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**PERIPHERAL LEUKOCYTE PROFILE IN PEOPLE WITH
TEMPORAL LOBE EPILEPSY REFLECTS THE ASSOCIATED
PROINFLAMMATORY STATE**

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ABSTRACT

Introduction: Markers of low-grade peripheral inflammation have been reported amongst people with epilepsy. The mechanisms underlying this phenomenon are unknown. We attempted to characterize peripheral immune cells and their activation status in people with temporal lobe epilepsy (TLE) and healthy controls.

Methods and Results: Twenty people with TLE and 19 controls were recruited, and peripheral blood lymphocyte and monocyte subsets evaluated *ex vivo* by multi-color flow cytometry. People with TLE had higher expression of HLA-DR, CD69, CTLA-4, CD25, IL-23R, IFN- γ , TNF and IL-17 in CD4⁺ lymphocytes than controls. Granzyme A, CTLA-4, IL-23R and IL-17 expression was also elevated in CD8⁺ T cells from people with TLE. Frequency of HLA-DR in CD19⁺ B cells and regulatory T cells CD4⁺CD25⁺Foxp3⁺ producing IL-10 was higher in TLE when compared with controls. A negative correlation between CD4⁺ expressing co-stimulatory molecules (CD69, CD25 and CTLA-4) with age at onset of seizures was found. The frequency of CD4⁺CD25⁺Foxp3⁺ cells was also positively correlated with age at onset of seizures. **Conclusion:** Immune cells of people with TLE show an activation profile, mainly in effector T cells, in line with the low-grade peripheral inflammation.

Key words: Human temporal lobe epilepsy; Immunophenotyping; Lymphocytes; Cell activation; Cytokines.

INTRODUCTION

Epilepsy is a major public health problem affecting around 1% of the population worldwide. Temporal lobe epilepsy (TLE) is the most common epileptic syndrome in adults, and is frequently associated with difficult control seizures. Its etiopathogenesis is complex, involving both genetic and environmental factors. Taking into account their role in plastic or structural changes in several organs, experimental studies have focused on the involvement of inflammatory mechanisms in epilepsy[1-3].

Inflammation has generally been regarded as harmful to the brain as local (microglia) and infiltrating immune cells (CD4⁺ and CD8⁺ T cells), as well as pro-inflammatory cytokines, such as interleukin (IL)-1 β , tumor necrosis factor (TNF), and IL-6, may exacerbate neuronal damage in epilepsy[2, 4]. Conversely, there is also evidence supporting a protective role for innate and adaptive immune cells as they can contribute to seizure-suppression in animal models of TLE[5, 6]. Whatever its effect or involvement, a growing body of evidence has supported a role for inflammatory mechanisms in epilepsy. This is mainly supported by the finding of high levels of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF, in the cerebrospinal fluid (CSF) or blood of people with epilepsy, notably TLE[7][8]. The meaning and/or the triggers of this low-grade inflammation in epilepsy is unknown. Only a few studies have characterized the peripheral immune cells in TLE[6, 9-14]. For instance, one study reported elevated frequency of monocytes and NK cells in people with refractory epilepsy to antiepileptic drugs (AEDs)[15].

As previous studies reported increased levels of circulating pro-inflammatory cytokines and/or mediators, suggesting a persistent peripheral low-grade inflammation in TLE, we hypothesized that cells of people with epilepsy would display an activated profile when compared with controls. To test this hypothesis, we evaluated the activation status and the production of cytokines by peripheral immune cells.

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Methods

Subjects: This study enrolled 20 people with TLE and 19 controls recruited from a tertiary referral center. The inclusion criteria for TLE were: diagnosis of TLE according to the ILAE criteria, age of more than 18 years, capacity to provide written informed consent, and seizure-free for at least 72 hours reported by the people with TLE and/or their companion. This period was chosen as previous studies showed that circulating cytokines returned to basal levels at least 24 hours after seizures [15, 16]. All patients had temporal mesial sclerosis on magnetic resonance imaging (MRI). Control group was recruited in Belo Horizonte, according to the following criteria: age more than 18 years and lack of any psychiatric disorder, any severe medical condition or neurological diseases including epilepsy. For both groups, the exclusion criteria were history of previous neurosurgery, use of anti-inflammatory and antibiotic drugs in the last two weeks, or cognitive impairment according to the Mini Mental State Examination and had other medical or neurologic diseases other than epilepsy. Sociodemographic (age, gender, ethnicity, marital status, occupational status and educational level) and clinical data (weight and height, age of onset, duration of epilepsy, seizure type, seizure frequency, medication use, AED regimen, MRI and EEG findings) were also collected for both groups. This study was approved by local ethics research committee under the protocol number 607.264-0.

Peripheral blood mononuclear cell isolation and cell surface staining: Blood was collected after clinical interview and immediately processed. Whole blood cells were

obtained from K3-EDTA venous vacuum tubes. Erythrocytes were lysed using ACK lysing solution (0.15 M NH_4Cl , 1 mM KHCO_3 and 0.1 mM Na_2EDTA) and washed twice with cold phosphate buffer saline (PBS) (1200 rpm, 4°C, 10 minutes). After erythrocyte lysis, white blood cell (WBC) were stained with a combination of fluorescein isothiocyanate (FITC), phycoerythrin (PE), cy5.5-chrome (Cy)-labeled or PerCP 5.5, allophycocyanin (APC), cy7- allophycocyanin (APC) and cy7-phycoerythrin (PE-Cy7) antibodies directed against the surface molecules anti-CD3, anti-CD4, anti-CD8, anti-CD25, anti-CTLA-4, anti-CD69, anti-IL23R, anti-CD56, anti-CD19, anti-HLA-DR, anti-CD14, anti-CD16 and anti-CD86 (eBioscience, San Diego, CA, USA; BD Pharmingen, San Diego, CA, USA and Invitrogen/Molecular Probes, Camarillo, CA, USA) for 20 minutes at 4°C and data acquired using a FACSCantoll (Becton & Dickinson, San Jose, CA, USA).

Intracellular cytokines and FoxP3 staining: White blood cells were analyzed for their surface profile and intracellular cytokine expression pattern. Briefly, cells were fixed with phosphate buffer saline (PBS) and formaldehyde (2%) (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes. Fixed cells were permeabilized using saponin 0.5% (Sigma-Aldrich) and stained using monoclonal antibodies for granzyme-A, FoxP3, CTLA-4, TNF, $\text{IFN-}\gamma$, IL-17A and IL-10 (Invitrogen/Molecular Probes and BD Pharmingen) conjugated with phycoerythrin (PE) or allophycocyanin (APC). PE and APC-labeled immunoglobulin control antibodies and a control of unstaining WBC were also included in all experiments. Preparations were acquired in FACSCantoll (Becton & Dickinson, San Jose, CA, USA). A minimum of 50,000 gated events

in lymphocyte population was acquired for analysis due to the low frequency of positive events being analyzed.

