in DAKO-buffer was made.
- EDTA-buffer:
    - EDTA 20x stock solution: 1.21 g of 10 mM Tris-buffer and 0.37g of 1 mM EDTA were dissolved in 50ml of a.d. The pH was adjusted to 8.5 or 9.0 depending on fixation of material.
    - EDTA working solution: 2.5 ml of stock solution was diluted in 50 ml of a.d.
- Peroxy conjugated Streptavidin 1:500 (Jackson Immunoresearch; # 016-030-08)

- DAB (3.3'-Diaminobenzidine): 1 ml of DAB (K3467 DAKO®) stock solution was mixed in 50 ml of PBS. The mixture was filtered with a paper filter into a jar and 16.5 µl of H_2O_2 (Merck Millipore) was added.
- Mayers Hemalaun (Merck # 1.09249): Mayer's Hematoxylin (Merck®)
- HCl-ethanol: 100 ml of 70% ethanol was mixed with 0.5 ml of concentrated HCl (37%).
- Scott's solution: 2 g of $KHCO_3$ and 20g of $MgSO_4$x 7H2O were dissolved in 1.000 ml of H_2O.
- Coverslips (Carl Roth, H8762)
- Eukitt (Kindler; R1339)
- Primary antibodies

Table 3| *Primary antibodies*

ANTIBODY	TYPE OF STAINING	ANTIBODY TYPE	TARGET	PRETREATMENT	DILUTION
γδ T cell receptor (γδTCR)	Single	Mouse monoclonal Ab	γδTCR	EDTA pH=9.0 (1h steamer)+ CSA	1:200
	Double			Citrate (1h steamer)+ CSA+ EDTA pH=8.5 (30 min steamer)	1:150
T-Bet	Single	Mouse monoclonal Ab		EDTA pH=8.5 (1h steamer)+ CSA	1:300
CD3	Single	Rabbit polyclonal Ab	Lymphocytes T	EDTA pH=9.0 (1h steamer)+ CSA	1:1000
Neu-N	Double	Mouse monoclonal Ab	DNA-binding neuron-specific protein NeuN	Citrate (1h steamer)+ CSA+ EDTA pH=8.5 (30 min steamer)	1:10000

- Secondary antibodies

Table 4| *Secondary antibodies*

ANTIBODY	TARGET	DILUTION
Bi-donkey-α-mouse	IgG (H+L)	1:500
Bi-donkey-α-rabbit	IgG (H+L)	1:1000

Method

The FFPE tissue was cut into 3-5 µm thick sections. For immunohistochemical staining the samples were deparaffinised two times for 15 minutes in xylene and rinsed twice in 96% ethanol. Next, endogenous peroxidase activity was blocked by 30 minutes incubation in H_2O_2-Methanol. Rehydration was performed by immersing the slides shortly (1min) in 96%, 70%, 50% ethanol and a.d. Antigen retrieval was done by heating the slides in EDTA (pH 8.5 or 9.0) or citrate-buffer (pH 6.0) in a household food steamer device (Gourmet FS20; Braun, Kronberg Taunus, Germany) for one hour. Depending on the antibody, the appropriate pretreatment solution was chosen (see Table 3). Next, the slides were cooled down at room temperature and were washed from 3 to 5 times with TBS buffer. Afterwards, unspecific background reactions were blocked by incubating the samples with FCS/DAKO buffer for 15-20 minutes in a humid chamber at room temperature. After the blocking step, the sections were incubated at 4°C overnight with the primary antibody diluted in 10% FCS/DAKO buffer in a humid chamber.

On the next day, the slides were rinsed in TBS for 3 to 5 times followed by 1h incubation with the biotinylated secondary antibody diluted in 10% FCS/DAKO buffer at room temperature in a humid chamber (Table 4). The samples were washed with TBS for 3-5 times followed by 1h incubation with peroxidase-conjugated streptavidin (1:500) diluted in 10% FCS/DAKO buffer in a humid chamber. The slides were developed under microscopic control in DAB until the colour reaction was satisfactory. The enzymatic reaction was stopped by changing the samples from DAB to deionized H_2O. Counterstaining was done by incubating the slides for 15-20 seconds in Mayer's haematoxylin, washed two times with H_2O and differentiated three times in HCl-ethanol. Next, slides were washed two times in H_2O, incubated 4 minutes in Scott's solution and rinsed with H_2O. Following, the slides were dehydrated through graded ascending ethanol steps (50%, 70%, 95%, 95% and 95%) ending with N-butyl acetate. At the end, the samples were mounted with coverslips using Eukitt® for embedding. Examinations under the microscope were performed the next day when the slides were dry.

Quantitative Analysis for light microscopy staining

Analysis of immunohistochemically stained sections was done by using Image-Pro software. The samples were first analysed by light microscopy to ensure that the staining was optimal, and then the samples were scanned. The area to be analysed was selected using NanoZoomer Digital Pathology (NDP) software, from each sample, 10 images were taken to a 10x amplification. Automatic counting was performed by using Image-Pro software in order to quantify the number of positive cells in the investigated sections. For statistical analysis GraphPad Prism 6 was used to process and interpret the data. For comparison of control cases and pooled RE cases Mann-Whitney U-test was used and Kruskal-Wallis test with post-hoc Dunn's multiple comparisons test was used to compare control cases and each stage of RE. In all the cases p-values below 0,05 were considered to be significant.

Confocal Laser Fluorescence Microscopy on FFPE Tissue Materials

- 3-5 µm thick FFPE sections mounted on normal glass slides.
- Xylene (Avator Materials; Ref. 3410)
- Ethanol (EtOH) 96%, 70% and 50% (Brenntag CEE GmbH, #442269)
- TBS-buffer:
    - TBS-stock solution pH 7.5 (1 L): 60.57 g of 25 mM Tris buffer, 180 g of 150 mM NaCl and 400 ml of 1N HCl were dissolved in deionized water (a.d.). The pH was adjusted to 7.5 by adding 1N HCl.
    - TBS working solution: the stock solution was diluted 1:20. 50 ml of stock buffer were diluted in 950 ml of a.d.
- PBS-buffer:
    - PBS-stock solution pH 7.4 (2.5L): 13.8 g of 0.04 M NaH_2PO_4, 71.2g of 0.16M Na_2HPO_4 and 90 g of NaCl were dissolved in a.d. The pH was adjusted to 7.4.
    - PBS working solution: the stock solution was diluted 1:4 250 ml of stock solution were diluted in 750 ml of a.d.
- 10% FCS/DAKO-Buffer: the commercial DAKO-buffer solution from Dako corporation 10x (#S3006) was diluted 1:10 with deionized water. For dilution of

primary, secondary antibodies and Avidin Peroxidase a solution with 10% FCS in DAKO-buffer was made.

