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Peripheral blood expression levels of inflammasome complex components in two different focal epilepsy syndromes

Canan Ulusoy[a], Ebru Nur Vanlı-Yavuz[b,c,*], Elif Şanlı[a], Özlem Timirci-Kahraman[d], Vuslat Yılmaz[a], Nerses Bebek[b], Cem İsmail Küçükali[a], Betül Baykan[b], Erdem Tüzün[a]

[a] Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey
[b] Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey
[c] Department of Neurology, Koc University, School of Medicine, Istanbul, Turkey
[d] Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Turkey

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ABSTRACT

Background: Although the role of inflammation in epilepsy pathogenesis has been extensively investigated, the inflammasome complex, a key component of neuroinflammation, has been understudied in epilepsy patients.
Methods: To better understand the involvement of this system in epilepsy, levels of inflammasome complex components (NLRP1, NLRP3, CASP1, ASC), end-products of inflammasome complex activity [IL-1β, IL-18, nitric oxide synthase (NOS) isoforms] and other inflammatory factors (NFκB, IL-6, TNF-α) were measured in peripheral blood of patients with focal epilepsy of unknown cause (FEoUC) (n = 47), mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) (n = 35) and healthy controls using real time qPCR and/or ELISA.
Results: Inflammasome complex associated factors were either downregulated or unchanged in epilepsy patients. Likewise, flow cytometry studies failed to show an increase in ratios of NLRP3-expressing CD3+ and CD14+ peripheral blood mononuclear cells (PBMC) in epileptic patients. Anti-neuronal antibody positive epilepsy patients showed increased NLRP1 and neuronal NOS mRNA expression levels, whereas patients under poly-therapy showed reduced serum inflammasome levels. FEoUC patients demonstrated increased PBMC NFκB mRNA expression levels and serum IL-1β and IL-6 levels. Both MTLE-HS and FEoUC patients displayed higher ratios of NFκB-expressing CD14+ PBMC than healthy controls.
Conclusions: Although previous clinical studies have implicated increased inflammasome complex expression levels in epilepsy, our results indicate suppressed inflammasome complex activity in the peripheral blood of focal epilepsy patients. Alternatively, the IL-6-NFκB signaling pathway, appears to be activated in focal epilepsy, suggesting that factors of this pathway might be targeted for future theranostic applications.

1. Introduction

Epilepsy is a chronic paroxysmal neurological disorder caused by various genetic and environmental factors. Animal and human studies have implicated the involvement of inflammation particularly in patients with temporal lobe epilepsy (TLE) and status epilepticus (SE) (Vezzani, 2014). Expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α is increased in TLE patients (de Vries et al., 2016). Moreover, inhibition of IL-1β and IL-6 have therapeutic effects in experimental animal models of epilepsy (Maroso et al., 2011; Iori et al., 2016).

Inflammasome complex and the associated nuclear factor kappa B (NFκB) pathway are essentially involved in initiation of neuroinflammation. The inflammasome complex is composed of NOD-like receptor protein (NLRP), caspase 1 (CASP1) and apoptosis speck-like protein (ASC) molecules. Activation of this complex in neurons and glia results in production of inflammatory cytokines (IL-1β, IL-18) and free radical producing nitric oxide synthase (NOS) isoforms (Kigerl et al.,

Abbreviations: AMPAR, Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; ASC, Apoptosis speck-like protein; CASP1, Caspase 1; CASPR2, Contactin-associated protein-like 2; FEoUC, Focal epilepsy of unknown cause; GABABR, Type B gamma aminobutyric acid receptor; GAD, Glutamic acid decarboxylase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; LGI1, Leucine-rich glioma inactivated 1; MTLE-HS, Mesial temporal lobe epilepsy with hippocampal sclerosis; NFκB, Nuclear factor kappa B; NLRP, NOD-like receptor protein; NMDAR, *N*-methyl-D-aspartate receptor; NOS, Nitric oxide synthase; PBMC, Peripheral blood mononuclear cells; SE, Status epilepticus; TLE, Temporal lobe epilepsy

* Corresponding author at: Department of Neurology, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey.
E-mail address: ebruvanli@gmail.com (E.N. Vanlı-Yavuz).

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2014; Lee et al., 2014). Neuroinflammation and oxidative stress lead to seizure-induced cell death and neuronal network alterations ultimately contributing to epileptogenesis (Ambrogini et al., 1865; Teocchi et al., 2013). Although the inflammasome complex appears to be located in the center of seizure induction in animal studies, it has been relatively understudied in epilepsy patients.

NLRP1 and CASP1 levels are increased in resected hippocampus of TLE patients (Tan et al., 2014) and NLRP3 is upregulated in peripheral blood mononuclear cells (PBMC) of children with febrile seizures (Liu et al., 2020). Cerebral NLRP3 levels have been found to be increased in animal models of epilepsy (Meng et al., 2014). Moreover, knockdown or inhibition of NLRP1 or NLRP3 has significantly reduced neuronal loss and severity of seizures (Meng et al., 2014; Shen et al., 2018; Rong et al., 2019; Sun et al., 2019; Mohseni-Moghaddam et al., 2019; Gao et al., 2018). Notably, inhibition of the purinergic receptor P2X7R, an inflammasome complex activator, has ameliorated the hippocampal inflammation and seizure severity of epileptic rats (Huang et al., 2017; Jimenez-Pacheco et al., 2016).