Flow cytometry data analysis: Natural killer, T and B lymphocytes were analyzed for their intracellular cytokine and costimulatory surface marker expression pattern and frequency using the FlowJo program (Tree Star, Ashland, OR, USA). Limits for the quadrant markers were always set based on negative populations and isotype controls. At least three different fluorochromes were combined for each analysis. Gating strategy is depicted in Figure 1. After gating specific cell subsets, histograms were generated for evaluating the frequency of cells expressing the given surface markers or cytokines. These cells were then analyzed for the expression (frequency and mean fluorescent intensity, MFI) of a given marker using histograms with control markers based on negative isotype controls.

Statistical analysis: Numerical variables were expressed as median or mean with standard deviation. The frequency of cells was compared using the nonparametric Mann-Whitney U test. Correlation between the frequency of immune cells and clinical parameters was performed with the Spearman's correlation test. Statistical analysis was performed using the SPSS 17.0 package (SPSS Inc., Chicago, IL, USA). All tests were two-sided with a significance level of $p < 0.05$.

RESULTS

Twenty people (12 men), aged 19 to 55 years (mean age \pm SD, 39 ± 11) were enrolled. Mean age at onset of seizures was 6.8 years. The mean frequency of seizures was 3.0 per month. For control, 19 people without epilepsy or any psychiatric disorder, aged 24 to 65 years (mean age \pm SD, 39 ± 9.3) were enrolled. Clinical features of subjects are shown in Table 1. There was no difference between controls and people with TLE regarding sex, mean age, body mass index and educational level (Table 1). However, people with TLE presented different marital ($p= 0.005$) and employment status ($p<0.001$) when compared with controls (Table 1).

T lymphocytes from people with TLE showed high expression of co-stimulatory molecules

There were no differences between people with TLE and controls regarding the percentage of T cells ($CD4^+$ and $CD8^+$), monocytes ($CD14^+$), B lymphocytes and regulatory T cells (Treg).

We next sought to investigate the expression of co-stimulatory molecules on peripheral lymphocytes. As shown in Figure 2, people with TLE showed higher expression of CD25 and HLA-DR on $CD4^+$ T cells as compared with controls (Figure 2A and B). The expression of CD69 and CTLA-4 on both $CD4^+$ and $CD8^+$ T cell subsets was also higher in people with TLE (Figure 2C and D).

Increased intracellular cytokine expression in T cells from people with TLE

Increased intracellular levels of IFN- γ , IL-6 and TNF in CD4⁺ T cells were found in people with TLE compared with controls (Figure 3). The expression of IL-17A was also higher in CD4⁺ and CD8⁺ T cells from people with TLE compared with controls (Figure 4A).

IL-23R is a receptor that plays a pivotal role in the induction of Th17-specific cells. Our results showed higher expression of IL-23R on CD4⁺ and CD8⁺ T cells from people with TLE compared with controls (Figure 4B).

Treg cells from people with TLE express high levels of IL-10

Regulatory T cells (Treg) may inhibit effector Th1, Th2 and Th17 cells by secreting IL-10 and transforming growth factor (TGF)- β [17].

The frequency of IL-10 producing CD8⁺ T lymphocytes was elevated in TLE compared with controls (Figure 5A). People with TLE and controls had similar frequency of Treg cells (Figure 5B), but IL-10 was increased 3-fold in Treg cells from people with TLE (Figure 5B).

Innate response and granzyme A production exacerbated in people with TLE

The total number of NK cells was lower in people with TLE in comparison with controls (Figure 6A), but the number of NK cells producing granzyme A was higher in the former (Figure 6A). The same pattern was observed for cytotoxic T cells producing granzyme A (Figure 6A). HLA-DR expression, a marker for lymphocyte activation, was higher in B cells from people with TLE than controls (Figure 6B).

Monocyte subpopulations were evaluated through the expression of the co-stimulatory molecules CD86 and the FcγRIII receptor activation marker CD16. While the frequency of monocytes expressing CD86 was higher (Figure 7A), the frequency of monocytes expressing CD16 was lower in people with TLE than controls (Figure 7B). These results suggesting activation of classical monocytes in TLE were confirmed by higher MFI for HLA-DR, IL-6, CD86 in monocytes from people with TLE compared with controls (Figure 8A). Also, a high frequency of IL-6 and TNF in monocytes was founded (Figure 8B).

Inflammatory association with clinical findings

To evaluate the clinical meaning of these findings, we correlated the frequency of leukocyte subpopulations with clinically meaningful parameters (*i.e.* age at onset of seizures, mean time since onset of seizures and mean frequency seizures/month). A negative correlation between CD4⁺ cells expressing CD25, HLA-DR and CTLA-4 with the mean time since onset of seizures was noticed (Table 2). There was positive correlation between Treg percentage and mean time since onset of seizures (Table 2). No other correlation between immune cells and clinical parameters emerged.

DISCUSSION

We characterized peripheral immune cells from people with TLE by assessing a comprehensive panel of cell-surface markers and intracellular cytokines. People with TLE had increased frequency of activated T cells (CD25, CD69, CTLA-4 and HLA-DR) compared with controls. Previous studies have already shown increased number of T cells [6, 15, 18, 19], but none has addressed the activation state of peripheral immune cells.

Costimulatory molecules are pivotal for cell activation, proliferation and differentiation, and for cytokine production. The findings of high expression of CTLA-4, CD25, and HLA-DR in T lymphocytes from people with TLE indicate that these lymphocytes are in a state of chronic activation. Moreover, as CD69 is an early activation marker, its increase in TLE indicates that these people present a continuous and/or persistent immune activation.

People with TLE also exhibited activation of innate immunity cells. Monocytes from people with TLE displayed a classical phenotype (CD14⁺CD16⁻), and increased expression of CD86, HLA-DR, IL-6 and TNF, further indicating a pro-inflammatory state. This finding in the periphery may be in line with the report of infiltration of monocytes/macrophages in areas of neuronal loss in human samples of hippocampal sclerosis and experimental models of TLE [5, 23].