- DAKO REALTM Antibody Diluent (code S2022).
- EDTA-buffer:
    - EDTA 20x stock solution: 1.21 g of 10 mM Tris-buffer and 0.37 g of I mM EDTA were dissolved in 50 ml of a.d. The pH was adjusted to 8.5 or 9.0 depending on fixation of material.
    - EDTA working solution: 2.5 ml of stock solution was diluted in 50 ml of a.d
- Citrate-buffer pH 6.0: 2.10g of citric acid is dissolved in 1 L at a.d.
- Catalysed signal amplification system (CSA):
    - Biotinylated tyramine stock (CSA-stock): 6 ml of borate buffer (pH 8) (Merck®) was mixed with 15 ml of sulpho-NHS-LCS-Biotin (Pierce, Illinois, USA) and 4.5 mg of tyramine (Sigma-Aldrich®). The mixture was left overnight at room temperature for stirring and after it was filtered and stored in small aliquots at -20°C.
    - CSA working solution: 10 µl, 20 µl or 50 µl of CSA aliquot (1:1500) and 16.5 µl of H_2O_2 were mixed with 10 ml, 30 ml or 75 ml of PBS. Both, CSA and PBS volumes were chosen according to the amount of slides.
- Streptavidin conjugated Cy2 (1:100) (Jackson ImmunoResearch; #016-220-084)
- ProlongTM Gold Antifade Mountant (Thermo Fisher Scientific P36930)
- Coverslips (Carl Roth; H8762)
- Confocal LEICA SP5 DMI 6000 CS laser scan microscope (Mannheim, Germany)
- Primary antibodies

Table 5| *Primary antibodies*

ANTIBODY	TYPE OF STAINING	ANTIBODY TYPE	TARGET	PRETREATMENT	DILUTION
γδ T cell receptor (γδTCR)	Double Triple	Mouse monoclonal Ab	γδTCR	EDTA pH=8.5 (1h steamer)+ CSA Citrate (30 min steamer)+ EDTA pH=8.5 (45 min steamer)	1:1000 1:50
CD3	Double	Rabbit	Lymphocytes T	EDTA pH=8.5	1:250

		polyclonal Ab		(1h steamer)	
Neu-N	Triple	Mouse monoclonal Ab	DNA-binding neuron-specific protein NeuN	Citrate (30 min steamer)+ CSA+ EDTA pH=8.5 (45 min steamer)	1:10000
Granzyme B (GrB)	Triple	Rabbit polyclonal Ab	GrB	Citrate (30 min steamer)+ EDTA pH=8.5 (45 min steamer)	1:25

- Secondary antibodies

Table 6 | *Secondary antibodies*

ANTIBODY	TYPE	DILUTION
Bi-donkey-α-mouse	IgG (H+L)	1:500
Cy3 donkey-α-rabbit	IgG (H+L)	1:200
Cy2 donkey-α-mouse	IgG (H+L)	1:100

Method for double and triple labelling with antibodies from different species

The FFPE tissue was cut into 3-5 µm thick sections. For fluorescence microscopy, the samples were deparaffinised two times for 15 minutes in xylene and rinsed in two changes of 96% ethanol. Rehydration was performed by immersing the slides shortly (1 min) in 96%, 70%, 50% ethanol and a.d. Next, endogenous peroxidase activity was blocked by 30 minutes incubation in H_2O_2-Methanol. Antigen retrieval was done by heating the slides in EDTA (pH 8.5) or Citrate (pH 6.0) in a household food steamer device (MultiGourmet FS20; Braun, Kronberg/Taunus, Germany) for one hour. Next, the slides were cooled down to room temperature and were washed 3-5 times with TBS buffer. Afterwards, unspecific background reactions were blocked by incubating the samples with 10% FCS/DAKO diluent for 15 minutes in a humid chamber at room temperature. After blocking, the sections were incubated at 4°C overnight with the primary antibody in 10% FCS/DAKO diluent in a humid chamber (Table 5).

On the next day, the slides were rinsed in TBS for 3-5 times, after they were incubated with biotinylated secondary antibody diluted in 10% FCS/DAKO buffer for one hour at room temperature in a humid chamber (Table 6). The samples were washed with TBS for 3-5 times followed by one hour incubation with peroxidase-conjugated streptavidin

(1:500) at room temperature in a humid chamber, followed by washing step in TBS. The staining proceeds with a signal enhancement step with biotinylated tyramine. The slides were incubated for 15 minutes at room temperature in CSA working solution followed by washing 3-5 times in PBS. Afterwards the samples were incubated with peroxidase-conjugated streptavidin (1:500) in 10% FCS/DAKO buffer in a humid chamber for 30 minutes followed by the washing step in TBS. Antigen retrieval was done by heating the slides in EDTA (pH 8.5 or 9.0) in a household food steamer device (Gourmet FS20; Braun, Kronberg Taunus, Germany) for half an hour. Next, they were cooled down to room temperature and washed with TBS buffer. Then streptavidin-Cy5 (1:500) diluted in 10% FCS/DAKO buffer was applied for one hour at room temperature in a humid chamber protected from the sun. Following, the samples were rinsed in TBS and the primary antibody diluted in 10% FCS/DAKO was applied. The sections were incubated at 4°C overnight at in a humid chamber protected from the sun. In case of performing a triple staining, in this step, two primary antibodies diluted in 10% FCS/DAKO were applied simultaneously.

