Peripheral T cells from multiple sclerosis patients trigger synaptotoxic alterations in central neurons

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Aims: The crucial step in the pathogenic events that lead to the development and the progression of multiple sclerosis (MS) is the infiltration of autoreactive T cells in the brain. Data from experimental autoimmune encephalomyelitis (EAE) mice indicate that, together with microglia, T cells are responsible for the enhancement of the glutamatergic transmission in central neurons, contributing to glutamate-mediated excitotoxicity, a pathological hallmark of both EAE and MS brains. Here, we addressed the synaptic role of T cells taken from MS patients. **Methods**: A chimeric model of human T cells and murine brain slices was established to record, by Patch Clamp technique, the glutamatergic transmission in the presence of T cells isolated from the peripheral blood of healthy subjects (HS), active (a) and nonactive (na) relapsing remitting MS patients. Intracellular staining and flow cytometry were used to assess tumour necrosis factor (TNF) expression in T cells. **Results**: Chimeric experiments indicated that, compared to HS and naMS, T cells from aMS induced an increase in glutamatergic kinetic properties of striatal neurons. Such alteration, reminiscent of the those induced by EAE T cells, was blocked by incubation of the slices with etanercept, a TNF receptor antagonist. Of note, T cells from aMS expressed more TNF than naMS patients and HS subjects. **Conclusion**: These data highlight the synaptotoxic potential retained by MS T cells, suggesting that during the inflammatory phase of the disease infiltrating T cells could influence the neuronal activity contributing to the TNF-mediated mechanisms of glutamate excitotoxicity in central neurons.

Keywords: glutamate-excitotoxicity, multiple sclerosis, striatum, synaptic transmission, T cells, tumour necrosis factor

Introduction

T cells are recognized to exert a crucial role in the pathogenic mechanisms leading to central nervous system (CNS) damage in multiple sclerosis (MS). Immunization of T cells against myelin epitopes by antigen

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presenting cells together with the blood brain barrier (BBB) breakdown is considered the first step towards the spreading of the autoimmune attack into the CNS with consequent neuronal and axonal damage as well as demyelination. Once in the brain, T cells interact with brain resident immune cells, namely microglia and macrophages, to amplify the inflammatory reaction [1].

In recent years, clinical, radiological and preclinical evidence have changed our vision of MS, supporting the notion that MS is both a white and a grey matter disease [2]. Neurons undergo significant structural and functional perturbations during MS, even in the absence of demyelination [3]. In this scenario, we hypothesized that T cells might play a role in neuronal alterations, by releasing molecules toxic to basic neuronal function, that is synaptic neurotransmission. Indeed, we demonstrated the synaptic effects of peripheral T cells taken from the spleen of mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS, which, although with some limitations, resembles well the neurological and the pathological features as well as the autoimmune nature of the disease [4]. Homologous *ex vivo* experiments consisting of the incubation of EAE T cells with murine brain slices of healthy mice revealed the synaptotoxic effect of T cells on glutamatergic transmission [5–7]. Notably, glutamatergic transmission is increased in MS and EAE brains and glutamate-mediated excitotoxicity is a well-recognized pathological mechanism of neuronal and oligodendroglial cell death in MS [8,9]. Several lines of evidence indicate that limiting glutamate-excitotoxicity is a therapeutic strategy to treat MS and immunomodulatory or immunosuppressant drugs, namely disease-modifying therapies, in addition to the anti-inflammatory effect, might exert neuroprotective role, by restraining T cells synaptotoxicity [3].

It is worth noting that other experimental approaches based on organotypic co-cultures of murine brain slices and antigen-specific activated $CD8^+$ cells have been developed to investigate the role of T cells in triggering long-term neuronal and oligodendroglial damage [10,11]. However, whether T cells from MS patients retain the potential to foster synaptic damage is entirely a speculative question. Here, we explored this issue using a heterologous chimeric model in which glutamatergic transmission was recorded from murine neurons in the presence of T cells taken from healthy subjects (HS) and relapsing remitting (RR) MS patients during either relapses or stable disease phases.

Materials and methods

Human subjects

This study was carried out in compliance with the Declaration of Helsinki principles and was approved by the Institutional Review Board of the University Hospital Tor Vergata, Rome, Italy. All subjects gave their written informed consent to participate in the study.

Seven healthy volunteers and 18 MS subjects were included in this study (Table 1). MS subjects were admitted to the neurological clinic of the University Hospital Tor Vergata of Rome and later diagnosed as suffering from RR-MS. After admittance, all patients underwent brain and spine MRI and cerebrospinal fluid (CSF) withdrawal within 24 h. Oligoclonal band analysis was performed on the CSF. No patient was treated with immunoactive drugs before lumbar puncture. Corticosteroids or other MS-specific therapies were initiated later when appropriate.

