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Increased production of inflammatory cytokines by circulating monocytes in mesial temporal lobe epilepsy: A possible role in drug resistance

C. Milano [a,*], M. Montali [b], S. Barachini [b], I.S. Burzi [b], F. Pratesi [c], L. Petrozzi [a], L. Chico [a], R. Morganti [d], G. Gambino [b], L. Rossi [b], R. Ceravolo [a], G. Siciliano [a], P. Migliorini [e], I. Petrini [c], C. Pizzanelli [a,*]

[a] Department of Clinical and Experimental Medicine, Neurology Unit, University of Pisa, Pisa, Italy
[b] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
[c] Department of Translational Research and New Technology in Medicine and Surgery, University of Pisa, Pisa, Italy
[d] Section of Statistics, University of Pisa, Pisa, Italy
[e] Department of Clinical and Experimental Medicine, Clinical Immunology and Allergy Unit, University of Pisa, Pisa, Italy

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ABSTRACT

We analyzed peripheral blood mononuclear cells (PBMCs) and serum inflammatory biomarkers in patients with mesial temporal lobe epilepsy (drug-resistant – DR, vs. drug-sensitive – DS). Patients with epilepsy showed higher levels of serum CCL2, CCL3, IL-8 and AOPP, and lower levels of FRAP and thiols compared to healthy controls (HC). Although none of the serum biomarkers distinguished DR from DS patients, when analysing intracellular cytokines after in vitro stimulation, DR patients presented higher percentages of IL-1β and IL-6 positive monocytes compared to DS patients and HC. Circulating innate immune cells might be implicated in DR epilepsy and constitute potential new targets for treatments.

1. Introduction

Epilepsy is a chronic brain disease characterized by a stable susceptibility of the affected brain to initiate seizures not provoked by any immediate central nervous system (CNS) insult (Fisher et al., 2005).

Epilepsy is among the most widespread neurological disorders, considering that it affects around 50 million people worldwide (Beghi, 2020). Currently available anti seizure medications (ASMs) are almost exclusively symptomatic drugs, thus they are able to prevent seizure's recurrence, but they do not act against the cause of epilepsy and they do not stop disease progression.

Moreover, in spite of a large number of available ASMs, about a third of patients with epilepsy (PWE) are drug-resistant (DR), i.e. continue to have seizures regardless of at least two therapeutic attempts with appropriate drugs at appropriate doses (Kwan et al., 2010).

The mechanisms determining resistance to ASMs are still not completely recognized. However, in the last decades, a number of experimental and clinical data have indicated that inflammatory mechanisms occurring within the CNS may be involved in the process of epileptogenesis and in the recurrence of epileptic seizures (Aronica et al., 2017; Iori et al., 2016; Terrone et al., 2016; van Vliet et al., 2018; Vezzani et al., 2019; Vezzani and Viviani, 2016; Vezzani et al., 2016).

In particular, the favorable response to steroids in some forms of drug-resistant epilepsy (DRE), the increased incidence of epilepsy in people suffering from systemic autoimmune diseases, as well as the most recent discovery of autoimmune encephalitis as a possible cause of seizures have suggested that immune system may be of critical concern in some cases.

Consistently, numerous inflammatory mediators and immune cells have been found in the brain tissue of DR patients undergoing epilepsy surgery, thus supporting the hypothesis that a chronic inflammatory response is also present in epilepsies not classically linked to immune system dysfunction, rather than being a specific feature of only inflammatory or autoimmune etiologies (Aronica and Crino, 2011; Aronica and Gorter, 2007; Ravizza et al., 2008; Vezzani et al., 2011a).

The process of inflammation that takes place in the CNS is called "neuroinflammation", and it is closely related to oxidative stress processes. These two phenomena are functionally interconnected and reinforce each other, both contributing to reduce epileptic threshold (Hsieh and Yang, 2013; Rowley and Patel, 2013; Vezzani et al., 2015).

* Corresponding authors at: Neurology Unit, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy.
E-mail addresses: chiara.milano@phd.unipi.it (C. Milano), c.pizzanelli@ao-pisa.toscana.it (C. Pizzanelli).

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Inflammatory mediators can be produced both by CNS resident cells, i.e., astrocytes, microglia, neurons, and endothelial cells of blood-brain barrier (BBB) – or by peripheral immunocompetent cells that are recruited into the inflamed epileptic focus from the blood circulation by continuous microglia activation (Banks and Erickson, 2010; Ransohoff et al., 2003; Rodríguez-Gómez et al., 2020).

To further sustain the hypothesis of peripheral immune cell recruitment in the epileptic focus, several anatomopathological studies demonstrated increased amounts of chemokines in the brain tissue of patients with epilepsy and of epileptic experimental animal models, i.e. molecules able to attract peripheral immune cells in inflammatory sites, to promote their adhesion to the endothelium of the BBB, and their invasion of the brain parenchyma (Bozzi and Caleo, 2016; de Vries et al., 2016).

Circulating cells of innate immunity, in particular monocytes, may therefore be able to penetrate the brain parenchyma and to differentiate into microglia-like cells, thus playing a part in the pathogenesis of seizures (Feng et al., 2019; Ginhoux et al., 2013; Varvel et al., 2016; Yamanaka et al., 2021a).

The adaptive immune system is also believed to play a role: T cell infiltrates have been documented in several forms of epilepsy, in particular in mesial temporal lobe epilepsy (MTLE), the most common DR focal form in adulthood (Bauer et al., 2017; Lu et al., 2017). Depending on the specific etiology of MTLE, a variable number of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells have been identified in resected hippocampi of patients, with the latter that seem to correlate with the degree of neuronal loss (Lu et al., 2017; Tröscher et al., 2021).

Consequently, a promising field of research is focused on the role of peripheral immune cells in epilepsy and drug-resistance. Some authors suggested that peripheral inflammatory mediators are differentially expressed in DR PWE compared to healthy subjects and, consequently, they may be utilized as biomarkers for diagnostic, prognostic, or therapeutic purposes (Balosso et al., 2005; Bauer et al., 2017; van Vliet et al., 2018; Yamanaka et al., 2021c; Yamanaka et al., 2021b).

To date, while the majority of data refers to DR form of epilepsy, only few information is available on drug-sensitive (DS) patients, and the differences of inflammatory biomarkers and peripheral immune cells within epilepsy population (DR vs. DS) remain only partially understood.

In this study, we analyzed an extended panel of serum inflammatory molecules and oxidative stress biomarkers in patients with MTLE, divided on the basis of their response to ASMs in DR and DS patients. Moreover, for a subgroup of patients, we isolated peripheral blood mononuclear cells (PBMCs) and we stimulated in vitro both monocytes and lymphocytes, to define the profile of intracellular cytokines expression of circulating immune cells by flow cytometry.

2. Materials and methods

2.1. Study population

Forty-seven patients diagnosed with MTLE (25 DR and 22 DS) were enrolled at the Epilepsy Clinic of the Azienda Ospedaliero-Universitaria Pisana from May to December 2022.