CD8⁺ and NK cells producing granzyme A were also increased in TLE compared with controls. Granzyme A is a serine protease involved in several immune functions, including cell death by non-apoptotic pathways [21, 24], and mediation of inflammatory cytokine release [25-27]. Granzyme A produced by CD8⁺ and NK cells could be involved in cytokine production and apoptotic

response in epilepsy. Interestingly, in Rasmussen encephalitis, granzyme B and TNF were increased in the CSF [19].

When assessing intracellular cytokine production, we found increased expression of IL-6, IFN- γ , TNF and IL-17A in lymphocytes and monocytes. This finding is in line with previous studies reporting elevated cytokine levels in serum/plasma and CSF in the interictal period, but especially after seizures, in TLE and other epileptic syndromes [9, 15, 16, 18, 28, 29]. The mechanisms underlying this low-grade inflammation, *i.e.* the elevated circulating levels of pro-inflammatory cytokines, in TLE remain unclear. For some, active epilepsy is accompanied by a chronically up-regulated stress response which peaks in the postictal period, leading to persistent elevated levels of pro-inflammatory cytokines [7, 9, 16]. It is tempting to hypothesize that brain-derived damage-associated molecular pattern (DAMPs) could be responsible for persistent immune activation and related low-grade inflammation. DAMPs generated in the brain after seizures would induce a cascade of events leading to local and systemic inflammation (Figure 9). Indeed experimental studies have demonstrated that there is continuous recruitment of peripheral cells into the brain following seizures, with a parallel between the activation of immune cells in the CNS and in the periphery [3, 19-22].

We showed that IL-17A production is also increased in CD4⁺ and CD8⁺ T cells in people with TLE. IL-17A is implicated in the development of autoimmune diseases in the central nervous systems such as multiple sclerosis, and was correlated with the severity of epilepsy [8]. Epidemiological studies showed that epilepsy is associated with autoimmune diseases [30, 31]. IL-23R is a receptor

involved in Th17 response skewing, being expressed on the surface of lymphoid cells, such as $\alpha\beta$ and $\gamma\delta$ T cells, innate lymphoid cells and myeloid-derived cells [32, 33]. Accordingly, we also found increased expression of IL-23R in T cells in TLE.

People with TLE presented increased IL-10 expression in Treg cells and CD8⁺ T cells. Treg cells inhibit both Th1 and Th2 effector cells, and also Th17-mediated inflammation and autoimmunity. Their inhibitory mechanisms include engagement of cell-surface inhibitory receptors and production of soluble molecules. Treg cells can secrete IL-10 and transforming growth factor (TGF)- β that inhibit pro-inflammatory responses. It is possible that Treg cells became active, producing more IL-10, in order to counterbalance the continuous pro-inflammatory response observed in TLE.

Treg cells are central for the maintenance of peripheral immune tolerance, but their role in the central nervous system is unclear. Besides secretion of IL-10, they can exert their immune suppressive function through cell contact-dependent mechanisms using inhibitory co-receptors such as CTLA-4 [41]. The correlation between the mean time since onset of seizures and the number of Tregs is intriguing, and might reflect the need of persistent compensatory mechanisms to counterbalance continuous pro-inflammatory stimuli.

Our study has several limitations. The sample size is relatively small. The cross-sectional design of the study prevents definite causal assumption. All people with TLE were medicated, and AEDs could influence immune parameters. Most of these drug-related effects were anti-inflammatory or immunosuppressive, and people with TLE exhibited an enhanced inflammatory immune profile despite the

use of AEDs. For instance, there was decreased secretion of pro-inflammatory cytokines, especially IL-1 β and TNF, in human PBMC cultivated with carbamazepine, lamotrigine, oxcarbazepine, phenobarbital, topiramate, and valproate[39]. CD8⁺ T cells secreting perforin reduced degranulation after culture with valproate and levetiracetam[40]. Accordingly, we could expect at most a moderating effect of these drugs on the findings, but not their explanation. One limitation regarding AEDs was that control for drug intake was not carried out. The current findings do not necessarily apply for TLE with controlled seizures, and further studies are warranted to confirm these results.

In conclusion, our study offers further evidence of increased activation of circulating immune cells of those with TLE, supporting the consistently reported low-grade inflammation in epilepsy.

Table 1: Demographic and clinical characteristics of the study sample

		Total (n=42)	
		TLE (n=20)	Controls (n=19)
Sex			
	Male	12 (60%)	11 (58 %)
	Female	8 (40%)	8 (42 %)
Mean age, years (SD)		38.7 (11.3)	39.8 (9.3)
Educational level, years of study (SD)		8.0 (3.8)	11.6 (7.5)
Marital status			
	Single	13 (65%)	4 (21.05%)
	Married	7 (35%)	13 (68.4%)
	Divorced	0 (0%)	2 (10.52%)
Body Mass Index (kg/m²)		26.12 ± 5.14	25.43 ± 9.78
Employment status			
	Employed	5 (25%)	17 (89.47%)
	Unemployed	9 (45%)	1 (5.26%)
	Retired	6 (30%)	1 (5.26%)
Seizure type			
	Simple partial	6 (30%)	
	Complex partial	20 (100%)	
	Partial evolving to secondary generalized	6 (30%)	
MRI			
	RMTS	6 (30%)	
	LMTS	9 (45%)	
	BMTS	3 (15%)	
	WMTS	2 (10%)	
Age at onset of epilepsy (years)		6.8 (7.7)	
Mean time since onset of seizures, years (SD)		26.2 (11.3)	
Mean frequency, seizures/month (SD)		3.0 (4.8)	
Seizure free for the last 6 months		3 (15%)	
AEDs, n (%)			
	Valproate	5 (25%)	
	Carbamazepine	15 (75%)	
	Oxcarbazepine	1 (5%)	
	Phenytoin	1 (5%)	
	Phenobarbital	1 (5%)	
	Lamotrigine	9 (45%)	
	Topiramate	7 (35%)	
	Benzodiazepines	19 (95%)	
AEDs therapy regime, n (%)			
	Monotherapy	0	
	Dualtherapy	4 (20%)	
	3 AEDs	14 (70%)	
	4AEDs	2 (10%)	

SD = Standard deviation; MRI = Magnetic Resonance Imaging; WMTS = Without MTS; RMTS = Right Mesial Temporal Sclerosis; LMTS = Left Mesial Temporal Sclerosis; BMTS = Bilateral MTS; AEDs = Antiepileptic Drugs.