On the next day, after rinsing the samples with TBS one or two, depending if performing a double or triple stain, fluorescence conjugated secondary antibodies diluted with 10% FCS/DAKO buffer were applied for one hour at room temperature in a humid chamber protected from the light. Finally, the slides were washed with deionized H_2O and mounted with coverslips using ProlongTM Gold Antifade Mountant for protecting fluorescent dyes from fading (photo bleaching) during fluorescence microscopy experiments. Fluorescent preparations were examined using the confocal LEICA SP5 DMI 6000 CS laser scan microscope.

RESULTS

Analysis of CD3 T lymphocytes in Rasmussen Encephalitis

One of the objectives of this project was to study inflammation in Rasmussen's encephalitis, which was done by analysing the population of T lymphocytes using antibodies against all T cells (CD3).

The RE samples were classified in three different stages (0, 1 and 2) according to the ones established by Pardo et al. All the case studies, RE as well as control samples, were stained for CD3 following the standard single staining procedure for light microscopy. With the help of a light microscope, it was possible to notice few T lymphocytes within the control group, besides these cells were mostly found around blood vessels (Figure 2 A and B). In contrast to the RE samples, where CD3 positive cells were found infiltrated in parenchyma, especially in microglial nodules and in areas with neuronal damage (Figure 2 C-E).

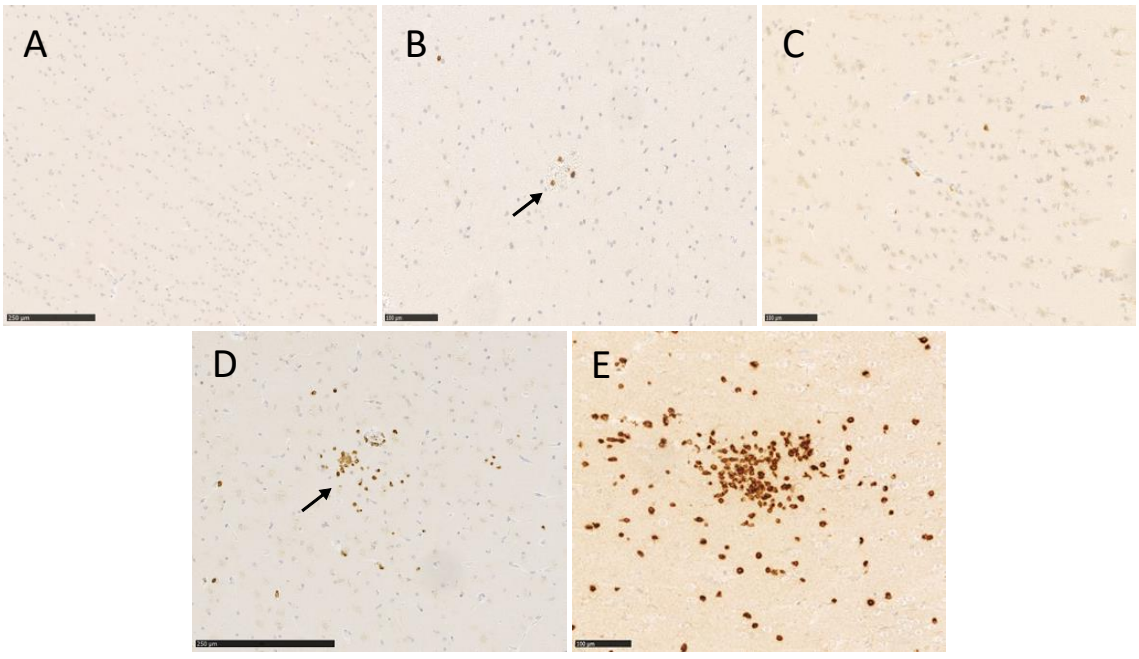

Figure 2| *Immunohistochemistry staining for CD3 in control samples and RE. (A) Negative control with no CD3 positive cells. (B) Negative control with CD3 positive cells in blood vessels. (C) Stage 0 RE sample with hardly any CD3 positive cells. (D) Stage 1 RE sample with CD3 positive cells forming a nodule. (E) Stage 2 RE sample with CD3 positive cells occupying a large area.*

The samples were then scanned and cellular quantification was performed by using ImagePro Premier Software. Once the results were obtained, they were statistically analysed with the program GraphPad Prism. Firstly, the relationship between the set of

RE cases and the control group was studied. By performing a Mann Whitney test, it was found that the two groups were significantly different (Figure 3 A). Data on CD3 T-lymphocyte concentration between stages 0, 1 and 2 and control was then analysed by doing a Kruskal-Wallis test. As a result a p-value of 0.0001 was obtained, according to which it can be said that there is significant difference between the four groups, but specifically between the stage 1 and 2 group and the control group (Figure 3 B). This proves that there is increased CD3 lymphocyte infiltration in patients with RE compared to the control group.

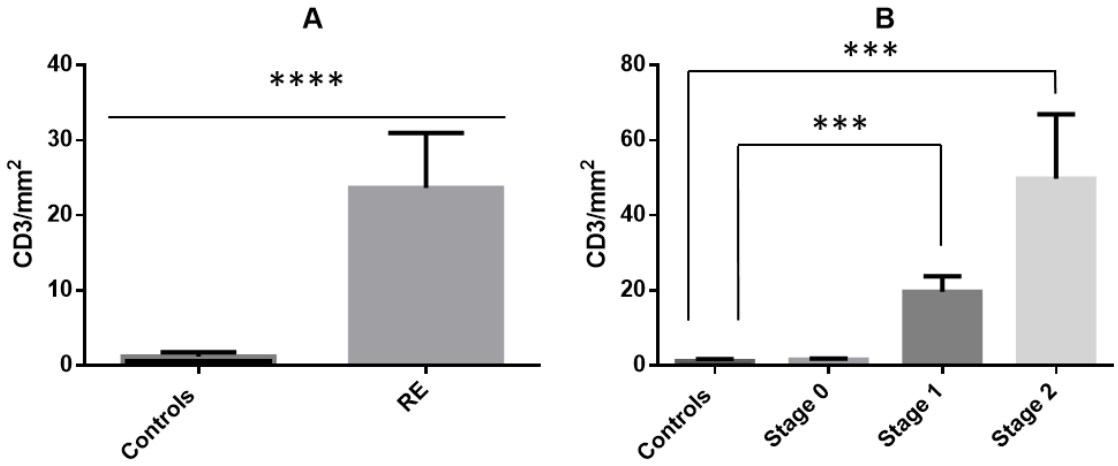

Figure 3 | Quantification of the number of CD3 positive cells in Rasmussen encephalitis. (A) Quantification of CD3 positive cells per mm2 in patients from the control group (n=19) and the pool of RE cases (n=18). The RE group is compared to the control group. (B) Representation of CD3 positive cells in the control group (n=19), stage 0 of RE (n=6), stage 1 (n=6) and stage 2 (n=6). Each RE stage is compared to the control group. Data is shown as mean ± SEM.