To identify the contribution of the inflammasome complex to the pathogenesis of focal epilepsy and to test the biomarker value of inflammasome complex proteins and their end-products in patients with focal seizures, we aimed to investigate the serum expression levels of several inflammatory factors in two different and frequent focal epilepsy syndromes, mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) and focal epilepsy of unknown cause (FEoUC).

2. Materials and methods

2.1. Epilepsy patients

Consecutive patients diagnosed with FEoUC ($n = 47$) or MTLE-HS ($n = 35$) as per International League against Epilepsy classification criteria, followed up at least one year, in our tertiary epilepsy center were enrolled. The mean age at onset and epilepsy duration of all patients were 16.30 years [11.45], 21.28 [12.00] years, respectively. All patients had at least one 1.5 or 3 Tesla magnetic resonance imaging with epilepsy protocol and diagnostic EEG and/or video-EEG investigations. MRIs were reviewed and presence of atrophy and hyperintense signal changes on T2 and FLAIR series in any one or more parts of the hippocampus were considered as major criteria to establish the neuroradiological diagnosis of HS. Epileptic patients with all other etiologies like limbic encephalitis and with dual pathologies were excluded. Clinical findings such as age, follow-up duration, gender, age of seizure onset, drug-resistance, history of status epilepticus were collected from the files of the patients systematically. Drug resistance was defined according to the ILAE criteria. As the control group, 47 healthy, age-matched volunteers (29 males, 18 females) were also enrolled after their examination and consent.

To test the impact of neuronal auto-antibody positivity on inflammation variables, previously described seven anti-neuronal antibody positive epilepsy patients with sufficiently available frozen PBMC samples were also included (Vanli-Yavuz et al., 2016). These patients were seropositive for glycine receptor ($n = 4$), *N*-methyl-D-aspartate receptor (NMDAR) ($n = 2$) and contactin-associated protein-like 2 (CASPR2) ($n = 1$). Twenty-two patients who were screened with the same protocol and found seronegative in the same study for glutamic acid decarboxylase (GAD), NMDAR, leucine-rich glioma inactivated 1 (LGI1), CASPR2, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), type B gamma aminobutyric acid receptor (GABABR) and glycine receptor antibodies were used as antibody negative patients. Antibody detection was done with a commercial kit (Euroimmun, Luebeck, Germany) containing HEK293 cells transfected with relevant plasmids.

The ethics committee approved the study protocol and all participants signed informed consent forms. The blood samples were taken under regular anti-epileptic medications except in nine patients who were in remission, due to ethical reasons in this cohort with established focal epilepsy. None of the patients experienced a seizure during blood collection.

2.2. ELISA

Serum levels of inflammasome factors (NLRP1, NLRP3, ASC, CASP1, IL-1β and IL-18), pro-inflammatory cytokines (IL-6, TNF-α), NFκB and NOS isoforms (inducible NOS, endothelial NOS, neuronal NOS) were measured with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA; Diaclone, Besancon Cedex, France; YHBioscience, Shanghai, China) according to the manufacturer's instructions.

2.3. Real time qPCR

PBMCs were separated by Ficoll density gradient centrifugation, resuspended in freezing solution and stored in liquid nitrogen (1×10^6 cells in fetal bovine serum with 10% dimethyl sulfoxide). Frozen PBMCs were thawed and washed in complete medium (enriched with 10% fetal calf serum, 1% minimum essential medium vitamin, 1% L-glutamine, 1% Na-pyruvate, 1% non-essential amino acids, 1% penicillin-streptomycin) at 1800 rpm at +4 °C for 10 min. RNA was isolated from PBMCs by using isolation kit (Jena Bioscience®, Total RNA Purification Kit, PP-210) as per manufacturer's recommendations and quality was measured by the A260/A280 and 260/230 ratio (Thermo Scientific Nanodrop 2000® Spectrophotometer). RNA was then converted to cDNA by using the SCRIPT cDNA Synthesis Kit (Jena Bioscience®, PCR511). Quantitative real-time PCR reactions were performed in Agilent Technologies Mx3005P QPCR System by using SYBR green master mix (Jena Bioscience®, qPCR GreenMaster UNG kit, PCR375) and primers obtained from DNA Technology® (DN-10) (Table 1). The relative mRNA expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression using the simplified comparative threshold cycle delta, cycle threshold (CT) method [2 − (ΔCT gene of interest − ΔCT GAPDH)].

NLRP, NOD-like receptor protein; CASP1, caspase 1; ASC, apoptosis speck-like protein; nNOS, neuronal nitric oxide synthase; NFκB, nuclear factor kappa B; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.4. NFκB and NLRP3 expression on peripheral T cells and monocytes

Frozen PBMCs were thawed and washed in complete medium. A total of 1×10^6 cells were stimulated with PMA (400 ng/ml, Adipogen,

Table 1
Forward (Frw) and reverse (Rev) primers used in real time PCR studies.