^a χ^2

^b Fisher's Exact test

^c Mann-Whitney U test

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Table 2: Ex vivo correlations between clinical aspects of seizure disorder and inflammatory characteristics and T regulatory cells from TLE

Correlation Analysis*	Spearman's rho	p
Mean time since onset of seizures (years) x CD4 ⁺ CTLA4 ⁺	-0.465	0.045
Mean time since onset of seizures (years) x CD4 ⁺ HLA-DR ⁺	-0.643	0.003
Mean time since onset of seizures (years) x CD4 ⁺ CD25 ⁺	-0.588	0.008
Mean time since onset of seizures (years) x CD4 ⁺ CD25 ⁺ FOXP3 ⁺	0.514	0.024

*The correlation analysis was performed with an "n" of 19, using the Spearman correlation coefficient and results were considered significant with a p<0.05.

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Figure Legends

Figure 1. Representative flow cytometry graphs of CD8⁺ T cells expressing granzyme A from control individual (CT) (B) and participant with temporal lobe epilepsy (TLE) (C). Flow cytometry dot-plots demonstrate the region of total lymphocytes (A) and the data analyzed in CD8⁺ T cells (B and C) expressing granzyme A and histograms (D) in controls (black line) and TLE (red dashed line).

Figure 2. CD25, HLA-DR, CD69 and CTLA-4 expression in T lymphocytes from temporal lobe epilepsy (TLE) and controls (CT) in *ex vivo* condition. Graphs show (A) CD25 and (B) HLA-DR expression on CD4⁺ T cells. Graphs show (C) CD69 and (D) CTLA-4 expression on CD4⁺ and CD8⁺ T lymphocytes. Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 3. IFN- γ , IL-6 and TNF expression by T lymphocytes from people with TLE and controls (CT) in *ex vivo* condition. Graph (A) IFN- γ show expression of this cytokine in T cells (CD4⁺ and CD8⁺). Graph (B) IL-6 and TNF show expression of these cytokines in CD4⁺ T lymphocytes. Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 4. IL-17A and IL-23R expression by T lymphocytes from people with TLE and controls in *ex vivo* condition. Data were collected using flow cytometry and analyzed using Flowjo software. Graphs show (A) IL-17A and (B) IL-23R expression in T cells (CD4⁺ and CD8⁺). Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 5. IL-10 expression by T lymphocytes and T regulatory cells from people with TLE and controls (CT) in *ex vivo* condition. (A) IL-10 expression in T cells (CD4⁺ and CD8⁺) and (B) IL-10 intracellular production by T regulatory cells (CD4⁺CD25^{high}Foxp3⁺). Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 6. Granzyme A expression by NK cells and CD8 T lymphocytes, and HLA-DR frequency in B cells from people with TLE and controls (CT) in *ex vivo* condition. (A) Granzyme A expression in CD56⁺ NK and CD8⁺ T cells and (B) HLA-DR frequency on CD19⁺ B cells. Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 7. CD86 and CD16 expression by CD14⁺ monocytes from people with TLE and controls (CT) in *ex vivo* condition. Graph (A) shows CD86 expression on different CD14⁺ monocytes subtypes. Graph (B) shows CD16 expression in different CD14⁺ monocytes subtypes. Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

Figure 8. CD86, HLA-DR, IL-6 and TNF expression by CD14⁺ monocytes from people with TLE and controls (CT) in *ex vivo* condition. Graph (A) shows HLA-DR, IL-6 and CD86 mean intensity fluorescence (MFI) in total monocytes. Graph (B) shows IL-6 and TNF expression in CD14⁺ monocytes. Significant differences were considered when $p < 0.05$ (Mann-Whitney U test).

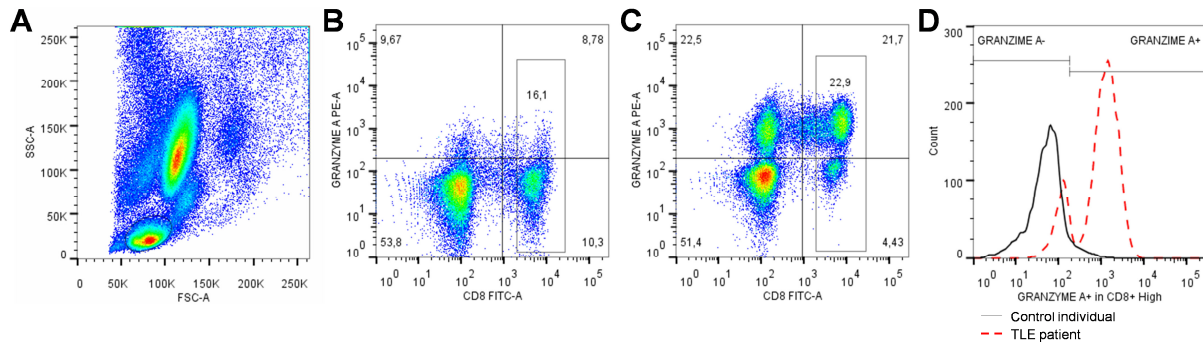
Figure 9. Peripheral inflammation could perpetuate brain damage in temporal lobe epilepsy (TLE) people. People with TLE with uncontrolled seizures may present continuous release of damage-associated molecular patterns (DAMPs) that activate microglia and astrocytes. These cells release mediator factors that induce changes in the blood brain-barrier (BBB), facilitating the infiltration of peripheral immune cells. In the blood, innate and adaptive immune responses can also be activated. Accordingly, it is observed increased expression of costimulatory surface markers and cytokines (IL-6 and TNF) by CD14⁺ monocytes, while NK and CD8⁺ cytotoxic cells produce more granzyme A, a molecule capable of inducing cell death. There is also a high frequency of CD4⁺ and CD8⁺ T cells expressing costimulatory markers and pro-inflammatory cytokines. All these immune cells might increase and/or support peripheral low-grade inflammation and brain tissue damage with further DAMPs generation. To modulate this exacerbated immune response, there is an elevated number of Treg cells producing IL-10 in TLE.