The diagnosis of RR-MS was established by clinical, laboratory and MRI parameters, and matched published criteria [12]. Demographic and clinical information was derived from medical records. MS disease onset was defined as the first episode of focal neurological dysfunction indicative of MS. Disease duration was estimated as the number of years from onset to the time of hospitalization. Disability was determined by a specially trained and certified examining neurologist using Expanded Disability Status Scale, a 10-point disease severity score derived from nine ratings for individual neurological domains [13]. Relapses were

Table 1. Demographical and clinical features of both MS and HS subjects included in the study

Variable	*MS patients*	*HS*
Gender (M/F)	7/11	2/5
Age (years)	32.47 ± 4.83	35.57 ± 3.55
Disease duration (years)	2.86 ± 2.07	
Pharmacological therapy	NO	
Disease activity (yes/no)	10/8	
EDSS	1.76 ± 1.03	

Values are expressed as mean ± SD.
EDSS, Expanded Disability Status Scale; F, female; M, male; MS, multiple sclerosis; HS, healthy subjects.

defined as the development of new or recurrent neurological symptoms not associated with fever or infection lasting at least 24 h. All patients were examined using 1.5 or 3 T MRI including dual-echo proton density, fluid-attenuated inversion recovery, T1-weighted spin-echo (SE), T2-weighted fast SE and contrast-enhanced T1-weighted SE after intravenous gadolinium (Gd) infusion. Disease activity was defined as the presence of either clinical relapse or Gd-enhancing (Gd+) lesions at MRI scan, at the time of hospitalization.

T cell isolation

After blood withdrawal, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll histopaque gradient centrifugation, according to standard techniques and soon frozen at −80. T cells were purified by magnetic immunosorting with FITC-CD3 antibody and microbeads-conjugated anti-FITC antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) from defrost PBMCs. Next, freshly isolated T cells (5×10^3) were both diluted in artificial CSF (ACSF) and put in culture (RPMI medium, 1% penicillin/streptomycin, 1% glutamine, 10% foetal bovine serum and 5% of autologous human serum) for 24 h to perform chimeric experiments.

T cell activation and flow cytometry

Peripheral blood mononuclear cells from HS, active MS (aMS) and nonactive MS (naMS) were stimulated with Dynabeads CD3/CD28 T-Cell Expander (1 bead/cell; Thermo Fisher Scientific, Waltham, MA, USA) for 8 h to allow cytokine synthesis. To measure the intracellular cytokine levels, secretion was inhibited by adding brefeldin A (10 mM; Millipore Sigma, Burlington, MA, USA) 5 h before the end of stimulation. At the end of the incubation period, cells were stained at cell surface with e780-conjugated anti-CD3 (1:100 dilution; eBioscience, San Diego CA, USA) and Pacific Orange Live/Dead Dye (Biolegend, San Diego, CA, USA). Cells were then made permeable with Cytofix/Cytoperm reagents (BD Biosciences, San Jose, CA, USA) and stained intracellularly with phycoerythrin-Cy7–conjugated anti–tumour necrosis factor (TNF)-a (1:100 dilution; eBioscience) at room temperature for 30 min. Intracellular cytokines were analysed by flow cytometry (FACS-Cyan ADP; Beckman Coulter, Brea, CA, USA). For each analysis, at least 300 000 events were acquired by gating on Pacific Orange–conjugated Live/Dead negative cells, as reported [14].

Animals

Six- to eight-week-old female C57BL/6 (Charles River, Milan, Italy) mice were used. Animal experiments described in this study were conducted according to the guidelines set by the Internal Institutional Review Committee, the European Directive 2010/63/EU and the European Recommendations 526/2007 and the Italian D.Lgs 26/2014. All efforts were made to minimize the number of animals used and their suffering.

Mouse brain slices preparation and electrophysiology

Mice were sacrificed by cervical dislocation and the brains were removed. Then, corticostriatal coronal slices (200 μm) were cut by means of a Vibratome (Leica VT1200 - Leica biosystems, Wetzlar, Germany) [5,6]. A single slice was then transferred to a recording chamber and submerged in a continuously flowing ACSF (32°C, 2–3 ml/min) gassed with 95% O_2–5% CO_2. The composition of the control ACSF was (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 11 Glucose and 25 $NaHCO_3$. Only data from putative medium spiny projection neurons (MSNs), which account for over 95% of the entire population of striatal neurons, were included in this study. MSNs were identified for their morphological and electrophysiological properties.