Gene	Primer sequence
IL1β_Frw	ACAGATGAAGTGCTCCTTCCA
IL1β_Rev	GTCGGAGATTCGTAGCTGGAT
IL18_Frw	TTCAAGACCAGCCTGACCAA
IL18_Rev	GCTCACCACAACCTCTACCT
NLRP1_Frw	CCGGCTCCCATTAGACAGAT
NLRP1_ Rev	ATGAGGTTCTGCAGAGCTGT
NLRP3_Frw	TCTCATGCTGCCTGTTCTCA
NLRP3_Rev	CAACACTCTCATCCCTGGGT
NNOS_Frw	CACTGTGTCCTTGGTGTTGG
NNOS_Rev	GAGCCAGGAGTGTGAGTCTT
NFκB_Frw	AGAAGCAGGCTGGAGGTAAG
NFκB_Rev	GCCATGGTTGAGCAAGGAAA
ASC_Frw	AGCCAGGCCTGCACTTTATA
ASC_Rev	CATCTTGCTTGGGTTGGTGG
CASP1_Frw	CCACATCCTCAGGCTCAGAA
CASP1_Rev	TCACATCTACGCTGTACCCC
GAPDH_Frw	GCCAT CAATGACCCC-TTCATT
GAPDH_Rev	TTGACGGTGCCATGG AATTT

Switzerland) and ionomycin (1 μg/ml, Santa Cruz, California, USA) for 20 minutes at 37 °C in 5% CO_2 at incubator. The Inside Stain Kit (Miltenyi Biotec) (for intracellular NFκB expression) and Cell Signaling Buffer Set A (Miltenyi Biotec) (for anti-NLRP3-APC expression) were used as manufacturer's recommendations on T cells (Anti human CD3-PE, BD Biosciences) and monocytes (Anti human CD45-FITC/CD14-PE, BD SimultestTM LeucogateTM). Thereafter, 6 color immunofluorescence staining was utilized (BD FACS Aria II, Becton Dickinson, Franklin Lakes, NJ). Cells were acquired for each sample and data were analyzed using the FlowJo software.

2.5. Statistics

For three group comparisons, ANOVA and Tukey's post-hoc test were used. ANOVA results were displayed in Results, whereas post-hoc test results were shown in related Figures. For comparison of anti-neuronal antibody positive and negative patient cohorts, Student's *t*-test was used. $p < .05$ was considered as statistically significant. Association of all laboratory results with clinical parameters like drug-resistance, history of status epilepticus, and current poly-therapy was assessed with independent samples test with Bonferroni correction ($p = .017$).

3. Results

3.1. Expression levels of the inflammasome complex associated factors are not increased in the peripheral blood of patients with focal epilepsy

To assess the activity of the inflammasome complex in epileptic patients, mRNA expression levels of the inflammasome complex (NLRP1, NLRP3, CASP1, ASC), the end-products of inflammasome complex activity (IL-1β, IL-18, neuronal NOS) and NFκB (required for inflammasome priming) were measured in PBMC of MTLE-HS patients, FEoUC patients and healthy controls. Notably, none of these factors showed increased expression levels in epilepsy patients in comparison to healthy controls. NLRP3 was significantly downregulated in MTLE-HS patients compared to healthy controls ($p = .002$). FEoUC patients showed similar trends although this reduction in NLRP3 expression levels did not attain significance. NFκB was significantly upregulated in FEuOC patients compared to MTLE-HS patients ($p = .036$), but not to healthy controls (Fig. 1, Table 2). mRNA expression levels of NLRP1 ($p = .367$), CASP1 ($p = .265$), ASC ($p = .445$), IL-1β ($p = .271$), IL-18 ($p = .609$) and neuronal NOS ($p = .371$) were comparable among epilepsy groups and healthy controls.

3.2. Serum levels of inflammasome factors are reduced in MTLE-HS and FEoUC patients

Levels of NLRP1 ($p = .001$), NLRP3 ($p = .002$), ASC ($p = .026$), IL-18 ($p = .004$), neuronal NOS ($p < .001$) and inducible NOS ($p < .001$) were significantly decreased in sera of MTLE-HS and/or FEoUC patients, as compared to healthy controls, as measured by ELISA. The exception was IL-6 ($p = .018$) and IL-1β ($p = .028$), which were significantly increased in both epilepsy groups as compared to healthy controls. These increases attained significance only in FEoUC-healthy control comparisons (Fig. 2, Table 2). Levels of CASP1 ($p = .636$), TNF-α ($p = .446$), NFκB ($p = .524$) and endothelial NOS ($p = .371$) were comparable among epilepsy patients and healthy controls.

3.3. MTLE-HS and FEoUC patients show increased NFκB expression in CD14+ cells

For more precise detection of immune cell-specific expression levels of inflammation pathways, we measured ratios of NLRP3 and NFκB producing CD3+ and CD14+ peripheral blood cells of randomly selected MTLE-HS patients ($n = 9$), FEoUC patients ($n = 8$) and healthy controls (n = 9) with sufficiently available PBMC samples. MTLE-HS patients showed significantly elevated CD3+ ($p = .005$) and significantly reduced CD14+ ($p = .034$) cell ratios than healthy controls (Fig. 3, Table 2). MTLE-HS and FEoUC patients showed trends towards displaying reduced ratios of CD3 + NLRP3+ ($p = .070$) and CD14 + NLRP3+ ($p = .148$) cell ratios than healthy controls. However, these differences did not attain statistical significance. While CD3 + NFκB+ cell ratios were comparable among study groups ($p = .195$), MTLE-HS and FEoUC patients had significantly higher CD14 + NFκB+ cell ratios ($p = .001$) than healthy controls (Fig. 4, Table 2).