Diagnosis of MTLE was based on International League Against Epilepsy (ILAE) classification of seizures and epilepsy (Fisher et al., 2014; Scheffer et al., 2017), and on clinical ictal features, i.e., seizures with manual and oroalimentary automatisms or prominent experiential phenomena, gustatory or olfactory hallucination, eventually followed by arrest and unresponsiveness (Proposal for revised classification of epilepsies and epileptic syndromes, 1989). Patients were categorized as DR if they continued to experience seizures despite at least two adequate trials of ASMs, and as DS if they had been seizure-free for at least 12 months or three times the longest pretreatment interseizure interval (whichever was longer), according to the ILAE definition (Kwan et al., 2010). The term "seizure-free" was referred to these patients who experienced freedom from all seizures types, including auras.

We included patients with a follow-up of at least 2 years, and a disease duration of at least 3 years. We excluded: (a) subjects with other concomitant neurological diseases, (b) subjects with systemic autoimmune diseases, (c) subjects with a history of infections, inflammations, immunizations or allergic reactions during the previous 3 weeks, (d) subjects who took immunomodulant or immunosuppressive therapies, antitumor drugs and antioxidant supplements during the previous 3 months, (e) pregnant or breastfeeding subjects.

Twenty-five healthy controls (HC) matched for sex and age were recruited among personnel of AOUP.

Demographic and clinical data were collected for each participant: the demographic features included sex and age, while the clinical features included age at seizure onset, duration of the disease, number of ASMs used, number of seizure/months, epilepsy etiology based on ILAE classification.

We defined as "structural etiology" those cases with structural brain abnormalities inferred to cause patients' seizures, i.e., hippocampal sclerosis (HS), temporal focal cortical dysplasia (FCD), temporal dermoid cysts, and dysembryoplastic neuroepithelial tumor (DNET).

All participants were required to provide written informed consent. All procedures were approved by the Research Ethic Committee of Azienda Ospedaliero-Universitaria Pisana (Protocol EPINOX_21341) and the study was carried out according to the Helsinki Declaration.

2.2. Blood collection

Venous blood samples were drawn between 7.30 and 9.00 AM to reduce the variability due to physiological circadian fluctuations in the immune activity. All PWE's samples were collected during the interictal period, i.e. after at least 72 h from the last reported seizure, since it has been documented that levels of circulating cytokines increase during seizures and return to their basal level between 24 and 72 h (Lehtimäki et al., 2007; Vieira et al., 2016).

The following biomarkers were measured:

- Serum cytokines and chemokines: IL-6, TNF-α, IL-33, IL-8, CCL2, IL-13, IL-1β, IFN-γ, IL-1Ra, CCL3, IL-4, CCL4, IL-5, IL-1α, IL-17 A, IL-18, IL-33r, IL-1RII, IL-1RI;
- Oxidative stress biomarkers: ferric reducing antioxidant power (FRAP), advanced oxidation protein products (AOPP), and thiols (SH);
- Intracellular cytokine: IL-1β, IL-6, IL-1Ra, IFN-γ, IL-10, TNF-α produced by monocytes, and IFN-γ and IL-17 A produced by $CD4^+$ and $CD8^+$ T lymphocytes.

Blood samples for serum cytokines and thiols were collected without anticoagulant, blood samples for AOPP and intracellular cytokines were collected with dipotassium ethylenediaminetetraacetic acid (EDTA), while blood samples for FRAP were collected in lithium heparin tubes. All the tubes for the analysis of serum cytokines and oxidative stress biomarkers were centrifuged at 3000 rpm for 10 min at 4 °C. The serum/plasma was collected and stored at −80 °C until the assay procedures, that were carried out within one month from the collection.

All the samples for intracellular cytokines detection were freshly treated and analyzed, according to the protocol described below.

While serum cytokines and oxidative stress biomarkers were evaluated in all the patients and HC enrolled in the study, intracellular cytokines were measured in a subgroup of patient, composed by 16 DR, 15 DS and 10 HC.

2.3. Analysis of serum cytokines and chemokines

Serum cytokines were measured using a R&D Systems Luminex Assay (Bio-Techne, Minneapolis, MN, USA), a magnetic bead-based sandwich immunoassay for cytokines, in accordance with the

manufacturer's instructions. The cytokines and chemokines expression panel was chosen from the available commercial products but customized for the specific needs of the project.

Two custom panels Human Magnetic Luminex Assay were purchased. Both panels allowed us with an ideal speed and sensitivity to simultaneously detect several analytes in technical duplicate, using FlexMap3D instrument (Luminex Corporation, Austin, TX).

Two different software (Xponent by Luminex Corporation, Austin, TX, and Belysa Immunoassay Curve-Fitting by Sigma-Aldrich, St.Louise, USA) were used for the analysis of the run samples.

2.4. Analysis of oxidative stress biomarkers

AOPP, FRAP and thiols levels were determined as previously described (Mancuso et al., 2010). Briefly, for AOPP evaluation, plasma was mixed with PBS, glacial acetic acid and potassium iodide. The absorbance was read spectrophotometrically at 340 nm and compared with a solution of chloramine T dissolved in the same buffer. Values were expressed as mmol/l of chloramine equivalents. For FRAP dosage, the FRAP reagent (sodium-acetate, tripyridyltriazine in hydrochloric acid, and ferric chloride, in a ratio 10:1:1), pre-warmed at 37 °C, was mixed with plasma; the absorbance was read after 4 min at 620 nm, and a calibration curve was established by a solution of iron sulphate in hydrochloric acid (Benzie and Strain, 1996). Values were expressed in mmol/l.

To determine thiols levels, plasma was mixed with tris-EDTA solution, 5,5′-dithiobis-2-nitrobenzoic acid, and absolute methanol. This mixture was incubated at room temperature for 20 min, samples were centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was assessed at 405 nm. Values were expressed in nmol/l.

2.5. Isolation of PBMCs and stimulation of cytokine production

PBMCs were isolated from whole blood by Ficoll-Paque density gradient centrifugation (Ficoll-Paque, Cytiva Europe GmbH, Friburg, Germany). In detail, whole blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline (D-PBS, Thermo Fisher Scientific, Massachusetts, USA) at room temperature, stratified on Ficoll-Paque, then centrifuged at 1800 rpm, for 30 min, with acceleration and deceleration set to zero.

PBMCs were collected from the Ficoll-Paque-plasma interface into a 15 mL tube, washed with D-PBS, then resuspended in 2 mL of RPMI + FBS 10% (Thermo Fisher Scientific, Massachusetts, USA) and counted in a Burker chamber after Trypan Blue staining. A total of 300.000 cells resuspended in 1 mL RPMI + FBS 10% were distributed in each 15 mL tube. For each patient, 3 tubes were used to assess cytokine production: two for monocytes (IL-1β, IL-6, IL-1Ra, IFN-γ, IL-10, and TNF-α analysis) and one for lymphocytes (IFN-γ and IL-17 A analysis).

Cells were incubated with REAfinity® anti-human CD14 Vioblue-conjugated antibody (Miltenyi Biotec GmbH, Bergish Gladbach, Germany), and with CD4 Pe-Cy7-conjugated antibody (BD Biosciences, San Diego, USA) for 15 min at room temperature in the dark, then washed.