Recording pipettes were advanced towards individual striatal cells in the slice under positive pressure and visual control (WinVision 2000; Delta Sistemi, Alessandria, Italy) and, on contact, tight GΩ seals were made by applying negative pressure. The membrane patch was then ruptured by suction and membrane current and potential monitored using Multiclamp 700B amplifier (Molecular Devices, Foster City, CA, USA). Whole-cell access resistances measured in voltage clamp were in the range of 5–20 MΩ. Whole-cell patch clamp recordings were made with borosilicate glass pipettes (1.8 mm o.d.; 2–3 MΩ), in voltage-clamp mode, at the holding potential of −80 mV. To study glutamate-mediated spontaneous excitatory postsynaptic currents (sEPSCs), the recording pipettes were filled with internal solution of the following composition (mM): K-gluconate (125), NaCl (10), $CaCl_2$, (1.0), $MgCl_2$ (2.0), 1,2-bis (2-aminophenoxy) ethane-N,N,N,

N-tetraacetic acid (BAPTA; 0.5), N-(2-hydroxyethyl)-piperazine- N′-(2-ethanesulfonic acid) (HEPES; 19), guanosine triphosphate (GTP; 0.3) and Mg-adenosine triphosphate (Mg-ATP; 1.0), adjusted to pH7.3 with KOH. Picrotoxin (50 μM) was added to the perfusing solution to block $GABA_A$-mediated transmission. Synaptic events were stored using PCLAMP (Axon Instruments - Molecular Devices, San Jose, CA, USA) and analysed offline on a personal computer with MINI ANALYSIS 6.0.7 (Synaptosoft, Leonia, NJ, USA) software. The detection threshold of sEPSCs was set at twice the baseline noise. The fact that no false events would be identified was confirmed by visual inspection for each experiment. For sEPSCs kinetic analysis, offline analysis was performed on spontaneous synaptic events recorded during fixed time epochs (1–2 min), sampled every 2–3 min [5,15] and events with peak amplitude between 10 and 50 pA were grouped, aligned by half-rise time, and normalized by peak amplitude. In each cell, all events between 10 and 50 pA were averaged to obtain rise times, decay times and half widths [5]. Pure T cells, 5×10^3, were then placed on the surface of single slice on the side where Patch Clamp experiments were performed after a 30–60 min incubation. To account for biological differences in glutamatergic transmission recordings from different animals, chimeric experiments were performed by incubating T cells of the same patient on slices from at least two animals. In a group of experiments corticostriatal slices were incubated with the TNF blocker etanercept (10 μg/ml) for 1 h and, after this period, T cells were added on slice (always in the presence of TNF blocker).

Statistical analysis

Comparisons between two groups were made by unpaired *t*-test. One-way ANOVA analysis followed by Tukey *post hoc* test was used to compare three groups. Throughout the text *n* refers to the number of recorded cells, unless otherwise specified. Data were presented as mean ± SEM. The significance level was established at $P < 0.05$.

Results

Chimeric MS model reveals synaptotoxic activity of T cells from active MS patients

To address the synaptic effect of autoreactive human T cells, we performed recordings of glutamatergic transmission from murine striatal neurons in the presence of T cells taken from MS patients and HS. Similar to the homologous *ex vivo* model, we chose the striatum for electrophysiological recordings, since this area is deeply involved in MS pathophysiology [16–18]. Demographic information about the population examined in this study are reported in Table 1. We recorded sEPSCs of MSN in the presence of HS and MS T cells (Figure 1). The kinetic properties of the sEPSCs, rise time and decay time were similar to sEPSCs recorded in basal condition (dot lines in the figure), irrespective of the source of T cells. In contrast, half width parameter was significantly increase in MS T cell condition (Figure 1**A-A″**: HS-T cells $n = 34$, rise time 0.96 ± 0.03 ms, decay time 4.63 ± 0.11 ms, half width 5.68 ± 0.93 ms; MS-T cells $n = 103$, rise time 0.96 ± 0.02 ms, decay time 4.85 ± 0.10 ms, half width 6.10 ± 1.11 ms; unpaired *T* test: $P > 0.05$ for rise time and decay time, $P < 0.05$ for half width).

Although the statistical analysis showed a slight difference between HS and MS, we asked whether the disease activity of the examined MS patients could highlight even more remarkable differences in terms of neuronal response elicited by T cells in the chimeric model. Our cohort of patients (18 in total) included 10 subjects with active disease (aMS) and 8 with nonactive disease (naMS), (see Materials and methods). By representing the data of rise time, decay time and half width using a dot plot configuration of each column of data (Figure 1**B-B″**), we observed that aMS and naMS were clearly separated groups within the MS population.