Analysis of γδ T lymphocytes in Rasmussen Encephalitis

Once the presence of inflammation in RE was confirmed, the next goal was to analyse the amount of γδ T cells in RE. As in the case of CD3 T lymphocytes, γδTCR staining was performed using the standard staining procedure for light microscopy. Using an optical microscope positive γδTCR lymphocytes were observed in RE samples. They were found in in areas with inflammation such as microglial nodules. Besides the presence of γδ T cells in nodules, they were also found spread throughout the cortex and in damaged areas, mainly areas with neuronal loss (Figure 4 C-D). As expected, in the control cases and stage 0 there was hardly any presence of γδ T lymphocytes (Figure 4 A-B).

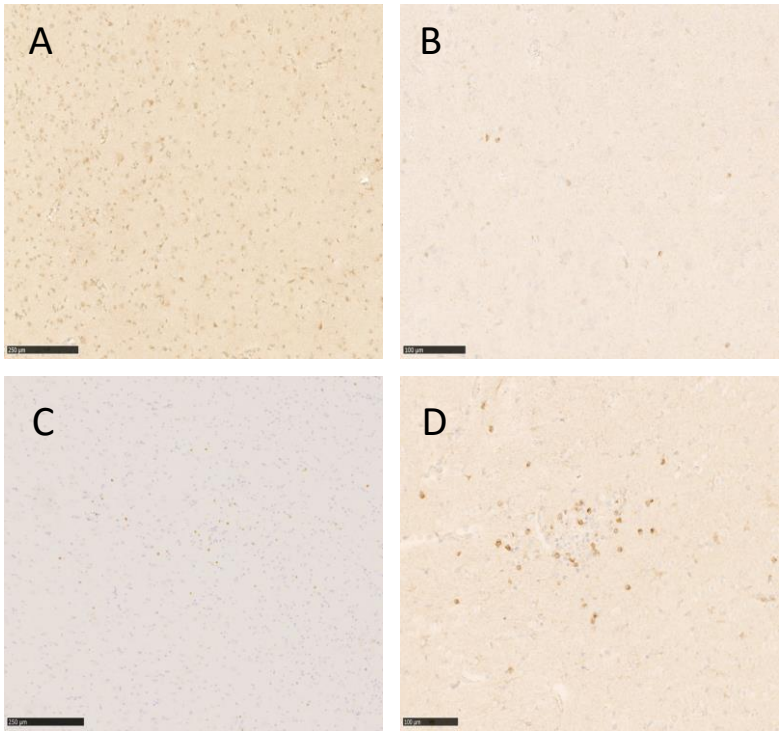

Figure 4 | Immunohistochemistry staining for γδTCR in control samples and RE. (A) Negative control with no γδTCR positive cells. (B) Stage 0 RE sample with hardly any γδTCR positive cells. (C) Stage 1 RE sample with γδTCR positive cells. (D) Stage 2 RE sample with γδTCR positive cells occupying a large area.

For the study of γδ T cell population, an analysis between the control group and the set of RE samples was first performed. The result of the Mann Whitney test showed that there was a significant difference between these two groups (Figure 5 A). A Kruskal-Wallis test was then carried out to study the relationship between the different stages of the disease and the controls. It was found that, as in the analysis of CD3 receptor, the control and stage 1 and 2 groups were significantly different (Figure 5 B).

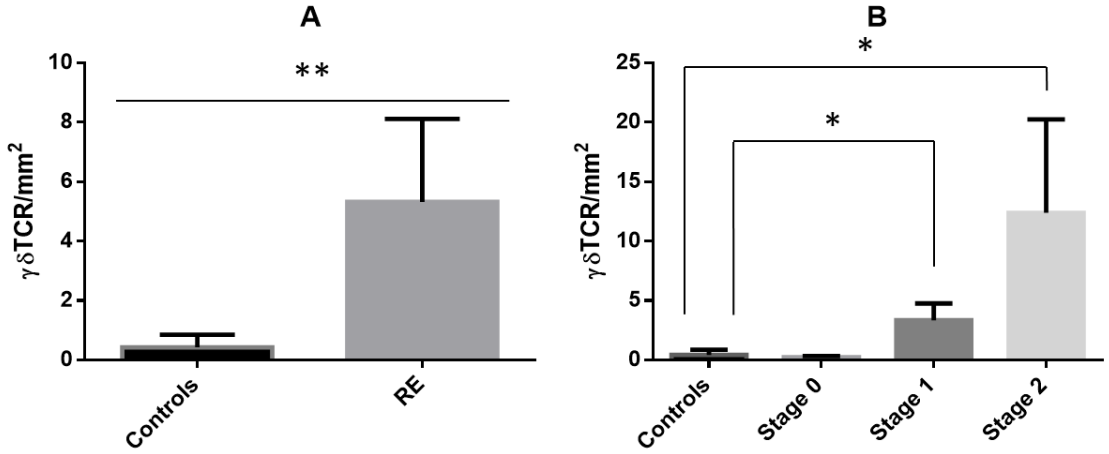

Figure 5 | Quantification of the number of γδ positive cells in Rasmussen encephalitis. (A) Quantification of γδ positive cells per mm2 in patients from the control group (n=7) and the pool of RE cases (n=18). The RE group is compared to the control group. (B) Representation of γδ positive cells in the control group (n=7), stage 0 of RE (n=6), stage 1 (n=6) and stage 2 (n=6). Each RE stage is compared to the control group. Data is shown as mean ± SEM.