The samples were stimulated using Lipopolysaccharide (LPS, 1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for monocytes stimulation, and Phorbol Myristate Acetate (PMA, 10 ng/mL; Sigma-Aldrich) plus ionomycin (1 μg/mL; Sigma-Aldrich) for lymphocytes stimulation.

Cells were then incubated at 37 °C for 2 h. After this time, brefeldin A (BioLegend, San Diego, CA, USA) was added at a concentration of 1 μg/mL, and cells were incubated at 37 °C for additional 3 h.

After 5 total hours, cells were washed with D-PBS, then fixed in 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, washed and resuspended in phosphate-buffered saline/bovine serum albumin (PBS/BSA) 0,5%. Cells were then left overnight at +4 °C.

2.6. Intracellular staining and cytofluorimetric analysis

Cells were permeabilized with saponin 0,5% and stained with REAfinity® anti-human CD3 APC-Vio770-conjugated, CD8 FITC-conjugated, IL-1β Vio515-conjugated, IL-6 APC-conjugated, IFN-γ PE-conjugated, TNF-α PE-Vio770-conjugated, IL-10 APC-conjugated, IL-17 A PE-Vio770-conjugated antibodies (Miltenyi Biotec), and IL-1Ra PE-conjugated antibody (BD Biosciences), for 15 min at room temperature in the dark.

Cells were then washed, resuspended in MACS Quant® Running Buffer (Miltenyi Biotec) and filtered with a 30 μm filter. Data were acquired using MACS Quant® Analyzer (Miltenyi Biotec).

Non-stimulated stained cells were used as negative controls. Unstained cells were used to monitor autofluorescence. The flow cytometer was set using cells stained with isotype controls. Frequencies of cell populations were calculated on total events, after exclusion of cell debris on FSC (forward scatter) vs. SSC (side scatter) density plots and doublets on FSC-A vs. FSC-H.

Monocyte population was gated to CD3-/CD14+ cells while specific lymphocyte populations were gated to CD14-/CD3+ T cells and then separated into CD4+/CD8- or CD4-/CD8+ T cells.

In order to analyze the production of cytokines, one-parameter histograms were created, and the production of cytokines after stimulus was compared with the production without stimulus, the latter representing background levels of cytokine production.

Results are expressed as the percentage of cytokine-producing cells within the $CD14^+$ population for monocytes, and within the $CD4^+$ and $CD8^+$ population for $CD4^+$ and $CD8^+$ T lymphocytes respectively.

2.7. Gating strategy

Data were analyzed by MACS Quantify® Analysis Software (Miltenyi Biotec).

Cell debris were excluded on FSC-A vs. SSC-A density plots, and doublets were excluded on FSC-A vs. FSC-H plots.

To analyze monocyte production of cytokines, all the singlets were displayed on $CD14^+$ vs. $CD3^+$ dot plot. $CD14^+$ cells were selected both in the stimulated and in non-stimulated samples.

To analyze $CD4^+$ and $CD8^+$ T lymphocytes production of cytokines, $CD3^+$ cells were selected. All the $CD3^+$ cells were displayed on $CD8^+$ vs. $CD4^+$ dot plot, and the $CD4^+$ and $CD8^+$ cells were individually selected, both in the stimulated and in non-stimulated samples. Gating strategy is depicted in Fig. 1.

In order to visualize the production of cytokines, one-parameter histograms were created, and the production of cytokines after stimulus was compared with the production without stimulus (negative control).

Results are expressed as the percentage of cytokine-producing cells within the $CD14^+$ population for monocytes, and within the $CD4^+$ and $CD8^+$ population for $CD4^+$ and $CD8^+$ T lymphocytes respectively.

2.8. Statistical analysis

Categorical data were expressed as absolute frequency, continuous data were summarized by median and interquartile range (IQR). To compare the group variable at three categories (DR, DS, HC) and at two categories (PWE, HC) with continuous predictive factors, Kruskal-Wallis test followed by multiple comparisons using Dunn method, and Mann-Whitney test were performed, respectively. Chi square test was applied to the comparison between group variables and categorical predictive factors. Thiols and FRAP variables were transformed into quartiles and successively all factors resulted significant to the univariate tests were analyzed together by binary logistic regression with stepwise method as multivariate analysis. Results of logistic regression were expressed with regression coefficient and OR (95% IC). Significance was fixed at 0.05 and all analyses were carried out by SPSS technology v.28.