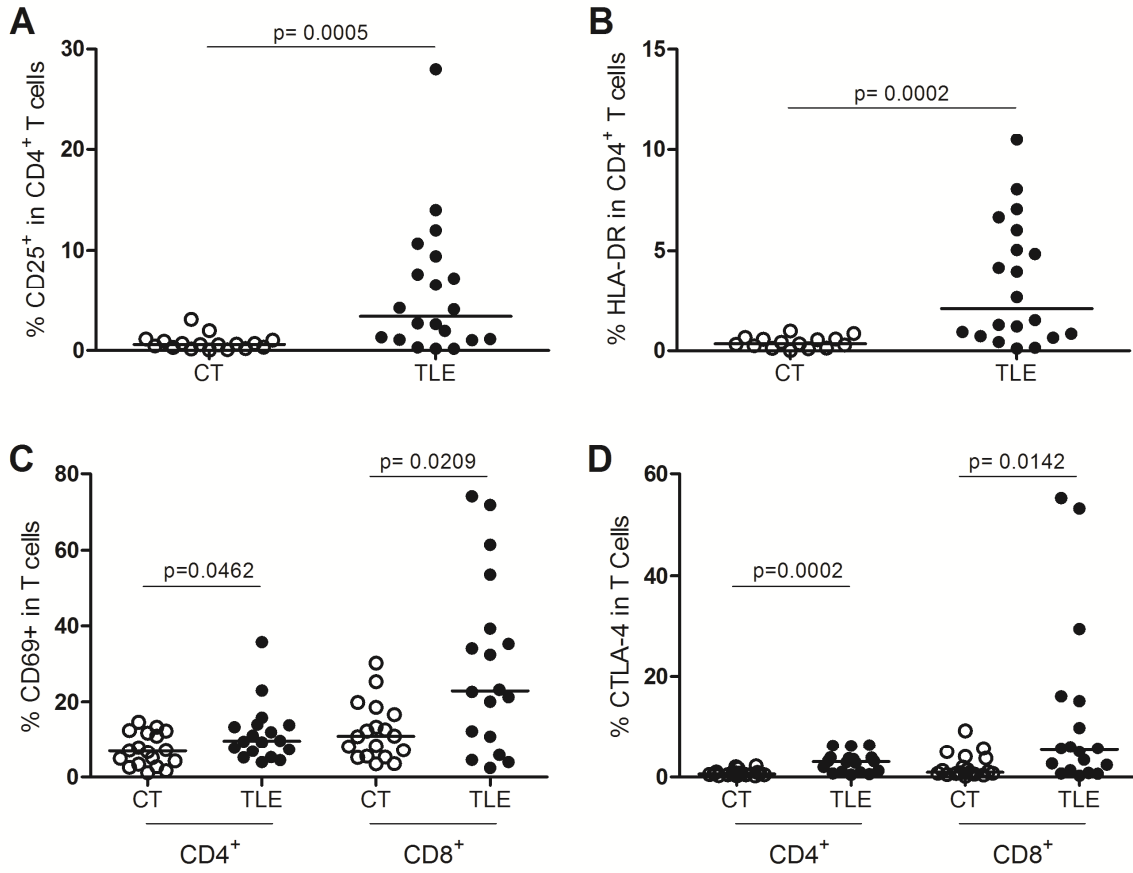
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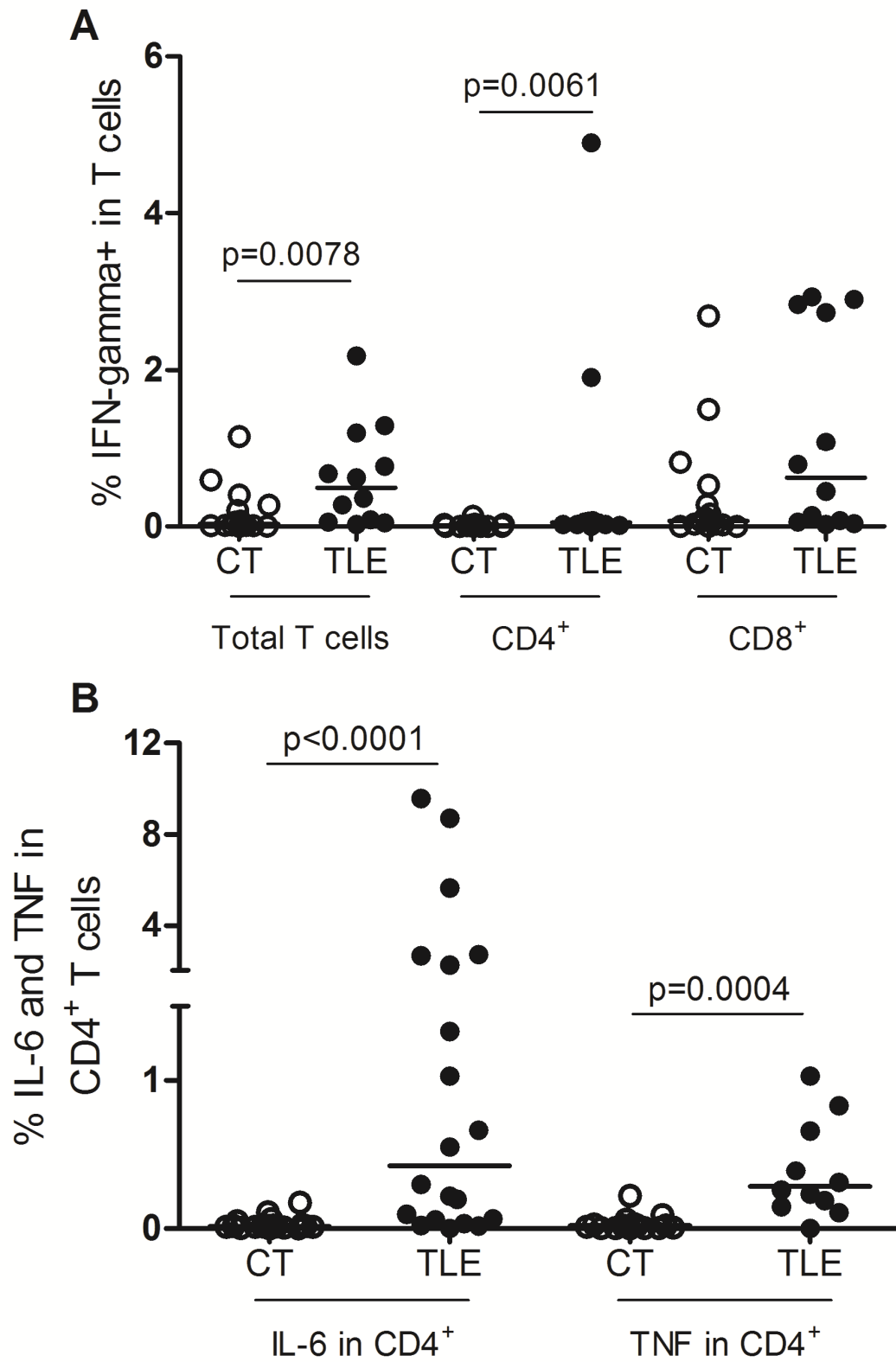
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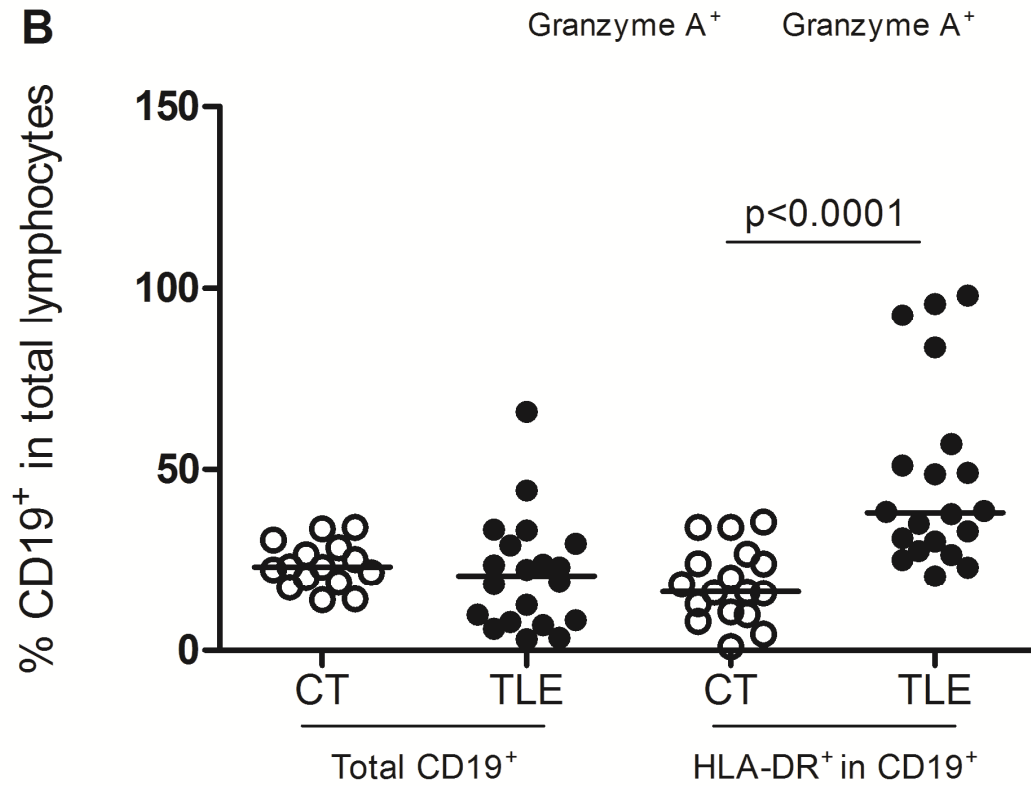