3.4. Comparison of anti-neuronal antibody positive and negative epilepsy patients

Levels of most of the above mentioned variables measured by real time qPCR, ELISA or flow cytometry were identical among antibody positive and negative epilepsy groups, indicating that anti-neuronal antibody status did not have an appreciable effect on inflammasome complex. As an exception, antibody positive epilepsy patients showed significantly increased NLRP1 and neuronal NOS PBMC expression levels than antibody negative ones. Anti-neuronal antibody positive patients also had significantly higher CD3+ PBMC ratios than antibody negative ones (Table 3).

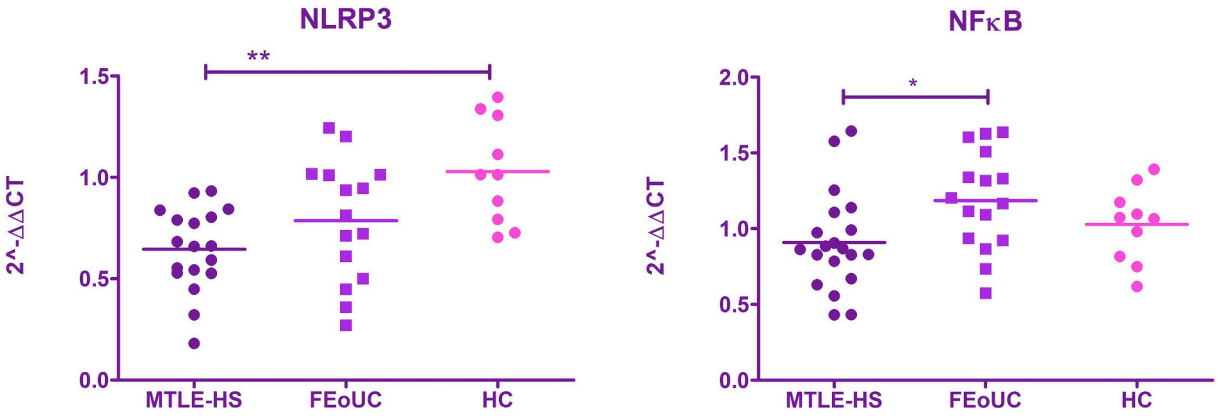

Fig. 1. Peripheral blood mononuclear cell NLRP3 and NFKB mRNA expression levels of healthy controls (HC), mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) patients and focal epilepsy of unknown cause (FEoUC) patients. Horizontal lines indicate mean values. *p* values above the horizontal lines are obtained by the Tukey's post-hoc test; *, $p < .05$, **, $p < .01$.

Table 2
Comparison of inflammation parameters among groups.

ELISA	MTLE-HS	FEoUC	Healthy controls	p value
NLRP1 (pg/ml)	83.03 ± 70.23	86.32 ± 66.95	138.3 ± 87.97	0.001
NLRP3 (pg/ml)	125.90 ± 74.16	139.90 ± 99.63	203.90 ± 137	0.002
ASC (ng/ml)	3.73 ± 2.32	5.70 ± 6.04	7.03 ± 6.12	0.026
CASP1 (ng/ml)	6.91 ± 6.66	7.25 ± 7.83	10.90 ± 10.86	0.636
IL-1β (pg/ml)	0.12 ± 0.18	0. 20 ± 0.28	0.05 ± 0.14	0.028
IL-18 (ng/L)	34.82 ± 37.44	39.96 ± 34.95	63.39 ± 50.10	0.004
IL-6 (pg/ml)	0.76 ± 14.01	0.84 ± 1.17	0.23 ± 0.55	0.018
TNF-α (pg/ml)	0.76 ± 5.10	0.0 ± 0.0	0.07 ± 0.48	0.446
NFkB (ng/ml)	2.65 ± 1.61	2.88 ± 2.21	3.79 ± 2.74	0.524
iNOS (U/L)	44.48 ± 34.51	48.22 ± 32.27	73.10 ± 42.95	< 0.001
nNOS (ng/ml)	5.82 ± 5.68	6.26 ± 5. 20	13.74 ± 11.70	< 0.001
eNOS (U/ml)	89.66 ± 74.08	96.03 ± 72.41	129.8 ± 84.73	0.371
RT-PCR (fold change)	MTLE-HS	FEoUC	Healthy Controls	p value
NLRP1	0.87 ± 0.44	0.96 ± 0.49	0.98 ± 0.29	0.367
NLRP3	0.66 ± 0. 20	0.79 ± 0.30	1.03 ± 0.26	0.002
ASC	1.76 ± 0.87	1.46 ± 0.50	1.29 ± 0.18	0.445
CASP1	0.96 ± 0.30	0.89 ± 0.29	1.02 ± 0.18	0.265
NFkB	0.91 ± 0.32	1.19 ± 0.32	1.03 ± 0.25	0.036
nNOS	1.40 ± 2.52	1.25 ± 1.11	1.15 ± 0.68	0.371
IL-1 beta	0.80 ± 0.52	0.69 ± 0.28	0.67 ± 0.52	0.271
IL-18	1.36 ± 1.39	1.23 ± 1.00	1.21 ± 0.71	0.609
Flow Cytometry	MTLE-HS	FEoUC	Healthy Controls	p value
CD3+ %	81.86 ± 6.50	75.34 ± 6.42	71.49 ± 4.21	0.005
CD14+ %	7.62 ± 2.12	10.27 ± 3.97	11.82 ± 3.14	0.034
CD3 + NLRP3+ %	0.32 ± 0.08	0.45 ± 0.22	0.63 ± 0.37	0.070
CD3 + NFkB+ %	12.43 ± 14.95	8.27 ± 9.78	2.04 ± 2.48	0.195
CD14 + NLRP3+ %	0.70 ± 0.46	0.94 ± 0.52	1.09 ± 0.81	0.148
CD14 + NFkB+ %	51.04 ± 32.38	41.19 ± 29.79	2.41 ± 3.00	0.001