On the basis of this observation, we performed a new analysis of the kinetic properties of sEPSCs to assess whether disease activity could affect the synaptic response of neurons to MS T cells. Interestingly, we found that compared to HS T cells, all the kinetic parameters of sEPSCs were significantly affected by aMS T cells ($n = 43$) and not by naMS T cells ($n = 60$) (Figure 1**C-C″**). Indeed, the rise time (1.07 ± 0.03 ms), the decay time (5.39 ± 0.14 ms) and the half width (6.69 ± 0.15) of sEPSCs recorded in the presence of aMS T were significantly enhanced in comparison to those recorded during the incubation of T cells from both HS and naMS (rise time: 0.87 ± 0.02; decay time: 4.47 ± 0.10 ms; half width: 5.67 ± 0.12) (rise time one-way ANOVA $P < 0.001$, $F = 17.36$, Tukey *post hoc* comparisons: HS *vs.* aMS $P < 0.01$, aMS

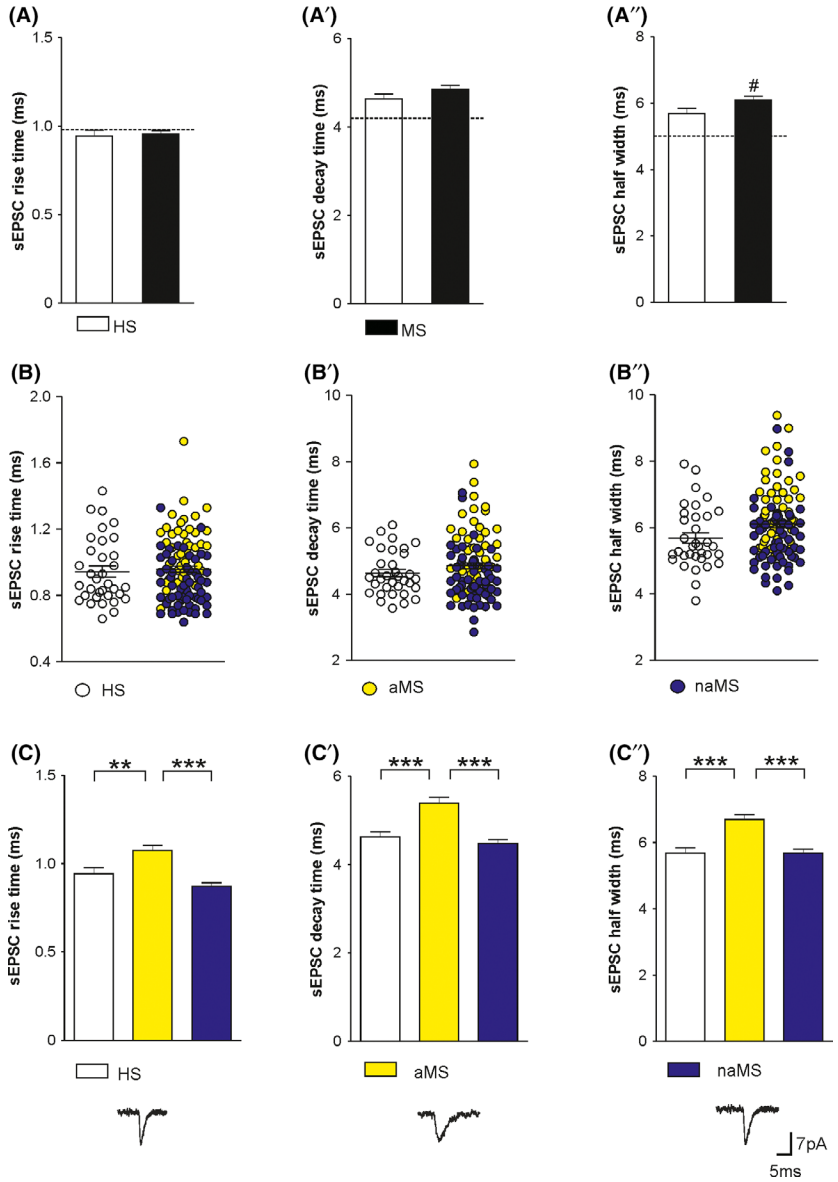

Figure 1. Disease activity modulates T cell synaptic effect in a chimeric model of multiple sclerosis (MS). Voltage-Clamp recordings were performed in the presence of T cells isolated from peripheral blood of healthy subjects (HS) and MS patients. The kinetic properties of spontaneous excitatory postsynaptic currents (sEPSCs), rise time (**A**) and decay time (**A′**) were similar between the two experimental conditions and also compared to recordings in the absence of T cells (dot lines in the graphs). Half width of MS T cells showed a significant difference compared to HS (**A″**). Data in A were represented as dot plot in the graphs in **B-B″**: each dot represents the value of a single cell time rise (**B**), decay time (**B′**) and half width (**B″**). The comparison of the kinetics of sEPSCs (**C-C″**) recorded from striatal neurons in the presence of HS, aMS and naMS T cells revealed a significant potentiation of the glutamatergic transmission induced by aMS T cells. Below the graphs, representative peaks of electrophysiological recordings in the three experimental conditions are shown. Statistical analysis was performed by unpaired *T* test ($^{\#}P < 0.05$) and by one-way ANOVA, followed by Tukey *post hoc* ($^{**}P < 0.01$, $^{***}<0.001$).