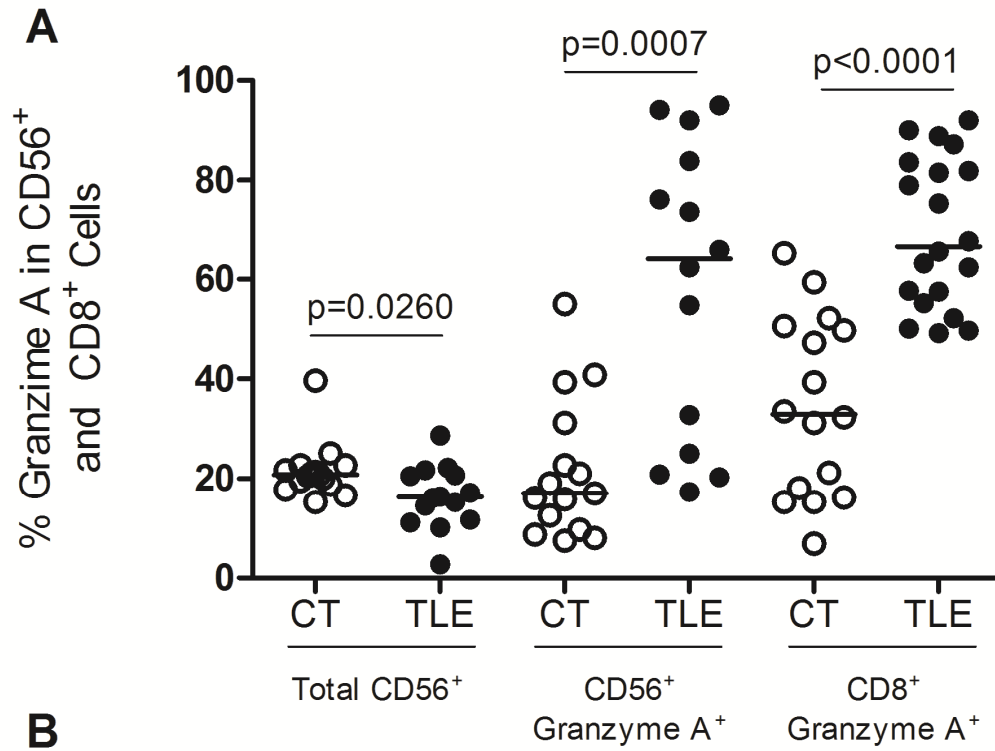
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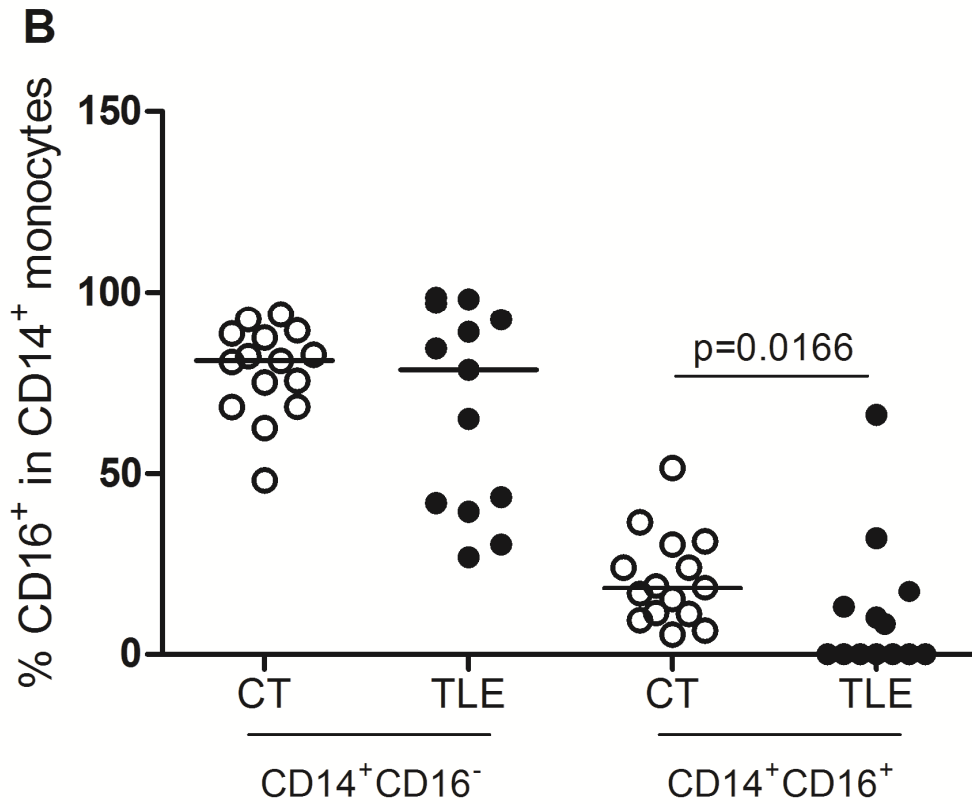
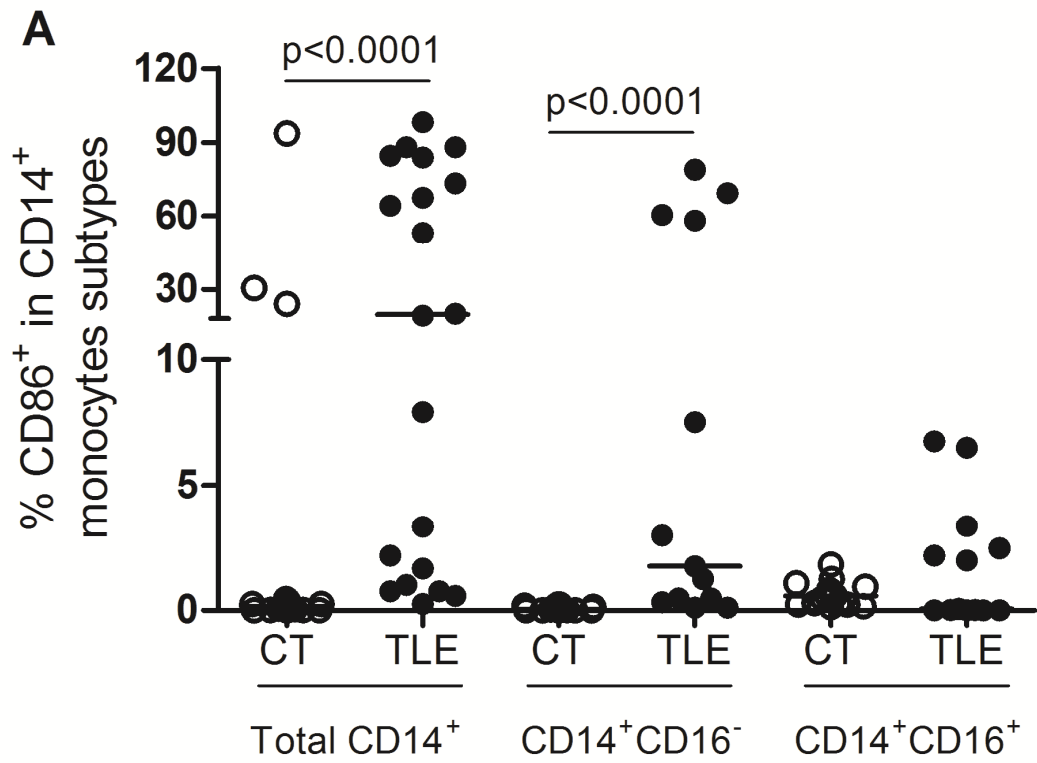