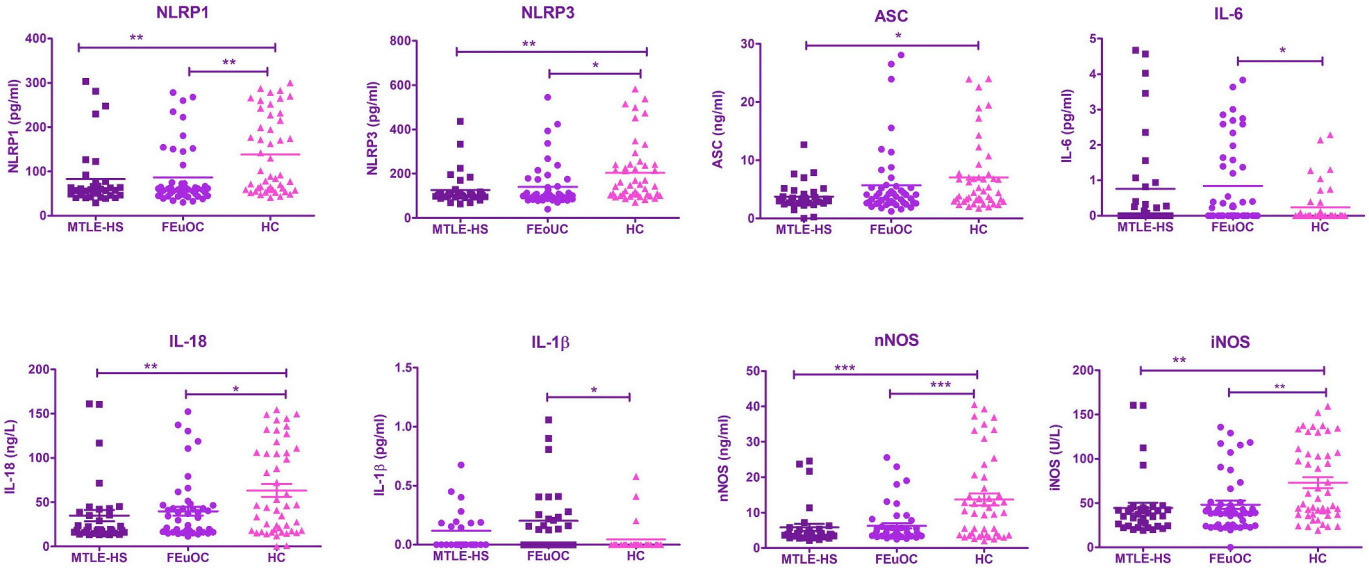

Fig. 2. Serum levels of inflammation factors significantly altered in mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) and focal epilepsy of unknown cause (FEoUC) patients as compared to healthy controls (HC). Horizontal lines indicate mean values. p values above the horizontal lines are obtained by the Tukey's post-hoc test; *, p < .05, **, p < .01, ***, *p* < .001.

3.5. Clinical associations with inflammasome parameters

Our clinical analysis showed that the group consisting of 37 patients under poly-therapy (using more than one anti-epileptic drugs) showed significantly lower serum levels of NRLP1 (p = .017), ASC (p = .007), IL18 (*p* = .007), neuronal NOS (0 = 001) and endothelial NOS (*p* = .003) levels as compared to those under mono-therapy. There were no significant differences between patients with or without status epilepticus, and with or without drug-resistance in any evaluated variables (not shown).

Furthermore, the results from flow cytometry as well as real time PCR analyses did not show any association with clinical parameters like drug-resistance, presence of status epilepticus, poly-therapy or type of current antiepileptic drug (not shown). While most of the patients were using sodium channel blockers, their doses and add-on therapies varied widely, making any conclusion related to antiepileptic medications unlikely.