It was also thought that it would be interesting to evaluate the presence of γδ T lymphocytes in other types of encephalitis to determine if it could be considered as a representative cell population of RE. For that purpose IHC stainings for γδTCR were performed in paraneoplastic encephalitis, non-paraneoplastic encephalitis, Herpes simplex encephalitis (HSV) and Cytomegalovirus encephalitis (CMV) samples. Using optical microscopy, it can be seen how γδ T cells are present in these cases but in a lower concentration than in RE. However, cell quantification with ImagePro program was not carried out due to the presence of background that made the staining not optimal enough.

Another aim of this thesis was to determine whether the percentage of γδ T lymphocytes in relation to total T lymphocytes increases with the progression of the disease. The IHC stainings for γδTCR and CD3 were used for this purpose. First of all it was done an analysis between the percentage of γδ T lymphocytes over CD3 T lymphocytes between the set of RE cases (n= 18) and the control group (n= 7). The Mann Whitney test confirmed that there was a significant difference between these two groups (Figure 6 A).

A study of the percentage of γδ T lymphocytes among the control groups, stage 0, 1 and 2, was therefore performed. In this case a Kruskal-Wallis test was carried out, the result of which was that there is a significant difference between the control group and stage 1 in RE. As shown in Figure 6B, the amount of γδ T lymphocytes as a percentage of total CD3-lymphocytes remains constant during the three different stages of RE, at about 15%.

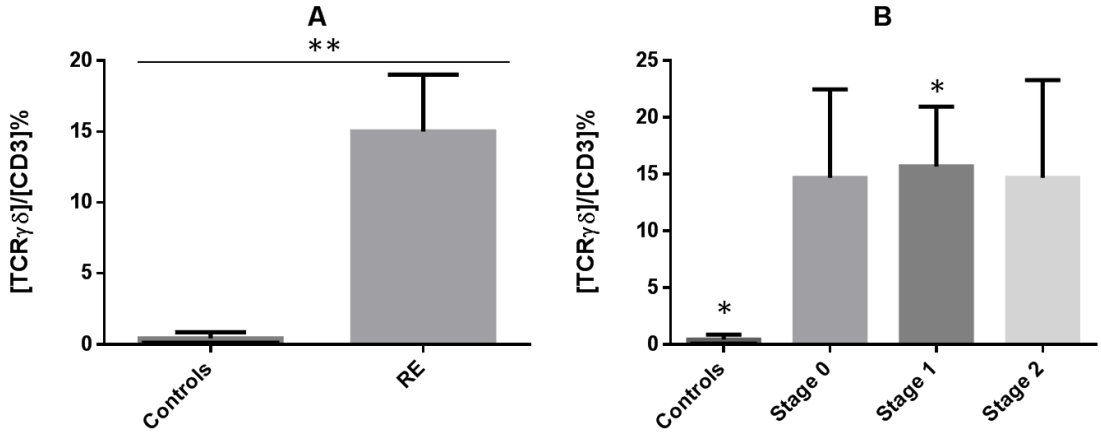

Figure 6 | *Quantification of the percentage of γδ positive cells in Rasmussen encephalitis. (A) Quantification of percentage of γδ positive cells per mm2 in patients from the control group (n=7) and the pool of RE cases (n=18). The RE group is compared to the control group. (B) Representation of percentage of γδ positive cells in the control group (n=7), stage 0 of RE (n=6), stage 1 (n=6) and stage 2 (n=6). Each RE stage is compared to the control group. Data is shown as mean ± SEM.*

Analysis of the expression of CD3 and γδTCR by confocal laser fluorescence microscopy in Rasmussen Encephalitis

Then the expression pattern of the CD3 and γδTCR receptors in RE was studied. The next step was to see if T lymphocytes could express both receptors simultaneously. To achieve this goal, the FFPE tissues (n=5) were stained for CD3 and γδTCR receptors following the double staining procedure for confocal laser fluorescence microscopy. This double staining for CD3 and γδTCR (Figure 7 A and B) showed that both receptors could be expressed in the same cell, although the number of positive cells for both markers was not very high. Which justifies the results obtained in the previous section, since, as can be seen in the figures 7 A and B, about 15% of T lymphocytes express γδTCR receptor.

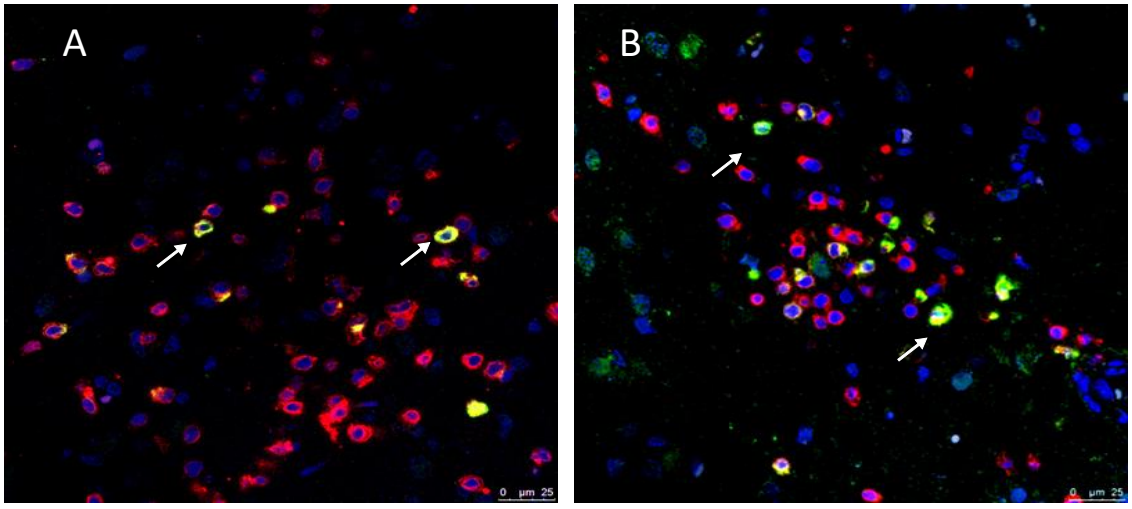

Figure 7 | *Confocal laser fluorescence microscopy of CD3 and γδTCR in RE. (A)(B) Microglial nodules. CD3 is shown in red (Cy3) whereas γδTCR is labelled in green (Cy2). Nuclei are labelled in blue. Overlay of the green and red fluorophores is shown in yellow. (Panels A-B used a scale bar of 25μm).*

Analysis of the cytotoxic capacity of γδ T lymphocytes in Rasmussen Encephalitis

The next step was to find out if this population of γδ T lymphocytes could attack neurons in the tissues of RE. For this aim, a double staining for light microscopy was performed, it was used NeuN as a marker for neurons and γδTCR for T lymphocytes. It was found that most of the γδ T lymphocytes were in apposition to neurons, suggesting that they could be attacking them (Figure 8).