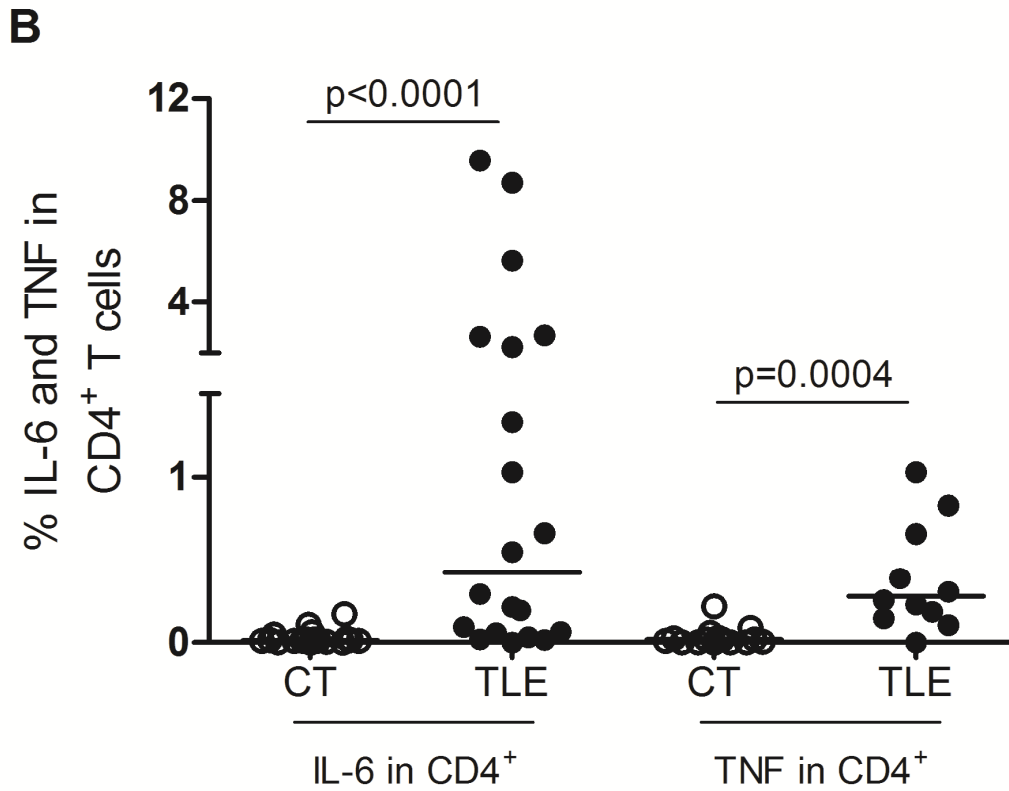
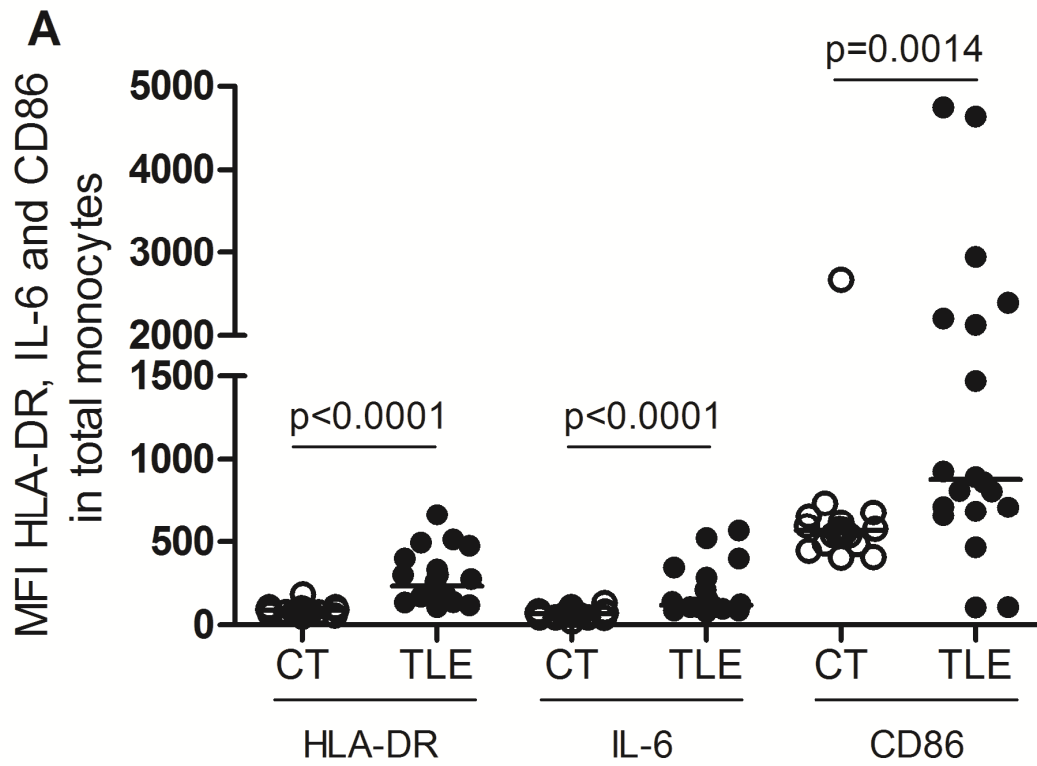