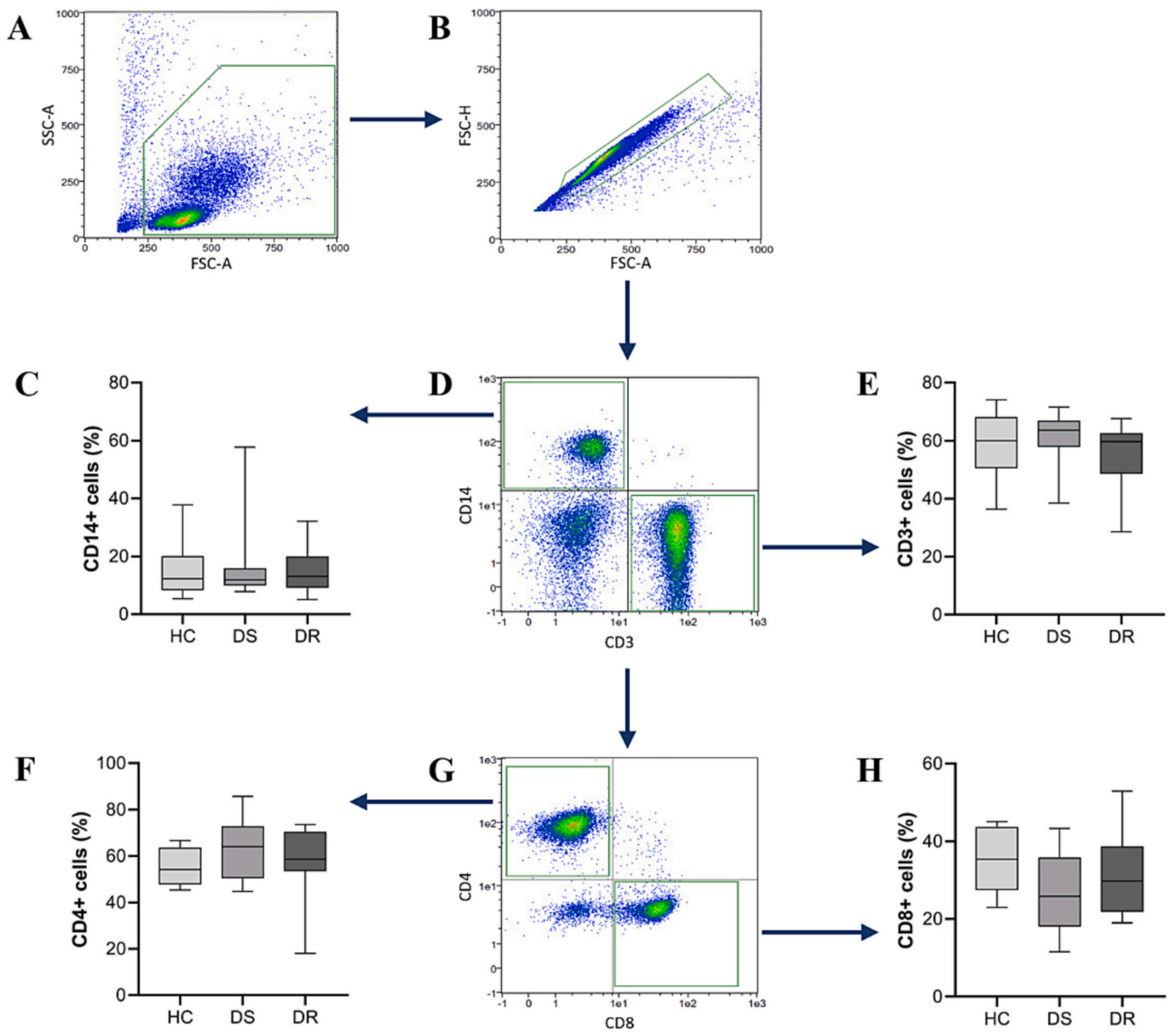

Fig. 1. Representation of gating strategy and total percentage of peripheral immune cells in the three groups (DR, DS, HC). Data generated by flow cytometry were visualized through density plots. Cell debris and aggregates were excluded on FSC-A vs. SSC-A density plots (A), then doublets were excluded on FSC-A vs. FSC-H plots (B). The obtained singlets were further distinguished into $CD14^+$ cells and $CD3^+$ cells (D). $CD3^+$ cells were then separated into $CD4^+$ and $CD8^+$ cells (G). The total percentage of each subpopulation was compared between the three groups (DR, DS, HC) and illustrated by box plots, showing the total percentage of $CD14^+$ (C), $CD3^+$ (E), $CD4^+$ (F) and $CD8^+$ cells (H) in the three groups. Statistical analysis was performed using Kruskal-Wallis test.

Graphs were realized using GraphPad Prism 9.5.1.

3. Results

3.1. Description of study population

Demographic and clinical features are presented in Table 1.

Age and sex were comparable among the three groups whereas, as expected, the number of ASMs used and the frequency of seizures were significantly higher in DR compared to DS patients.

All the DS patients had been, by definition, seizure-free for at least one year, with a median time interval from last seizure to sample withdrawn of 45 months (IQR 27–80 months).

There were no differences in age at onset and duration of the disease. Etiology of epilepsy (symptomatic vs. cryptogenic) was similar between DR and DS.

3.2. Serum cytokines and chemokines

Levels of CCL2, CCL3 and IL-8 were more elevated in the serum of PWE compared to HC ($p = 0.032$, $p = 0.038$ and $p = 0.044$ respectively), without differences between DR and DS patients ($p = 0.359$, $p = 0.359$ and $p = 0.676$ respectively).

No significant differences were detected in all the other cytokines and chemokines between PWE and HC. Data are reported in Fig. 2 and detailed in Supplementary Table 1.

3.3. Oxidative stress biomarkers

PWE (DR and DS together) showed higher levels of AOPP, a marker of oxidative damage to proteins, in comparison to HC ($p = 0.0002$) and lower amounts of both FRAP and thiols, which represent measures of nonenzymatic antioxidant properties, in comparison to HC ($p = 0.004$

Table 1
Demographic and clinical features of DR patients, DS patients and HC.

Serum cytokines, chemokines, and oxidative stress biomarkers analysis				
	DR patients (*n* = 25)	DS patients (*n* = 22)	Controls (n = 25)	*P* value
Age	53 (43.5–63.5)	53 (39.5–59.25)	49 (38.5–53)	0.209
Sex (F/M)	14/11	12/10	15/10	0.925
Age at MTLE onset	25 (15.5–44)	26,5 (13.5–45.25)		0.983
Disease duration	21 (7.5–40.5)	15 (9.75–24.75)		0.364
ASMs	3 (2–4)	1.5 (1–2)		**<0.0001***
Seizures/month	2 (0.55–5)	0 (0–0)		**<0.0001***
Etiology of epilepsy				
Structural	16	11		0.386
- HS	10	8		0.999
- Temporal FCD	1	1		0.999
- Temporal dermoid cyst	2	1		0.999
- DNET	3	1		0.612
Cryptogenic	9	11		0.386
Intracellular cytokines analysis				
	DR patients (*n* = 16)	**DS patients (*n* = 15)**	**Controls (*n* = 10)**	**P value**
Age	48.5 (42.25–56.75)	54 (44–61)	43 (34–54)	0.410
Sex (F/M)	8/8	10/5		0.473
Age at MTLE onset	24,5 (16.25–41.25)	23 (14–45)		0.922
Disease duration	19,5 (7.25–39)	19 (8–35)		0.770
ASMs	3 (2–4)	1 (1–2)		**<0.0001***
Seizures/month	2.5 (1.25–8.5)	0 (0–0)		**<0.0001***
Etiology of epilepsy				
Structural	11	9		0.716
- HS	6	6		0.999
- Temporal FCD	1	1		0.999
- Temporal dermoid cyst	2	1		0.999
- DNET	2	1		0.999
Cryptogenic	5	6		0.716

Statistical analysis was performed using Kruskal-Wallis test and Mann-Whitney U test for quantitative variables, and chi square and Fisher exact test for categorical variables. Results are expressed as median (lower and upper quartiles) for quantitative variables, and as frequency for categorical variables. Bold black character with * denotes significance ($p < 0.05$). Abbreviations: ASMs = anti-seizure medications; dysembryoplastic neuroepithelial tumor (DNET); DR = drug-resistant; DS = drug-sensitive; FCD = focal cortical dysplasia; MTLE = mesial temporal lobe epilepsy; PWE = patients with epilepsy.

and $p < 0.0001$, respectively).

When comparing the three groups (DR vs. DS vs. HC), both DR and DS patients showed higher AOPP levels and lower thiols levels than HC ($p = 0.005$ and $p = 0.005$, respectively), while only DR patients exhibited lower levels of FRAP ($p = 0.016$). Statistical testing showed lack of significance between DR and DS patients.

Data are reported in Fig. 3 and detailed in Supplementary Table 2.

3.4. Intracellular cytokines

The total percentages of monocytes ($CD14^+$), $CD3^+$T cells, $CD4^+$ T cells and $CD8^+$ T cells were similar between HC, DS and DR patients (Fig. 1, Supplementary Table 3).

Compared with HC and DS group, the DR group showed a significantly increased proportion of monocytes positive for IL-1β ($p = 0.04$ and $p = 0.01$ respectively) and IL-6 ($p = 0.03$ and $p = 0.01$ respectively) after LPS stimulation. No differences were detected in percentages of monocytes positive for IL-1RA, TNF-α, IFN-γ and IL-10 between the three groups. Moreover, there were no differences in percentages of IFN-γ and IL-17 A positive $CD4^+$ and $CD8^+$ T lymphocytes after PMA/ionomycin stimulation between the three groups. Data are shown in Fig. 4 and reported in detail in Supplementary Table 4.