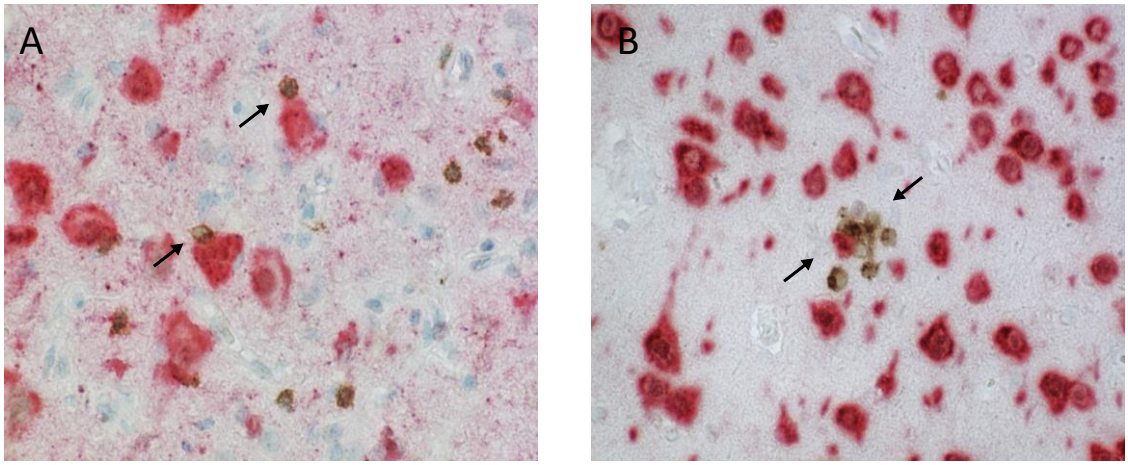

Figure 8 | *Immunohistochemistry staining for NeuN and γδTCR in RE. (A)(B) NeuN positive neurons are shown in red and γδTCR positive cells are shown in brown. γδ T lymphocytes in apposition to neurons are pointed out with arrows. (B) Cluster of γδ T lymphocytes surrounding a neuron.*

The cytotoxic capacity of γδ T lymphocytes was then evaluated. For this purpose, a triple staining for fluorescence microscopy was performed, in which neurons; γδ T lymphocytes and granzyme B were labelled. As a result, it was seen how γδ T lymphocytes polarized cytotoxic granules towards neurons in the neighbourhood, leading to the idea that they could be one of the causes of neuronal loss.

Qualitative analysis of the expression of T-bet in RE

As mentioned in the introduction, IFN-γ producing lymphocytes can play an important role in supporting CD8+ T cell activity in RE. This is due to the fact that, among others, IFN-γ can induce the expression of MHC-I molecules on the surface of neurons, making them more vulnerable to cytotoxic attack of T cells. Also, it can stimulate the production of several chemokines by microglial cells and astrocytes, which by chemotaxis again may indirectly attract further T cells.

IFN-γ producing lymphocytes express the T-bet transcription factor. That is why this transcription factor has been used as a marker to determine whether the lymphocytes found in the microglial nodules produced IFN-γ.

For this purpose, an immunohistochemical staining was performed using a monoclonal mouse antibody against the antigen T-Bet. T-Bet-positive cells were found in stage 1 and 2 of RE. In this case, stage 0 RE was not evaluated because it wasn't expected to find a high concentration of positive cells. Stages 1 and 2 were studied and two new subgroups called stages 0-1 and 1-2 were generated. These consisted of samples that

contained areas that could be classified in both group 0 and group 1, the same happened with stage 1-2. That is why it was specified two intermediate groups between stages 0 and 1 as well as 1 and 2.

In stage 0-1 hardly any cellular infiltration was observed, only a few cells around the blood vessels. While in stage 1 it was possible to see the presence of some T-Bet positive cells in the parenchyma, but where they were most notable in the microglial nodules, mostly in the smaller ones, although they were also observed in the larger ones. In stage 1-2 samples, positive cells were present in the parenchyma and in higher numbers in the microglial nodules. Finally, in stage 2, these IFN-γ producing cells were found distributed in the parenchyma, in microglial nodules and in areas where neuronal damage was seen (Figure 9).