vs. naMS $P < 0.001$, HS *vs.* naMS $P > 0.05$; decay time one-way ANOVA $P < 0.001$, $F = 17.96$, Tukey *post hoc* comparisons: HS *vs.* aMS $P < 0.001$, aMS *vs.* naMS $P < 0.001$, HS *vs.* naMS $P > 0.05$; half width one-way ANOVA $P < 0.001$, $F = 17.07$, Tukey *post hoc* comparisons: HS *vs.* aMS $P < 0.001$, aMS *vs.* naMS $P < 0.001$, HS *vs.* naMS $P > 0.05$. Degree of freedom (df) = 136 for all parameters).

We also analysed the frequencies and the amplitude of the sEPSCs, which, however, were similar in all the experimental groups (frequency: HS = 2.72 ± 0.25 Hz, aMS = 2.58 ± 0.19 Hz, naMS = 2.77 ± 0.19 Hz; F = 0.25; amplitude: HS = 12.79 ± 0.45 pA, aMS = 11.55 ± 0.36 pA, naMS = 13.00 ± 0.55, F = 0.24; one-way ANOVA $P > 0.5$ and df = 76 for both parameters, data not shown).

The above results raised from the statistical analysis of the total number of murine neurons recorded with T cells of different MS subtypes (aMS/naMS). To prove the biological relevance of these results, we analysed the data considering each patient as a statistical independent unit, calculating the mean values of the sEPSCs recorded for each subject. The dot plots in Figure 2A-A″ highlight that differences among groups (number of HS = 7, number of aMS = 10, number of naMS = 8) were almost completely maintained for each kinetic parameter. Indeed, while Tukey post analysis of the one-way ANOVA did not detect significant differences between the rise time values recorded with HS (0.95 ± 0.04 ms) and aMS (1.10 ± 0.05 ms), it confirmed that aMS T cells induced an increase in this parameter compared to naMS (0.88 ± 0.02 ms; one-way ANOVA $P < 0.01$, F = 6.76, Tukey *post hoc* comparisons: $P < 0.01$). The decay time and half width of sEPSCs recorded from striatal neurons were found again significantly increased by the presence of aMS (decay time: 5.26 ± 0.17 ms; half width: 6.56 ± 0.20 ms) compared both HS (decay time: 4.58 ± 0.11 ms, half width: 5.59 ± 0.14 ms) and naMS T cells (decay time: 4.51 ± 0.12 ms, half width: 5.72 ± 0.16 ms) (decay time one-way ANOVA $P < 0.01$, F = 8.47, Tukey *post hoc* comparisons: HS *vs.* aMS $P < 0.01$, aMS *vs.* naMS $P < 0.01$, HS *vs.* naMS $P > 0.05$; half width one-way ANOVA $P < 0.01$, F = 9.45, Tukey *post hoc* comparisons: HS *vs.* aMS $P < 0.01$, aMS *vs.* naMS $P < 0.01$, HS *vs.* naMS $P > 0.05$. df = 24 for all comparisons). These results clearly indicate that the synaptic activity of MS T cells is influenced by the disease phase.

TNF is involved in the aMS T cell-mediated glutamatergic transmission exacerbation

The proinflammatory cytokine TNF has been proposed as an important modulator of basal synaptic activity in both healthy and pathological conditions with variable effects according to the brain area analysed [19]. Increasing concentrations of the cytokine switch its