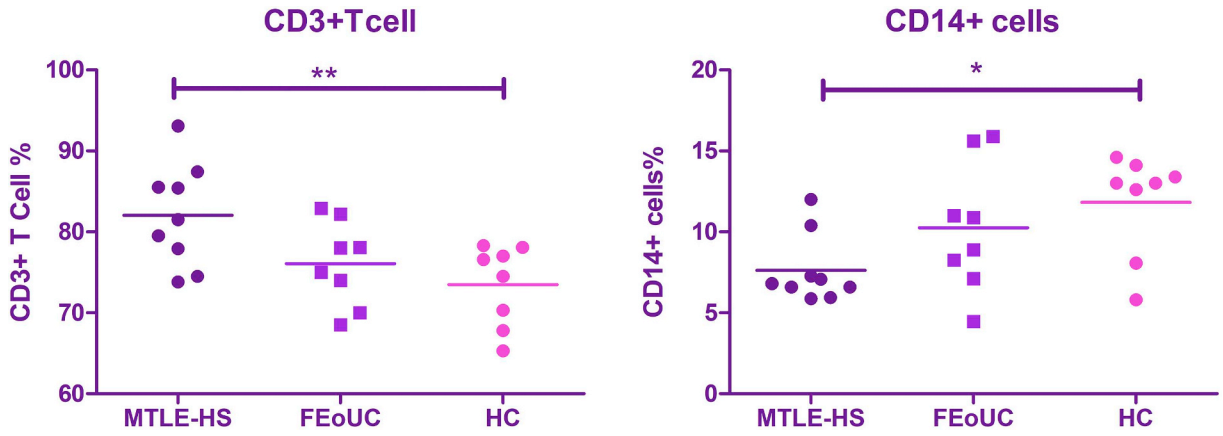

Fig. 3. Flow cytometric analysis of CD3 + and CD14 + peripheral blood mononuclear cells of healthy controls (HC), mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) patients and focal epilepsy of unknown cause (FEoUC) patients. Horizontal lines indicate mean values. p values above the horizontal lines are obtained by the Tukey's post-hoc test; *, p < .05, **, p < .01.

4. Discussion

Various studies have revealed that immunological disturbance is one of the fundamental contributing factors, if not the main culprit, of epilepsy and placed immune etiology as one of the five main etiological factors in the ILAE classification (Rong et al., 2019; Leal et al., 2017; Rana and Musto, 2018). Several recent animal studies have implicated the seminal role played by inflammasome complex, the pioneer pathway of inflammation, in the pathogenesis of epilepsy (Meng et al., 2014; Shen et al., 2018; Rong et al., 2019; Sun et al., 2019; Mohseni-Moghaddam et al., 2019; Gao et al., 2018). Moreover, in a single paper, TLE patients with drug-resistant epilepsy have been shown to display increased cerebral NLRP1 and CASP1 expression as compared to patients with no history of epilepsy (Tan et al., 2014).

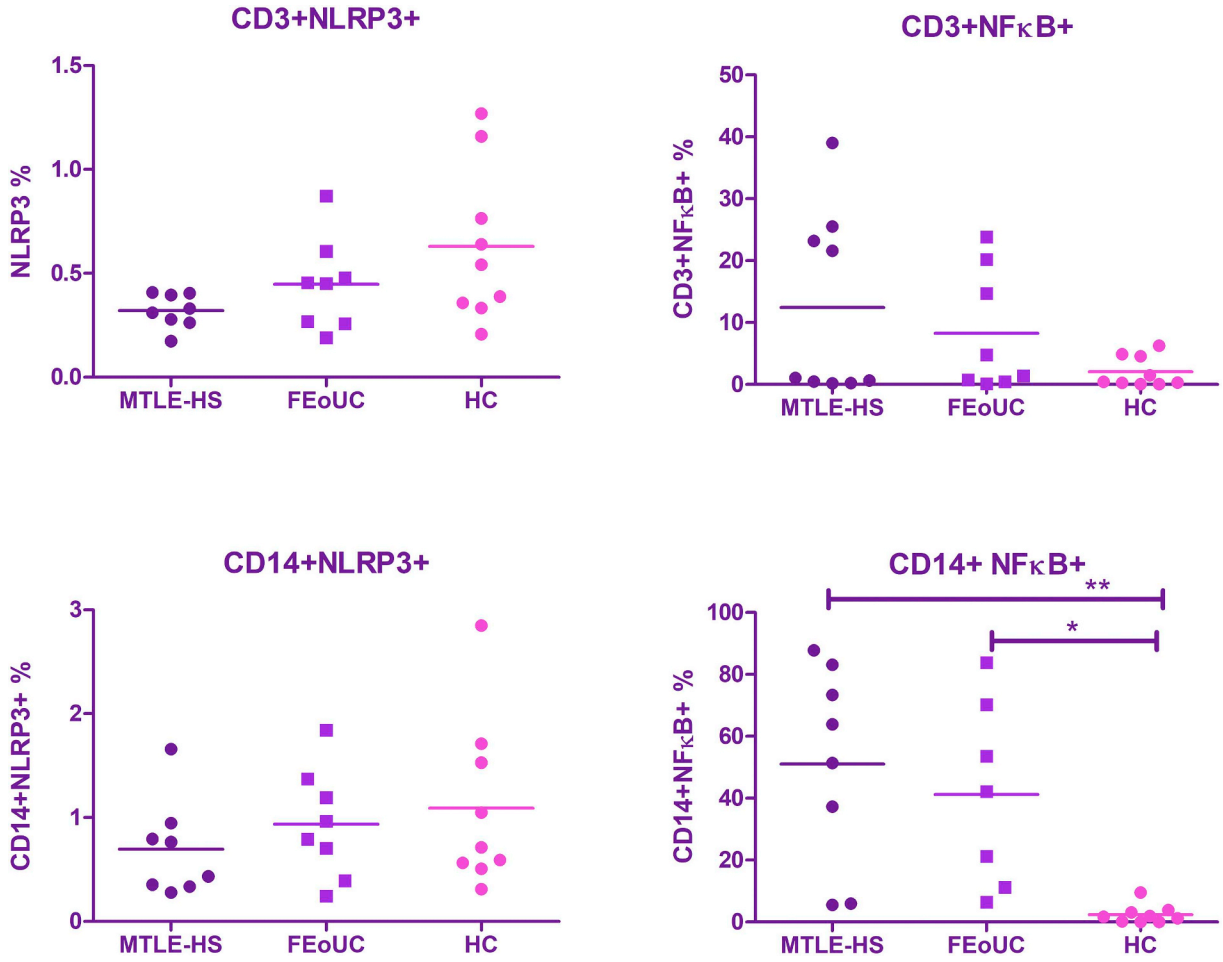

Fig. 4. Flow cytometric analysis of NLRP3 + and NFKB + peripheral blood CD3 and CD14 + cells of healthy controls (HC), mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS) patients and focal epilepsy of unknown cause (FEoUC) patients. Horizontal lines indicate mean values. p values above the horizontal lines are obtained by the Tukey's post-hoc test; *, p < .05, **, p < .01.