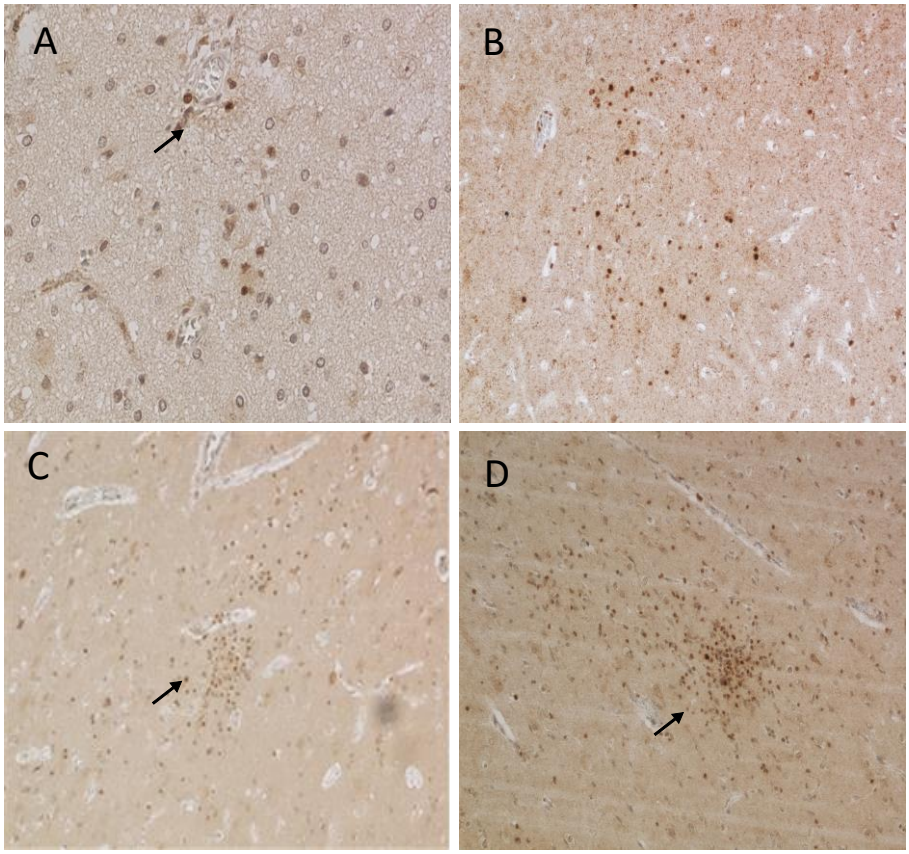

Figure 9 | *Immunohistochemistry staining for T-Bet in RE. (A) Stage 0-1 RE sample with hardly any T-Bet positive cells. (B) Stage 1 RE sample with T-Bet positive cells. (C) Stage 1-2 RE sample with a cluster of T-Bet positive cells. (D) Stage 2 RE sample with T-Bet positive cells occupying a large area.*

Qualitative analysis of the expression of T-bet and γδTCR in RE

It was also studied whether γδ T lymphocytes were capable of producing IFN-γ. A double staining was performed using the antibodies against T-Bet and γδTCR. As can be seen in Figure 10, positive cells were found in areas with high T cell infiltration. Cell quantification was not performed since the staining was not optimal in all samples due to the presence of background.

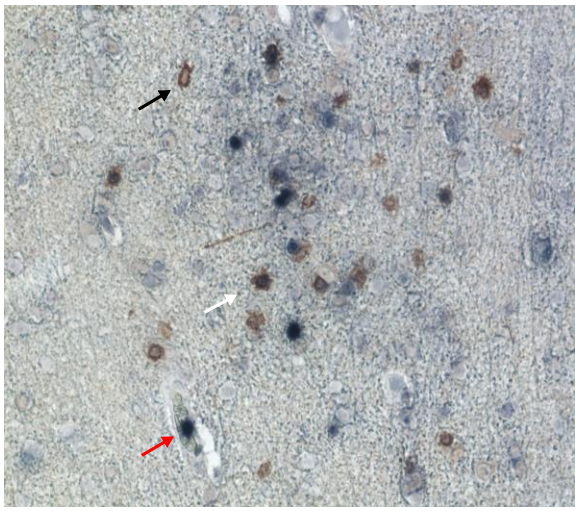

Figure 10 | *Immunohistochemistry staining for T-Bet and γδTCR in RE. γδTCR shown in brown and T-Bet in dark blue. Positive cell for γδTCR is pointed out by a black arrow. Positive cell for T-Bet is pointed out by a red arrow. Double positive cell for γδTCR and T-Bet is pointed out by a white arrow.*

DISCUSSION

The aim of this study was to investigate γδ T lymphocyte population in order to determine whether they constituted a specific population of Rasmussen's encephalitis with the ability to attack neurons. Special attention was also paid to the production of interferon γ by T cells in order to validate whether it plays a role in the innate immune response in RE.

As mentioned in the introduction, it is believed that autoimmunity is one of the causes of RE. Despite not knowing the mechanism that produces it, it is clear that deregulation in the T cell population may be a key component because of its constitutive role in immune surveillance.(Pilli et al., 2017) During the course of the disease, it is characteristic to see increased infiltration of T lymphocytes, in addition to astrogliosis and microglial activation reactions in the cerebral cortex. Based on this, Pardo et al. established some stages in which to categorize the progression of the disease.(Pardo et al., 2004)

To study the T-lymphocyte population, IHC stainings were performed in samples of human biopsies preserved by FFPE, as it is an excellent method for long-term preservation of most pathological specimens, being a great resource for molecular studies. Eighteen samples of RE were evaluated, divided into three groups of six, from the stage 0 to 2, and 19 control samples. To determine the basic composition of inflammatory infiltrates antibodies against all T cells (CD3) were used.

The data shown in this study is corroborated with those presented by Pardo et al. As expected, in the control group as well as in stage 0 of RE, no infiltration of T lymphocytes was observed. Some T cells were noticed but, mostly in the vicinity of the blood vessels, which can be explained as a usual event due to the extravasation of cells from the peripheral blood. However, as the disease progresses, it can be appreciated how the concentration of T lymphocytes increases. T cells are arranged in microglial nodules and in areas where greater neuronal damage is seen.

The predominance of T cells in RE is consistent with published immunohistochemistry results, but the presence of γδ T cells is a new finding and has implications for the immune response in RE. Seizures are one of the main defining characteristics of RE, which have been shown to promote an inflammatory reaction in the brain. γδ T cells are considered to be among the first immune cells to cross the blood brain barrier in response to pro-inflammatory cytokines released by inflammasomes. In response to inflammatory cytokines, γδ T cells can act as an innate immune cell and release inflammatory cytokines without TCR engagement potentially perpetuating an inflammatory reaction. (Owens et al., 2015)

γδ T lymphocytes constitute about 5% of the total peripheral T cells. Despite their low representation they can be considered as an interesting subpopulation of T cells in the study of RE. The reason for this is that they are able to recognize a wide range of antigens without the need to interact with the MHC molecule of APC cells. In addition, unlike conventional αβ T cells, simple cytokine stimulation is sufficient for the activation of γδ T cells. This is why they can be described as strong and potent mediators of inflammation. (Edwards et al. 2015). Since they are able to react to inflammation in a shorter time than αβ T lymphocytes, they can be expected to be over-represented in the early stages of the disease. These facts led us to hypothesize that γδ T lymphocytes are especially present in the early stages of RE.