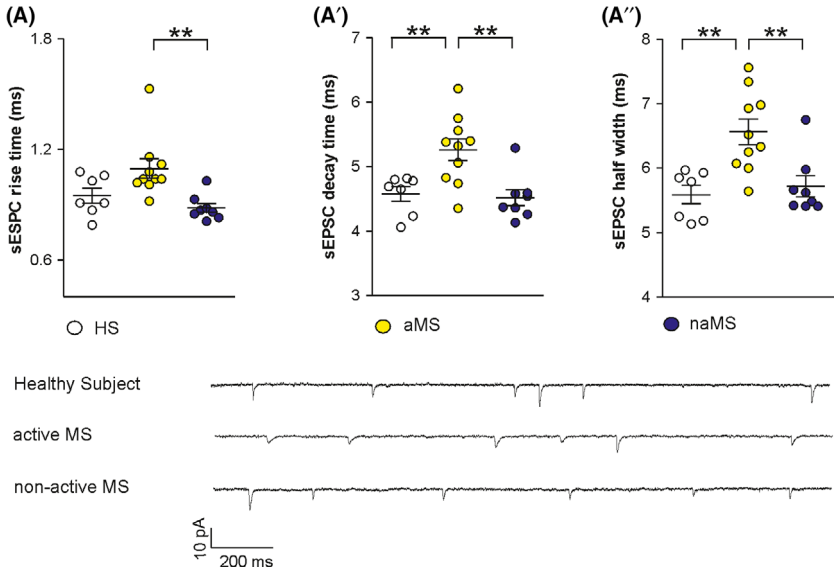

Figure 2. T cells from multiple sclerosis (MS) patients in active phase of the disease alters the kinetics of spontaneous excitatory postsynaptic currents (sEPSCs) without affecting frequency and amplitude. Single dot in histograms in **A-A″** refers to the mean value of sEPSCs kinetic parameters recorded from striatal neurons incubated with T cell of the same patient. This analysis confirms that active MS (aMS) T cells exacerbate the glutamatergic transmission in murine striatal slices. Below the graph, representative electrophysiological traces recorded in the three different experimental conditions are shown. Statistical analysis was performed by one-way ANOVA, followed by Tukey *post hoc* $^{**}P < 0.01$. HS, healthy subjects; naMS, nonactive MS.

physiological function to a pathological one, as it occurs in MS [20].

In EAE striatum, the concentrations of TNF raise significantly [5,21] and the potentiation of the kinetic properties of the glutamatergic transmission has been linked to its deleterious effects at postsynaptic sites [5,21]. Indeed, the synaptic alterations observed by *in vitro* and *in vivo* application of TNF were mimicked by the incubation of control murine slices with EAE T cells or *in vitro* activated microglia [5].

Thus, we evaluated the expression of TNF in the activated T cell population obtained from aMS and naMS patients and HS subjects by flow cytometry. The intracellular staining for TNF on CD3 + gated T cells revealed that the percentage of $CD3^+$ T cells expressing TNF was significantly higher in aMS patients (16.46 ± 1.00%) compared to naMS (10.72 ± 0.63%), whose levels were comparable to healthy controls (9.57 ± 0.49%) and ($F = 27.17$, df = 27; one-way ANOVA Tukey *post hoc* comparisons: $P < 0.001$ for all comparisons) (Figure 3**A-A′**).

Next, we investigated whether the excitotoxic effect elicited by MS T cells on murine slices could be reversed by blocking the TNF signalling. As shown in Figure 3**B-B″**, the co-incubation with etanercept, a decoy receptor that binds to soluble TNF [21], completely rescued the synaptic effects induced by aMS T cells in terms of rise time (aMS-etanercept 1.00 ± 0.04 ms), decay time (aMS-etanercept 4.34 ± 0.12 ms) and half width (aMS-etanercept 5.76 ± 0.17 ms) to normal values (rise time 0.94 ± 0.03 ms; decay time 4.63 ± 0.11 ms, half width 5.68 ± 0.93 ms) (unpaired *T* test aMS *vs*. aMS-etanercept: $P > 0.05$ for rise time, $P < 0.001$ for decay time and half width; one-way ANOVA among HS, naMS and aMS-etanercept rise time $P > 0.1$, $F = 0.94$; decay time $P > 0.1$, $F = 1.49$; half width $P > 0.1$, $F = 0.11$, df = 142) (Figure 3**B-B″**). Of note, etanercept did not exert any effect on glutamatergic currents in chimeric experiments with naMS- or HS- T cells, showing values similar to data obtained in the absence of etanercept (unpaired *T* test naMS *vs*. naMS-etanercept: $P > 0.1$ for all parameters; HS *vs*. HS-etanercept: $P > 0.1$ for all parameters) (Figure 3**B-B″**).

Discussion

Data shown in the present report demonstrate for the first time that during the inflammatory phase of the disease, MS T cells have the potential to foster neuronal damage, by enhancing the glutamatergic transmission in a mechanism involving TNF signalling. These results extend our previous findings on the role of EAE T cells in the synaptic pathology occurring during the disease course and translate into novel hints for human pathology. Notably, the present data strengthen the link between peripheral inflammation and neuronal damage, since only T cells from aMS and not those from naMS induced synaptic alterations in our chimeric model.

Recent advances in the field of neuroimmunology have revealed important physiological and pathological functions of the adaptive immune response in the crosstalk between the immune and the nervous system. Under normal circumstances, T cells home to the cerebral meninges where, being continuously replenished by the circulation from the blood and the lymphatic system and vice versa, they influence neuronal activity and brain functioning by releasing cytokines and growth factors [22]. During MS, the meninges remain an important reservoir of both B and T cells, but the breakdown of the BBB is considered the major input to the extravasation of autoreactive T cells and infiltration into the brain parenchyma, causing neuronal damage [1]. T cell infiltrates have been described in the white and, to lesser extent, in the grey matter of *post mortem* brain of MS patients [2,23] and have been proposed to influence neuronal function via direct interaction and/or indirect mechanisms, including the release of proinflammatory cytokines and/or the activation of microglia. Of note, complex interactions can occur among T cells, neurons and microglia, as recently demonstrated by Di Liberto and colleagues [24]. In a murine model of a CD8-mediated encephalomyelitis, the authors demonstrated that neurons under $CD8^+$ attack orchestrate the synaptic stripping mediated by microglia. Moreover, T cells can alter the synaptic transmission through a paracrine action, that is the release of proinflammatory cytokines [5]. Cytokines have been shown to profoundly affect synaptic activity during neuroinflammation and TNF pathological levels have been linked to aberrant mechanisms of synaptic scaling and excitotoxicity [19]. Synaptic loss and/or dysfunction in EAE and MS brains occur independently of demyelination but follows inflammatory reaction [3]. It is worth noting that synaptic pathology is emerging as a crucial driver of neurodegenerative mechanisms in MS:

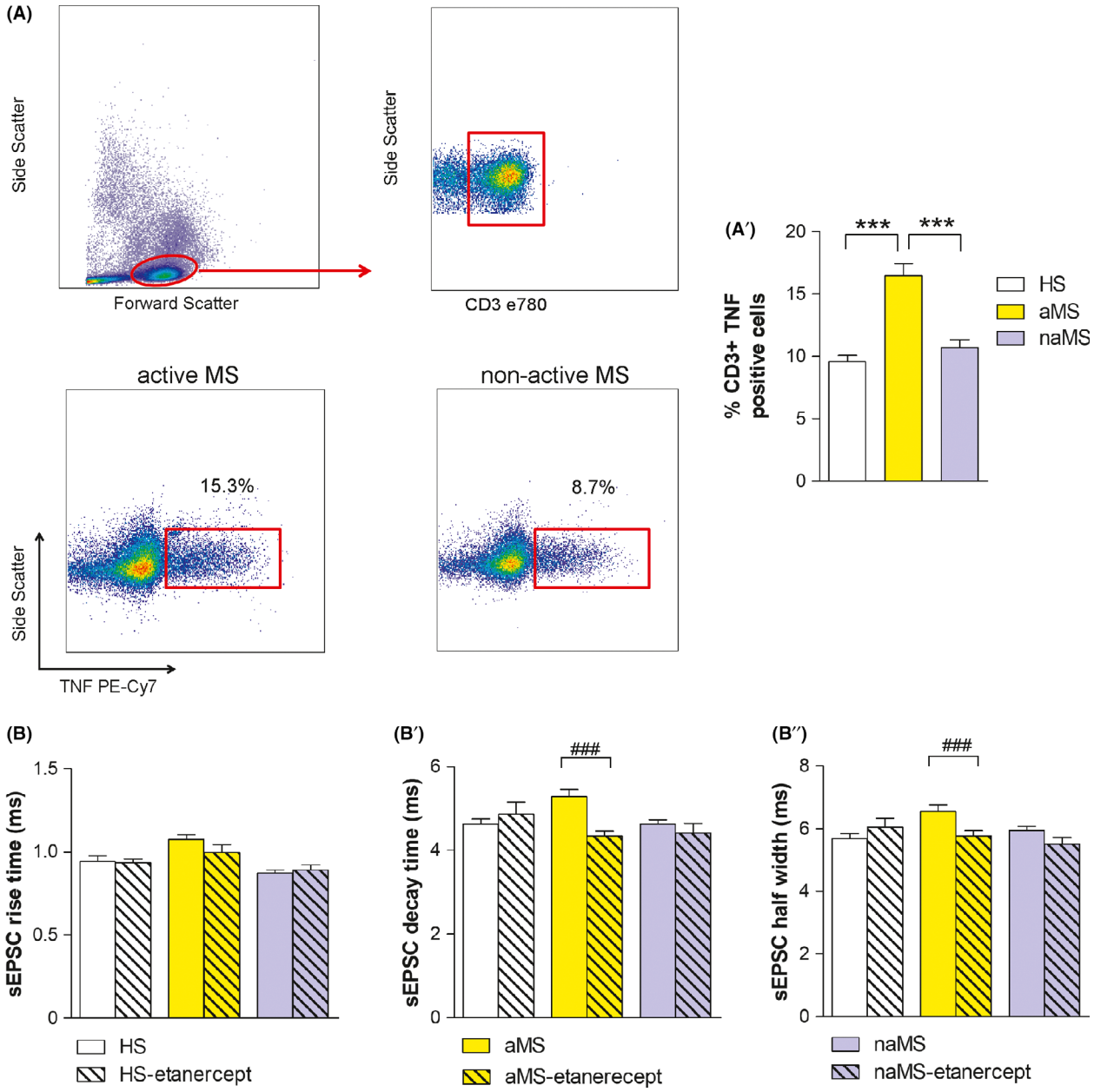

Figure 3. The blockade of the tumour necrosis factor (TNF) signalling prevents the excitotoxic effects of active multiple sclerosis (aMS) T cells. (**A**,**A′**) Representative cytofluorimetric plots of TNF intracellular expression on anti-CD3/anti-CD28-activated $CD3^+$ T cells of aMS and nonactive MS (naMS) patients (**A**), whose levels are shown as mean values of the percentage of $CD3^+$ T cells expressing TNF (**A′**). (**B**, **B′**,**B″**) healthy subjects (HS) T cells, aMS T cells and aMS T cells with and without etanercept were analysed. Etanercept rescued normal values of both decay time and half width, contrasting the deleterious effects of aMS T cells on glutamatergic transmission. Etanercept did not exert any effect on glutamatergic currents in experiments with naMS- or HS-T cells. Statistical analysis was performed by unpaired *T* test comparing each experimental condition (HS, aMS, naMS with etanercept) with its control (without etanercept) ($^{\#\#\#}P < 0.001$).

synapses sense the environment and easily respond and adapt to external stimuli by changing the synaptic strength. Moreover, synaptic plasticity assessed by transcranial magnetic stimulation in MS patients has been found deeply subverted and this alteration not only correlated with brain inflammatory burden [25]

but also underlined neurologic disability progression [26].

The above data highlight the clinical relevance of synaptic plasticity changes associated to MS, making synapses a valuable therapeutic target for MS, but do not clarify the mechanisms of T cell-mediated synaptopathy occurring in the brain of living MS patients. While significant advances have been made in the understanding of T cell-mediated neuronal damage in EAE model by *in vivo* two photon experiments [27,28], the use of *ex vivo* settings based on single-neuron electrophysiology still remains a unique opportunity to study the influence of T cells on synaptic transmission [5,7,25]. In this respect, other models based on homologous organotypic cell cultures of T cells and brain slices have been used to address this issue [10,11], providing insightful tools to investigate the communication between the immune and the nervous systems. However, none of these models have been used to study the synaptic activity of human T cells. Currently, compared to organotypic co-cultures the proposed chimeric model is the first and easiest *ex vivo* system to study the synaptic role of human T cells.

In the present investigation, the effect induced by aMS T cells on glutamatergic transmission suggests that, during the acute inflammatory phases of the disease, infiltrating T cells in the CNS might bear neurotoxicity in the brain of MS patients. The lack of synaptic effects by naMS T cells opposed to the observed potentiation of glutamatergic transmission by aMS T cells underlies the clinical relevance of our results, suggesting that the disease activity affects the neuron-T cell interaction. Additional studies will clarify the role of adaptive immunity in other clinically distinctive phenotype of the disease, like the progressive one.