3.5. Binary logistic regression

We performed a binary logistic regression with a stepwise approach on serum and intracellular biomarkers which had been identified as significantly different between the groups in the univariate analysis (Figs. 2, 3, and 4). Concerning serum cytokines, CCL2 was the only significant factor able to distinguish between epileptic subjects (DR and DS together) and HC (Table 2).

Concerning oxidative stress biomarkers, both higher levels of AOPP and lower levels of thiols were significant factors able to distinguish between epileptic subjects (DR and DS together) and HC (Table 2).

Finally, regarding intracellular cytokines, the percentage of IL-1β positive monocytes after LPS stimulation was the only significant factor able to distinguish between DR and DS patients, with higher values of IL-1β being associated to a higher risk of being DR (Table 2).

4. Discussion

In this work, we investigated the role of PBMCs and serum biomarkers of inflammation and oxidative stress in a well-characterized cohort of patients, diagnosed with MTLE and distinguished based on their response to ASMs (DR vs. DS).

One of the most appealing hypotheses to explain drug resistance in human epilepsy brings into play a possible role of neuroinflammation and oxidative stress, two phenomena closely related to each other. Inflammatory and oxidative stress molecules released locally in the epileptic focus may be able to induce, enhance, and perpetuate epileptic activity (Soltani Khaboushan et al., 2022; Vezzani et al., 2019). These molecules can be produced both by resident cells and by peripheral immune cells, which are in turn attracted by inflammatory substances produced in the epileptic focus, thus creating a vicious circle able to perpetuate and sustain epileptic activity. Interestingly, some of the biomarkers responsible for the neuroinflammatory response, can be measured also in patients' serum, where they undergo modifications that are similar to those occurring inside the CNS (van Vliet et al., 2018; Vezzani et al., 2019).

In our cohort, PWE showed significant higher levels of serum CCL2 and CCL3 compared to HC, with CCL2 being able to distinguish between PWE and HC also at multivariate analysis. CCL2 and CCL3 are two chemotactic cytokines, i.e., chemokines, able to induce migration and activation of leukocytes into inflammatory sites (Deshmane et al., 2009). In particular, CCL2 exerts its function by binding a G protein-coupled receptor called chemokine C-C motif receptor 2 (CCR2), which is expressed by microglia, astrocytes, neurons, and also by monocytes, thus acting as a potent chemoattractant also for this cell lineage (Izikson et al., 2002; Yamanaka et al., 2021a). A possible explanation for the increased serum levels of CCL2 and CCL3 in our PWE may be that these molecules, produced in the epileptic focus by hyperactive microglial cells and neurons, and that induce the recruitment of circulating inflammatory cells into the epileptic focus, are also partially released into the bloodstream (Foresti et al., 2009; Wu et al., 2008; Yamanaka et al., 2021a).

Increased CCL2 amounts in both serum and CSF have already been documented in drug resistant childhood epilepsies (Česká et al., 2023), as well as in animal models of status epilepticus (SE) (Arisi et al., 2015; Varvel et al., 2016), and in the brain of patients with DR epilepsy who underwent epilepsy surgery or who died after SE (Broekaart et al., 2018; Wu et al., 2008).

Of note, in all these contexts, not only the levels of CCL2 in the CNS were elevated, but also monocytes infiltrates were abundant, thus supporting the hypothesis that also peripheral innate immune cells may be