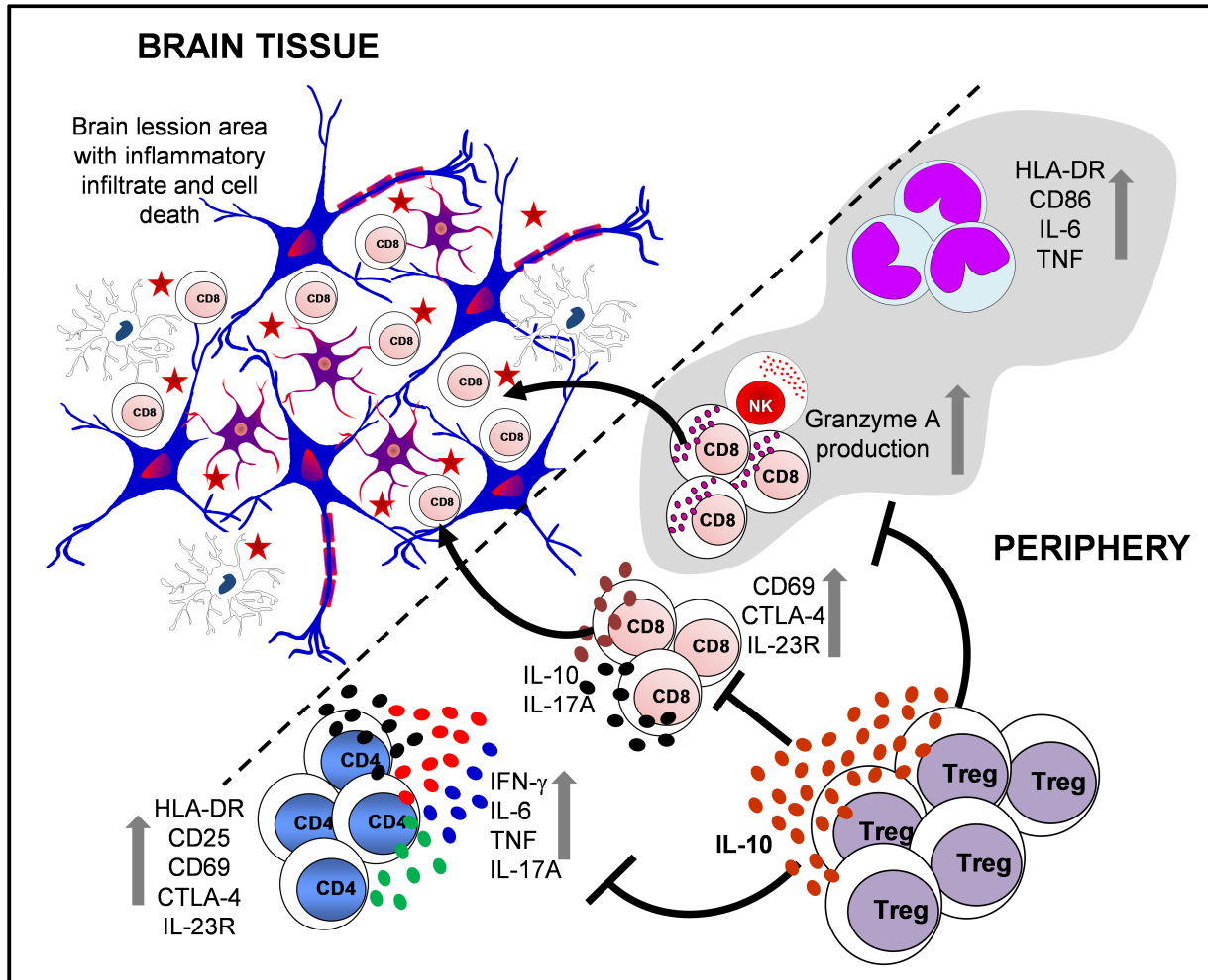
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Highlights

- 1 - Immune cells of temporal lobe epilepsy (TLE) people show an activation profile.
- 2- Regulatory T cells produced significantly more IL-10 in people with TLE whereas number of Treg cells was comparable.
- 3- Effectors T cells are involved in activation profile in TLE people.

ACCEPTED MANUSCRIPT