Table 3
Comparison of inflammation parameters among anti-neuronal antibody (Ab+) and negative (Ab-) epilepsy patients.

ELISA	Ab +	Ab -	p value
NLRP1 (pg/ml)	68.49 ± 26.66	81.92 ± 68.96	0.533
NLRP3 (pg/ml)	120.00 ± 27.68	111. 20 ± 38.49	0.487
ASC (ng/ml)	4.03 ± 2. 20	3.53 ± 1.44	0.387
CASP1 (ng/ml)	7.65 ± 6.09	4.57 ± 3.89	0.059
IL-1β (pg/ml)	0.10 ± 0.18	0. 20 ± 0.28	0.424
IL-18 (ng/L)	26.71 ± 11.34	40.56 ± 41.34	0.281
IL-6 (pg/ml)	0.75 ± 1.58	1.96 ± 3.66	0.319
TNF-alpha (pg/ml)	0.0 ± 0.0	0.0 ± 0.0	> 0.999
NFkB (ng/ml)	2.67 ± 1.34	2.36 ± 1.44	0.529
iNOS (U/L)	38.60 ± 16.06	45.59 ± 33.96	0.514
nNOS (ng/ml)	4.44 ± 1.06	6.21 ± 6.11	0.346
eNOS (U/ml)	71.44 ± 15.17	96.89 ± 82.27	0.316
RT-PCR (fold change)	Ab +	Ab -	p value
NLRP1	1.83 ± 1.49	0.85 ± 0.47	0.024
NLRP3	0.72 ± 0.18	0.83 ± 0.53	0.524
ASC	1.36 ± 0.64	1.05 ± 0.38	0.233
CASP1	1.18 ± 0.31	0.98 ± 0.35	0.244
NFkB	1.05 ± 0.38	1.03 ± 0.36	0.877
nNOS	2.42 ± 1.60	1.05 ± 1.08	0.017
IL-1 beta	0.53 ± 0.27	0.60 ± 0.29	0.656
IL-18	2.16 ± 1.67	1.24 ± 0.99	0.086
Flow Cytometry (%)	Ab +	Ab -	p value
CD3+	83.9 ± 5.7	75.9 ± 5.5	0.017
CD14+	11.16 ± 3.87	9.10 ± 2.90	0.259
CD3 + NLRP3+	0.48 ± 0.25	0.32 ± 0.12	0.134
CD3 + NFkB+	10.92 ± 11.86	10.08 ± 14.61	0.905
CD14 + NLRP3+	0.69 ± 0.42	0.79 ± 0.48	0.692
CD14 + NFkB+	44.16 ± 30.03	38.93 ± 36.31	0.768

All results are shown as mean ± standard deviation.

Inflammasome complex molecules, particularly NLRP3, is mainly expressed by glial cells in the brain (Thawkar and Kaur, 2019). Since several peripheral blood cell types such as monocytes belong to the same mononuclear phagocytic system as microglia, we reasoned that inflammasome activity could also have been increased in peripheral blood of epilepsy patients and thus expression levels of inflammasome complex components could be used for monitoring disease activity of epilepsy. We selected patients with focal epilepsy for this purpose, since neuroinflammation is believed to be more frequently observed in this epilepsy type (Yeshokumar and Pardo, 2017). In contrast with our assertion, serum, PBMC, CD3+ T cell and CD14+ phagocytic cells of these patients showed reduced or unaltered inflammasome expression levels, as confirmed by different complementary methods. The only exception was IL-1β, which showed a marginal increase in FEoUC patients only in serum but not in PBMC. However, this cytokine can be produced by non-immune system cells without necessarily requiring activation of the NLRP3 complex.

A potential reason of the discrepancy between our study and previous ones could be commencement of counteractive compensatory mechanisms to ease the intensity of neuroinflammation in epileptic brain. Neuronal excitability may enhance the activity of inflammasome complex (Zheng et al., 2019). In return, both IL-1β and IL-18, end-products of inflammasome activation, may enhance neuronal excitability thereby worsening epileptic seizures (Richter et al., 2017; Kanno et al., 2004). Thus, our results may be a reflection of compensatory downregulation of the inflammasome system to decrease the production of excitotoxic inflammatory factors. Another potential explanation could be the suppression of the inflammasome complex due to chronic exposure to anti-epileptic medications. Our clinical correlations showing that the group under poly-therapy showed significantly lower levels of inflammasome parameters may also support this assumption. However, to our knowledge, there are no reports exhibiting this kind of suppressive effect of medications frequently used in focal epilepsy patients, such as carbamazepine and levetiracetam.