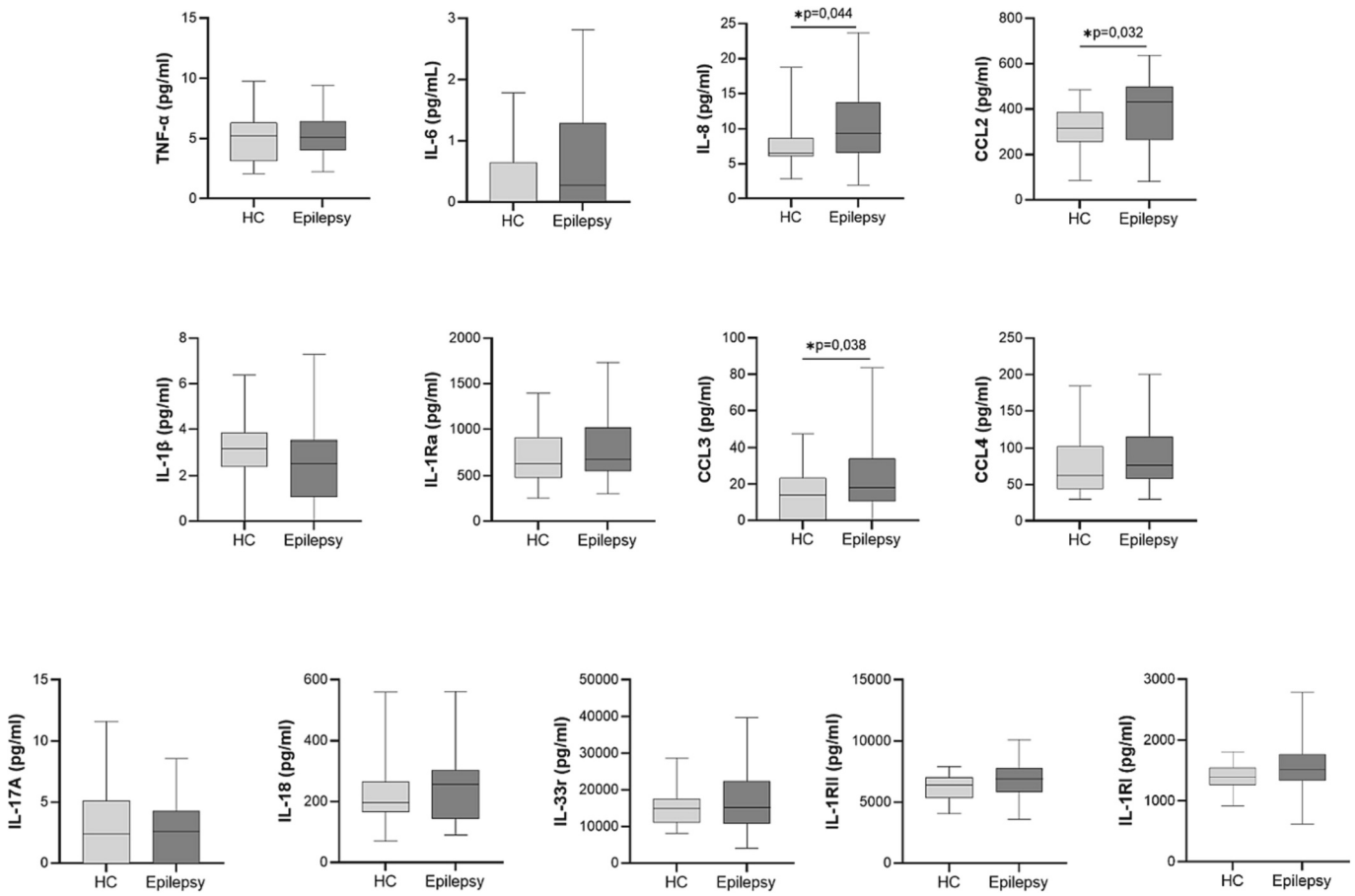

Fig. 2. Box plots showing circulating levels of pro- and anti-inflammatory cytokines and chemokines in PWE vs. HC. Levels of IL-8, CCL2 and CCL3 were more elevated in PWE compared to HC. Statistical analysis was performed using Mann Whitney *U* test. * $p < 0.05$. Outliers excluded, TNF-α: 4 PWE, p non significant (n.s.) with or without exclusion; IL-6: 1 HC, p n.s. with or without exclusion; IL-8: 2 HC and 5 PWE, $p = 0.046$ without exclusion; IL-1β: 1 epileptic patient, p n.s. with or without exclusion; IL-1Ra: 1 HC, p n.s. with or without exclusion; CCL4: 2 PWE, p n.s. with or without exclusion; IL-17 A: 2 HC, p n.s. with or without exclusion; IL-18: 1 epileptic patient, p n.s. with or without exclusion. Cytokines whose levels were close to 0 (IL-33, IFN-γ, IL-1a, IL-4, IL-13, IL-5) were not shown in the figure (see Supplementary Table 1).

implicated in neuroinflammation in epilepsy.

Concerning the adaptive immune cells, infiltrates of both $CD8^+$ cells and $CD4^+$ T cells were described in different forms of epilepsy and particularly in MTLE, with a direct correlation of T cell numbers in resected hippocampi and the degree of neuronal loss (Lu et al., 2017; Tröscher et al., 2021).

This background prompted us to further examine the role of circulating monocytes and T lymphocytes by reproducing in vitro activation of these cells, as it may occur when they are attracted in the epileptic focus, and analysing their ability to produce cytokines after stimulation, using flow cytometry.

We found that DR patients showed higher percentages of monocytes positive for IL-1β and IL-6 compared to DS patients and HC, while there were no significant differences between DS patients and HC. Moreover, binary logistic regression revealed that the percentage of IL-1β positive monocytes could predict ASMs responsiveness in our PWE, since more elevated values were able to identify DR patients.

IL-1β is a fundamental inflammatory cytokine, thought to be involved in the pathogenesis of epilepsy, and it is mainly produced by monocytes among peripheral immune cells (Minami et al., 1991; Roseti et al., 2015; Vezzani et al., 2011b; Vezzani and Viviani, 2016; Yamanaka et al., 2021c).

If the role of IL-1β is quite clear within the epileptic focus, with higher amounts in MTLE patients in comparison to autopsy controls (Fiala et al., 2013; Omran et al., 2012; Ravizza et al., 2008), many human studies investigating IL-1β serum levels failed to demonstrate differences between PWE and HC. In line with previous literature, in our study we did not find any difference in serum IL-1β levels between patients and HC. Moreover, we found comparable levels of IL-1 receptors and inhibitors in patients, and HC and also IL-18 levels were similar. IL-1β is a potently bioactive molecule, tightly regulated for its high inflammatory activity, and its levels often result within the standard interval, even in the case of active systemic autoinflammatory diseases (Yamanaka et al., 2021c).

As for IL-1β, similar considerations apply also to IL-6, another cytokine with important inflammatory properties, whose serum levels, in our study, were comparable between PWE and HC.

Few studies also focused on the role of inflammasome complex and its associated nuclear factor pathways in PWE; inflammasome complex is a key component of innate immunity responses and its activation in neurons and glia results in the production of inflammatory cytokines and oxidative stress molecules (Kigerl et al., 2014). A previous study, aimed at analysing peripheral blood expression levels of inflammasome complex components in patients with HS and patients with focal epilepsy of unknown cause, demonstrated that PWE do not show upregulation of inflammasome complex associated factors (Ulusoy et al., 2020).

Overall, these data suggest that no ongoing activation of the inflammasomes and production of inflammatory cytokines is detectable in epileptic subjects.

Conversely, IL-1β and IL-6 were produced at higher levels after stimulation of monocytes from DR patients compared to DS and HC, indicating that DR monocytes are primed for the production of