To evaluate its implication in the pathogenesis of RE, IHC staining for γδTCR antigen was performed on FFPE samples; 18 samples of RE and 7 control samples were studied. As seen in the results (Figure 5) the concentration of positive cells for γδTCR was higher in stages 1 and 2 of the disease compared to stage 0, where there is little cell infiltration, or control where there was scarcely any γδ T lymphocytes present. On the other hand, when studying the percentage of γδ T cells in relation to the total number of T cells previously evaluated, it was observed that they always constituted a proportion of 15% in relation to the total number of CD3 T cells. These data was corroborated with the results of the double staining for fluorescence microscopy for CD3 and γδTCR receptors. In which it was possible to see how the cells that expressed both receptors

simultaneously were in a lower proportion, being this, as in the previous case, approximately 15%.

Previously it has been shown that CD8+ T cells positive for granzyme B are present in the brain parenchyma surrounding neurons and astrocytes. It is believed that these cytotoxic T cells react against self-antigens presented by neurons and astrocytes in the affected brain hemisphere. Consequently, brain-resident macrophages trigger a focused innate immune response, promoting the recruitment of non-resident immune cells unrestricted by MHCs, such as natural killer cells and γδ T cells. (Owens et al., 2015)

For this reason we wanted to determine whether this population of γδ T lymphocytes could attack the neurons in the RE tissues. Based on our results, it was found that γδ T lymphocytes, which themselves are CD8 negative, were in the same areas as the CD8 T cells described above, suggesting that they, like the traditional cytotoxic CD8+ cells, might be targeting the neurons. That is why the cytotoxic capacity of γδ T lymphocytes was then evaluated, obtaining as a result that they were positive for granzyme B and potentially cytotoxic.

Based on the results obtained, it should be commented that it was surprising to find that γδ T lymphocytes during the course of the pathology had a constant representation of 15%. This cell population was expected to increase significantly in the early stages of RE over total T lymphocytes, thus obtaining a higher participation. Since, as mentioned in the introduction, because of their role as rapid and powerful mediators of inflammation, it was condescending to think that they might be especially involved in the early stages of the pathology. However, we can state that γδ T lymphocytes constitute a subpopulation of total T lymphocytes, but are not particularly involved in mediating inflammation in RE during the early stages. Nevertheless, γδ T cells expresses granzyme B, indicating that they can induce death of astrocytes and neurons that express MHC class I through direct cell-cell contact and release of granzyme B.

In RE, γδ T cells can provide a link between inflammation in the brain and an adaptive immune response involving antigen-specific CD8+ T cells. On the other hand, they can also act as an adaptive immune cell and produce cytokines. (Owens et al., 2015)

As shown in this study, γδ T cells are commonly represented at stage 2 than at the onset of RE. Being this stage the one where the microglia starts to become more active. By performing simple staining for T-Bet we have confirmed that IFN-γ producing cells are found in microglial nodules, being more present in the late stages of the disease. Throughout a double staining for IHC we then confirmed that γδ T lymphocytes are as well IFN-γ producing cells.

It may be suggested that γδ T lymphocytes might be partially responsible for microglial activation due to the production of IFN-γ. In RE, microglia and macrophages have been established as the main cells that exhibit inflammasome cellular machinery. (Ramaswamy et al., 2013) More specifically, microglial cells, using a wide range of immune receptors, are able to recognize IFN-γ and therefore become active. After that they are able to respond by producing inflammatory cytokines such as IL-1β, promoting an innate immune response.(Cherry et al., 2014)

It is also worth mentioning that Th1 cytokines such as IFN-γ released by γδ T cells would be expected to promote a cytotoxic CD8+ αβ T cell response. IFN-γ increases MHC class I on neurons, which would make them vulnerable to cytotoxic T cells.

CONCLUSION

Analysis of the results obtained in the study of γδ T lymphocytes in Rasmussen's encephalitis led to the following conclusions:

- γδ T cells, unlike the initial hypothesis suggested, do not constitute a specific population of the early stages of RE. However, we have been able to prove that they can generate neuronal loss, since they have been found in the neighbourhood of neurons. It has also been elucidated that γδ T lymphocytes constitute a cytotoxic population.

- It has been verified that γδ T lymphocytes are IFN-γ cells producing, however, it has not been possible to elucidate exactly what their involvement in RE is.

Several questions remain opened for further investigations, such as which antigens do γδ T cells identify and clarify if these are self or foreign antigens and if molecular mimicry is involved. Another interesting question would be to know which role plays IFN-γ produced by γδ T lymphocytes. If IFN-γ produced by γδ T lymphocytes actually played a part in microglia activation, it could be a good molecular target for the design of antiepileptic drugs, which might not only inhibit the epileptic seizures, but, since IFN-γ is a pro-inflammatory cytokine, might also influence the immunological response.

SELF-ASSESSMENT

I would like to conclude this thesis with a personal assessment of my internship experience and of my knowledge acquired during these four years as a bachelor student in Biotechnology, and how all this has been reflected when writing this thesis.

My passage through the university has allowed me to form myself as a researcher, providing me with a wealth of theoretical knowledge in many fields of science. However, one of the skills I most value having acquired is the ability to learn how to use all the tools at my disposal in order to search for information in a rigorous and critical way, knowing how to understand and interpret it. As a result, I have been able to overcome one of the difficulties I have encountered during the development of my thesis. As I have focused on the field of Neuroimmunology, an area that is under-represented throughout the degree due to the fact that it is a very specific subject, it has been difficult for me to understand the part about the nervous system.

Concerning the improvements he would make to this university studies in a forward-looking perspective and in order to improve learning, I believe that it would be appropriate to introduce some assignments in English. I am aware that I have written this thesis in this language on a voluntary basis, but I really believe that it is more than essential to learn to express oneself in English in a correct way and in a scientific framework, so that the language does not constitute a barrier when developing a project.

With regard to my internship at CBR I would describe it as a unique experience. Over the course of these five months I have been able to consolidate the knowledge that I have acquired during my studies in the field of immunology, as well as to put into practice the theoretical notions of IHC and fluorescence microscopy. I have also been able to understand the importance of statistics and I have learned to interpret results based on the literature, which is fundamental in research. This is why this first contact with the labour field has consolidated itself as a very satisfactory and enriching learning process.

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