Our results do not entirely exclude the possibility that the inflammasome complex is activated during epileptic seizures. As a limitation of our study, we did not intend to take ictal serum samples, which would be difficult for most of the patients with low seizure frequency and all samples were taken during the interictal periods. The time between the last seizure and blood collection was also very different between individuals and not precisely available in some patients' medical records. Therefore, the impact of seizure occurrence on peripheral blood inflammasome activation pends to be delineated in future studies. Secondly, while displaying reduced baseline levels of inflammasome factors, immune cells may give exaggerated inflammatory responses to specific stimulants, some of which, such as ATP, can be produced in exceedingly high levels during seizures. Since our primary goal in this study was to determine the baseline levels of inflammasome factors in epilepsy patients during remission, we did not utilize classical stimulators of the inflammasome complex (e.g. ATP) in flow cytometry assays. Therefore, the response of immune cells of focal epilepsy patients to different extracellular stimuli needs to be further characterized.

Since neutralization of the inflammasome complex is known to dampen innate immune responses (de Rivero Vaccari et al., 2009), it is important to understand why focal epilepsy patients do not exhibit increased liability to infections. As shown in our flow cytometry studies, MTLE-HS and FEoUC patients do not display significantly decreased immune cell ratios or decreased immune cell expression of NLRP3/NFκB molecules per se. It is tempting to speculate that, reduced inflammasome activity might be compensated by enhanced expression of alternative inflammation pathways.

In this context, to better grasp the expression alterations of inflammasome components, we assessed relative expression levels of other inflammation factors (NFκB, IL-6 and TNF-α) that function in concert with the inflammasome network. Notably, in contrast with inflammasome factors, IL-6 and NFκB levels were increased in the peripheral blood of FEoUC patients at protein and mRNA levels, respectively. Association between increased IL-6 production and seizures has long been established (Alapirtti et al., 2018). Binding of IL-6 to the IL-6 receptor activates NFκB activity, which consecutively induces production of proinflammatory cytokines, including IL-6 (Damsker et al., 2016; Abualsunun et al., 2020). This exclusive IL-6-NFκB signaling loop appears to be activated in our focal epilepsy patients. Inhibition of inflammasome-induced IL-1β and IL-18 cytokines, in parallel with increase of NFκB-induced IL-6 cytokine further verifies divergent responses of different inflammation systems in focal epilepsy patients and emphasizes that these patients do not exhibit a total shut down of inflammation.

An intriguing finding was the enhanced NLRP1 and neuronal NOS (end-product of NLRP1 activation) mRNA expression in PBMC of anti-neuronal antibody positive epilepsy patients. Since several autoantibodies have previously been shown to enhance inflammasome activity by directly interacting with their target molecules expressed by mononuclear phagocytic cells (Labzin et al., 2019; Zhang et al., 2016), it is tempting to speculate that anti-neuronal antibodies may have directly caused this effect. This assertion needs to be further tested by assessment of NLRP1 expression levels in antibody-positive patients before and after depletion of serum antibodies by immunosuppressive medications. Detection of increased ratios of peripheral blood CD3+ cells in antibody positive patients may be a reflection of enhanced inflammation in these patients and is congruent with the finding that antibody positive patients display a higher abundance of infiltrating CD3+ cells in their hippocampi (Vanli-Yavuz et al., 2016; Ünverengil et al., 2016).

Both mRNA expression levels of PBMC and levels of molecules in the whole serum might be influenced by inflammasome components produced by non-immune cells. Therefore, to delineate more precisely the inflammasome activity of immune cells, we assessed the PBMC ratios of NLRP3 producing CD3+ T cells and CD14+ cells. With this third assay, we once again confirmed that NLRP3 expression is not upregulated even in the immune cells of focal epilepsy patients. CD14+ cells are expressed mainly by the mononuclear phagocytic cells such as macrophages, monocytes and dendritic cells (Simmons et al., 1989). T cells and macrophages are the predominant infiltrating cell types in the affected brain regions of focal epilepsy patients (Vanli-Yavuz et al., 2016; Ünverengil et al., 2016). Therefore, it is notable that both MTLE-HS and FEoUC patients display significantly increased proportions of NFκB-producing mononuclear phagocytic cells in their circulation. Thus our results suggest that the inflammation system that is genuinely associated with focal epilepsy syndromes without a well-defined etiology is NFκB-producing CD14+ cells. To our knowledge, this association with CD14+ cells and focal epilepsies has never been reported.

5. Conclusion

In summary, inflammasome system is not upregulated in the peripheral blood possibly due to its interaction with potential confounders such as anti-epileptic treatment. Animal studies of epilepsy may not be accurately reflecting the inflammasome status since, in contrast with most epileptic patients, epileptic animals are drug-naïve and have a short duration of seizure activity. On the other hand, the IL-6-NFκB signaling pathway might be more directly associated with clinical severity of epilepsy and neuroinflammation. For this reason, components of this pathway might be useful as biomarkers and therapeutic targets.

Authors contributions

CU, ENV-Y, ET, BB and CIK designed the study and wrote the manuscript. ENV-Y, BB and NB collected data. ES and OTK were responsible for RT-PCR experiments. CU and ET were responsible for ELISA experiments. CU and VY were responsible for flow cytometer experiments. All authors revised and approved the final manuscript.

Availability of supporting data

All raw data used and analyzed in the current study are available from the corresponding author on reasonable request.

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Ethical approval and consent to participate

The study protocol was approved by the Istanbul University Istanbul Medical Faculty Clinical Research Ethics Committe (date: 11/11/2016 number: 2016/1295). The written informed consents were obtained from all patients.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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