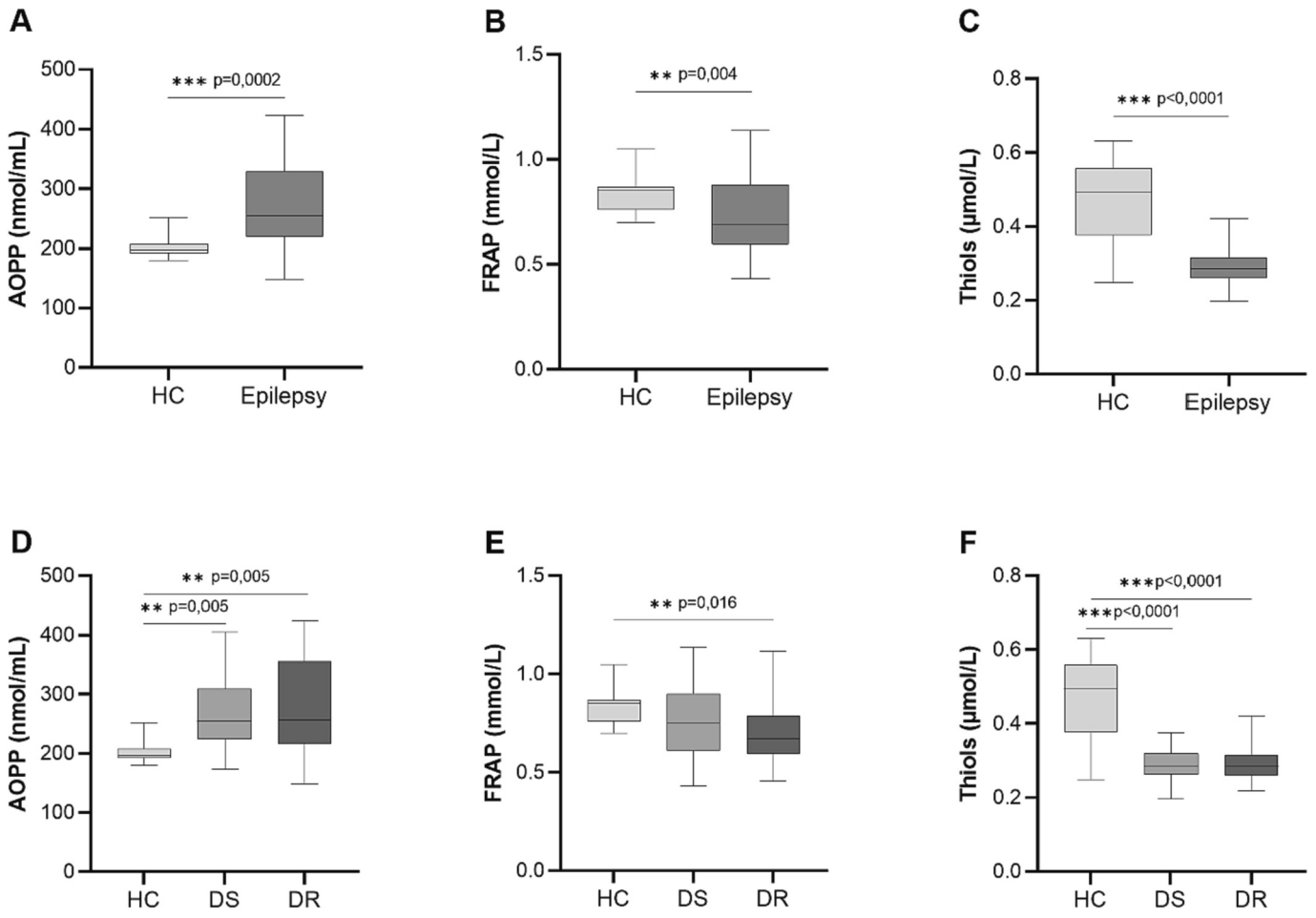

Fig. 3. Serum concentrations of oxidative stress biomarkers in PWE and HC. Box plots showing serum concentration of AOPP, FRAP and thiols in PWE vs. HC (A, B, and C, respectively), and in DR vs. DS vs. HC (D, E, and F, respectively). PWE all together showed higher levels of AOPP, a marker of oxidative damage, and lower levels of both FRAP and thiols, molecules with antioxidant properties. Distinguishing PWE in DR and DS, higher levels of AOPP and lower levels of thiols were found both in DR and in DS in comparison to HC, while only DR but not DS patients showed lower levels of FRAP compared to HC. Statistical analysis was performed using Mann Whitney *U* test (PWE vs healthy controls) and using Kruskal-Wallis test followed by Dunn multiple comparisons test (drug-resistant vs. drug-sensitive vs. healthy controls). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Abbreviations: AOPP = advanced oxidation protein products; FRAP = ferric reducing antioxidant power; HC = healthy controls; DS = drug-sensitive; DR = drug-resistant; PWE = patients with epilepsy.

inflammatory cytokines.

When analysing PBMCs and their ability to produce IL-1β after different in vitro stimulations, previous authors documented increased amounts of IL-1β in paediatric patients with febrile convulsion or with severe DRE of childhood, compared to HC (Dundar et al., 2013; Helminen and Vesikari, 1990; Matsuo et al., 2006; Nur et al., 2012; Pacifici et al., 1995; Straussberg et al., 2001).

In adults with epilepsy, the specific role of circulating monocytes and their ability to produce an inflammatory response after stimulation, has been poorly characterized. A study by Vieira and colleagues proved that peripheral immune cells in epileptic subjects are switched toward a low-grade chronic inflammatory phenotype, mainly involving effector T cells (Vieira et al., 2016). Moreover, a recent study took into account adult PWE with distinct responses to ASMs, investigating differences in peripheral immune cells subclasses, serum cytokines levels, and neurodegenerative biomarkers in subjects with epilepsy vs. HC, and in DR patients compared to DS patients (Ouédraogo et al., 2021). Nonetheless, in this study the authors did not consider monocytes, but they mainly concentrated on $CD4^+$ T cells, demonstrating that the frequency of $CD4^+$ T cells expressing inflammatory but not anti-inflammatory cytokines is elevated in the blood of DR patients compared to DS patients (Ouédraogo et al., 2021). Regarding $CD4^+$ T cells, no differences were found in our cohort between DR and DS patients, but this may be due to our reduced sample size.

Thus, in our study we demonstrated that the stimulation of monocytes generates a more pronounced inflammatory response in DR patients with MTLE.

Another interesting result is the increased level of circulating IL-8 in PWE compared to HC. IL-8 is another inflammatory chemokine, whose levels appeared to increase in patients with refractory epilepsy according to previous studies (Li et al., 2011).

Although we did not detect any differences in other serum cytokines between PWE and HC, we obtained some interesting results from the analysis of oxidative stress biomarkers. All the three biomarkers were significantly different between PWE and HC: PWE showed higher levels of AOPP (marker of oxidative damage) and lower levels of FRAP and thiols (measure of antioxidant properties) compared to HC. Moreover, the multivariate statistical analysis highlighted that both increased levels of AOPP and lower levels of thiols were able to distinguish patients with epilepsy from HC. However, none of the three molecules was able to distinguish between DR and DS patients, who presented similar levels of all the three biomarkers.

Increased oxidative stress levels were detected in the blood of subjects after SE, but also in subjects with epilepsy without SE (Cárdenas-Rodríguez et al., 2014; Pauletti et al., 2017). In particular, in a study by López and colleagues, the redox status of DR patients was assessed