Finally, the synaptotoxic effect of aMS T cells was inhibited by co-incubation with etanercept, an antagonist of soluble TNF. Such cytokine is involved in both MS and EAE pathogenesis [3]. Raises in TNF concentrations have been described in both serum and CSF of MS patients [29–33]. Noteworthy, T cells isolated from the CSF of MS patients have been shown to express TNF [34]. Here we show that a higher percent of T cells expresses TNF in MS subjects during the relapsing phase of the disease compared to both HS and MS patients in the remitting phase. Notably, the expression of TNF was comparable between naMS- and HS-T cells. These results, together with the electrophysiological data, suggest that only T cells expressing levels of TNF that are over a crucial threshold can induce synaptotoxic effects in striatal neurons. Such hypothesis is in line with the idea that only critical raises of TNF induce pathological mechanisms in the brain [20]. In addition to this, several direct or indirect mechanisms not explored in this study may be implicated in the synaptotoxicity exerted by T cells and, of course, demand future clarification. For example it cannot rule out the possibility of a kind of 'domino mechanism' in which T cell-derived TNF activates local microglia that in turn amplify the neuronal response. However, the significance of our results stems from several factors, including the strong statistical power of the multiple analysis performed and the replication of the data on murine brain slices from different animals to account for biological differences in recorded neurons.

In conclusion, this study highlights the synaptotoxic effects of MS T cells during inflammatory reactivation of the disease, providing first evidence of the role of human T cells in the potentiation of the glutamatergic transmission, a process known to contribute to excitotoxic damage in MS brains. This study opens new roads of investigation on the connection between adaptive immunity and neurons in an animal model-free system.

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Author contributions

AG, FDV, DF, FRR, SB, LG, VV, VC and AL performed experiments and analysed data. FB, MSB and GAM enrolled patients and HS for the study. AG, FDV, GM, DC and AM designed the study. AG, FDV, DC and AM wrote the paper. All authors read and approved the final manuscript.

Ethics approval

All procedures in this study were in accordance with the ethical standards of the institutional and/or

national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All subjects gave their written informed consent to participate in the study.

Conflict of interest

The authors declare that they have no conflict of interests.

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