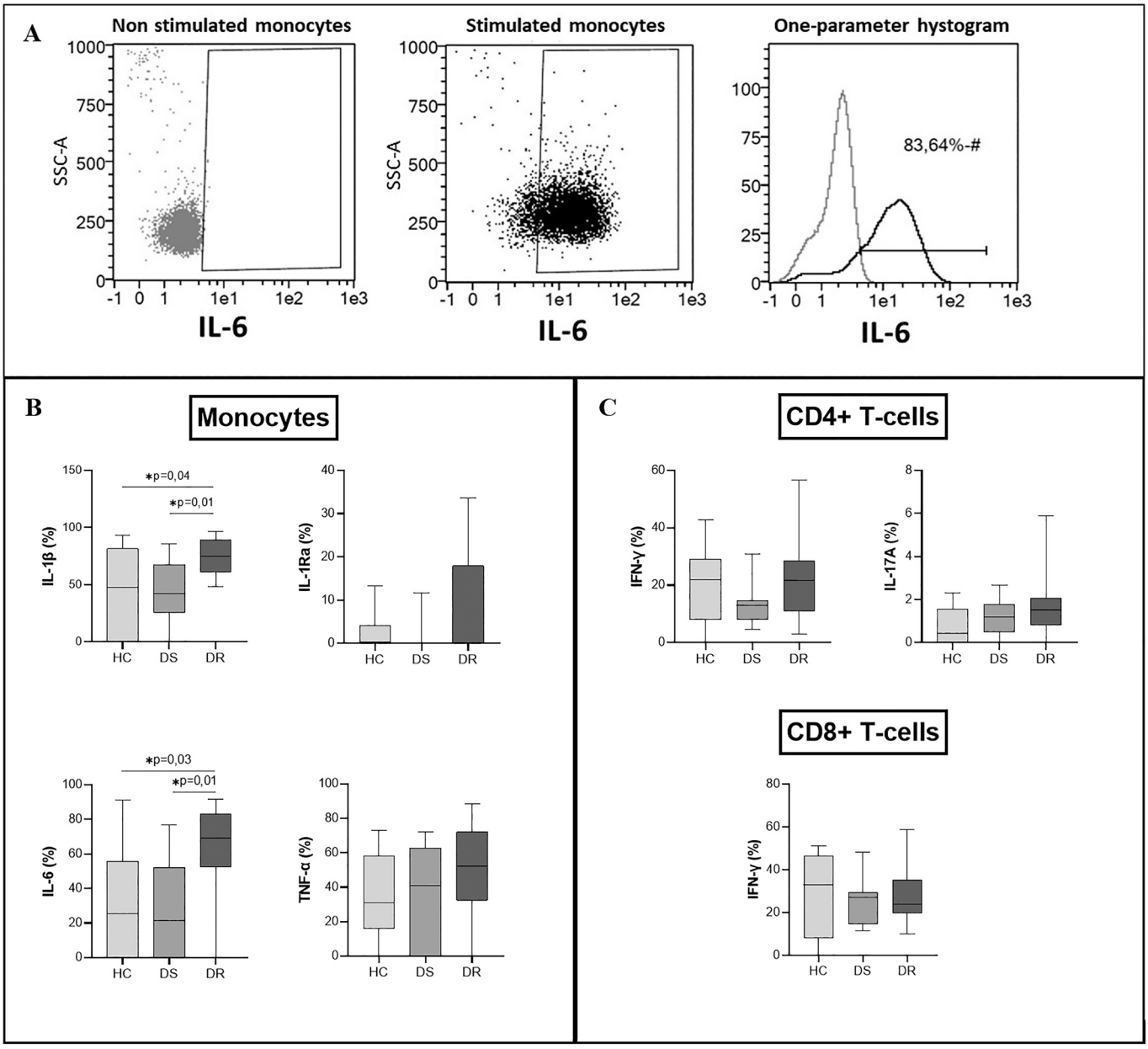

Fig. 4. Representation of intracellular cytokines analysis strategy, with boxplot showing the percentages of monocytes positive to IL-1β, IL-1Ra IL-6, and TNF-α, and of $CD4^+$ and $CD8^+$ T cells positive to IFN-γ and IL-17 A in DR vs. DS patients vs. HC.
Box A: Example of intracellular cytokine analysis (IL-6) in monocytes: representative dot plots of IL-6 expression after gating on CD14+ cells, in non-stimulated sample (left) and in LPS-stimulated sample (center). Quantification of intracellular IL-6 production with one-parameter histogram (right).
Box B: Boxplots showing the comparison of monocytes positive to IL-1β, IL-1Ra, IL-6, and TNF-α between the three groups: DR patients showed a significantly increased proportion of monocytes positive for IL-1β and IL-6 after stimulation. No production of IFN-γ and IL-10 by monocytes was observed (data not shown in the figure, see Supplementary Table 4).
Box C: Boxplots showing the comparison of $CD4^+$ and $CD8^+$ T cells positive to IFN-γ and IL-17 A among the three groups: there were no significant differences. No production of IL-17 A by $CD8^+$ T cells was observed (data not shown in the figure, see Supplementary Table 4).
Statistical analysis was performed using Kruskal-Wallis test followed by Dunn multiple comparisons test (drug-resistant vs. drug-sensitive vs. healthy controls).
* $p < 0,05$. Abbreviations: HC = healthy controls; DS = drug-sensitive; DR = drug-resistant.

before and after epilepsy surgery: pre-surgery, PWE showed increased oxidative stress markers (AOPP and malondialdehyde) and alterations in antioxidant substances activity (superoxide dismutase, catalase and glutathione peroxidase), while after surgery, all these molecules tended to normalize, except for the superoxide dismutase (López et al., 2007). More recent studies confirmed these results, and found correlations between increased oxidative stress biomarkers and other comorbidities often associated with MTLE, such as depression and anxiety (Fukuda et al., 2008; Kalita et al., 2019; Pedre et al., 2018; Rumià et al., 2013).

Our study has indeed some limitations: first of all, the reduced sample size, which did not allow us to assess the possible influence of the different structural etiologies and single ASMs on immune parameters, and secondly the self-reported seizure frequency, that may have induced inaccurate seizure counts in some cases.

For these reasons, our results need to be confirmed in larger studies, and eventually include also patients with other forms of epilepsy.

Table 2
Multivariate analysis by stepwise method on the factors influencing epilepsy and drug-resistance.

Factors	Regression coefficient	Odds ratio (95% CI)	P value
	Serum cytokines		
CCL2	0.004	1.004 (1.001–1.008)	**0.038***
constant	*−0.854*	*0.426*	*0.248*
IL-8			0.311
CCL3			0.117
	Oxidative stress biomarkers		
Thiols in quartiles §	−1.326	0.266 (0.130–0.542)	**<0.001***
AOPP	0.013	1.013 (1.002–1.025)	**0.025***
constant	1.242	*3.464*	*0.488*
FRAP in quartiles ∞			0.081
	Intracellular cytokines		
IL-1β	0.066	1.068 (1.020–1.119)	**0.005***
constant	*−3.920*	*0.020*	*0.010*
IL-6			0.121

Data were analyzed using binary logistic regression with stepwise method. For serum cytokines and oxidative stress biomarkers, the groups "PWE" and "HC" were considered as dependent variables. For intracellular cytokines, the groups "DR" and "DS" were considered as dependent variables. Bold black character with * indicates significance ($p < 0.05$).
§ Thiols values were divided into quartiles: (1) <0.27; (2) 0.27–0.31; (3) 0.31–0.45; (4) >0.45. ∞ AOPP values were divided into quartiles: (1) <0.66; (2) 0.66–0.76; (3) 0.76–0.86; (4) >0.86.

5. Conclusion

In this work, we showed that patients with MTLE – both DR and DS – have more elevated levels of oxidative stress molecules, lower levels of antioxidant biomarkers, and increased levels of CCL2 and CCL3, i.e., chemokines able to attract monocytes and other immune cells from the periphery to an inflamed site.

Moreover, when analysing cytokines profile in PBMCs, a significantly higher percentage of monocytes of patients with DR MTLE were activated by in vitro stimulation to produce IL-1β and IL-6 compared to DS patients and HC, suggesting a more prompt inflammatory response in this group of patients, which may be responsible for drug-resistance.

Our current results highlight the importance of considering the role of peripheral immune cells in epilepsy and particularly in DR forms, to provide potential new approaches in DRE treatment and biomarkers identification.

CRediT authorship contribution statement

C. Milano: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **M. Montali:** Conceptualization, Data curation, Formal analysis, Investigation. **S. Barachini:** Conceptualization, Data curation, Formal analysis, Investigation. **I.S. Burzi:** Conceptualization, Data curation, Formal analysis, Investigation. **F. Pratesi:** Conceptualization, Data curation, Formal analysis, Investigation. **L. Petrozzi:** Data curation, Investigation, Methodology. **L. Chico:** Data curation, Investigation, Methodology. **R. Morganti:** Formal analysis. **G. Gambino:** Data curation, Formal analysis. **L. Rossi:** Data curation, Formal analysis. **R. Ceravolo:** Conceptualization, Supervision. **G. Siciliano:** Conceptualization, Supervision. **P. Migliorini:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **I. Petrini:** Conceptualization, Methodology, Supervision. **C. Pizzanelli:** Conceptualization, Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2023.578272.

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