Tissue plasminogen activator worsens experimental autoimmune encephalomyelitis by complementary actions on lymphoid and myeloid cell responses

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Abstract

Background

Tissue plasminogen activator (tPA) is a serine protease involved in fibrinolysis. It is released by endothelial cells, but also expressed by neurons and glial cells in the central nervous system (CNS). Interestingly, this enzyme also contributes to pathological processes in the CNS such as neuroinflammation by activating microglia and increasing blood-brain-barrier permeability. Nevertheless, its role in the control of adaptive and innate immune response remains poorly understood.

Methods

tPA effects on myeloid and lymphoid cell response were studied *in vivo* in the mouse model of multiple sclerosis experimental autoimmune encephalomyelitis and *in vitro* in splenocytes.

Results

$tPA^{-/-}$ animals exhibited less severe experimental autoimmune encephalomyelitis than their wild type counterparts. This was accompanied by a reduction in both lymphoid and myeloid cell populations in the spinal cord parenchyma. In parallel, tPA increased T cell activation and proliferation, as well as cytokine production by a protease-dependent mechanism and *via* plasmin generation. In addition, tPA raised the expression of MHC-II and the co-stimulatory molecule CD80 and CD86 at the surface of dendritic cells and macrophages by an effect dependent of the proteolytic activity of tPA and of the activation of epidermal growth factor receptor.

Conclusions

Our study provides new insights into the mechanisms responsible for the harmful functions of tPA in multiple sclerosis and its animal models: tPA promotes the proliferation and activation of both lymphoid and myeloid populations by distinct, though complementary, mechanisms.

Background

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) characterized by lymphoid and myeloid cell infiltration and microglial activation, leading to demyelination and progressive neurodegeneration [1]. Tissue plasminogen activator (tPA), a serine protease involved in fibrinolysis [2], is mainly produced by endothelial cells of vessels [3], whereas this protease is also secreted by several cell types within the CNS such as oligodendrocytes [4], astrocytes [5] or neurons [5, 6]. tPA influences neuroinflammatory and neuroimmune processes [7]. Indeed, its effects are particularly relevant to the

context of MS: tPA activity is increased by tenfold in the cerebrospinal fluid (CSF) of MS patients [8] and the protease is found in the perivascular spaces associated to active MS plaques [9].

Myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$-induced experimental autoimmune encephalomyelitis (EAE), a well-established animal model of MS, is characterized by the recruitment and entry of CD4 + encephalitogenic T cells into the CNS parenchyma [10]. Interestingly, pioneer studies reported that tPA deficient mice ($tPA^{-/-}$) declare EAE later than their wild type (WT) counterparts, and show less severe symptoms in the early phase of the disease, with reduced demyelination, axonal damage, microglial activation and T cell presence in the parenchyma [11], while later studies in slightly different experimental designs drew opposite conclusions, showing earlier and more severe disease in $tPA^{-/-}$ mice [12]. This indicates that tPA can provide different effects in EAE course, depending on the phase of the disease and the experimental design.

Several actions of tPA can participate in its controversial effects in EAE. In addition to its protease activity, tPA exerts various functions due to its five distinct functional domains: finger domain, Epidermal Growth Factor (EGF)-like domain, kringle 1, kringle 2 and protease domain [13]. We have previously shown that tPA, by activating the EGF receptor (EGFR), has an anti-apoptotic effect over oligodendrocytes [4] and a chemotactic effect on their progenitors that helps remyelination after chemically-induced white matter lesions [14]. tPA is also able to increase blood brain barrier (BBB) permeability and helps leukocyte diapedesis [15] by several mechanisms, including the potentiation of endothelial N-Methyl-D-Aspartate receptors (NMDAR) [16] or tight junction disruption by the modification of occludin phosphorylation [17]. Our group has shown that Glunomab®, a monoclonal antibody which prevents the binding of tPA on the GluN1 subunit of NMDAR [18], inhibits leukocyte entry into the CNS, thus blocking demyelination and EAE progression [16].

In addition to these actions, controversial data indicate that tPA may directly act on cells of the innate immunity. tPA can regulate myeloid cell functions, but the pro- or anti-inflammatory nature of this action is still a matter of debate [19, 20]. On the other hand, the possible role of tPA on lymphoid cell activity is still poorly understood [7]. Therefore, the purpose of this study is to investigate the respective actions of tPA on lymphoid and myeloid cells in the context of EAE. Interestingly, we report a deleterious role of tPA in EAE, that may be due to an increase in T-cell proliferation, combined to a potentiation of dendritic and macrophage antigen presenting function. These combined effects of tPA converge to the promotion of pro-inflammatory T cell adaptive immune response in EAE.

Methods

Animals. Transgenic $tPA^{-/-}$ (C57BL/129 background) [21] and C57BL/129 control mice, aged 8–12 weeks, were provided by the *Centre Universitaire de Ressources Biologiques* (CURB, University of Caen, France). C57BL6/J mice aged 6–12 weeks were obtained from Janvier Laboratories. Mice were housed in our local conventional animal facilities. All procedures were performed according to the guidelines of the institutional ethics committee (*Comité Normand d'éthique en matière d'expérimentation animale*,

CeNomExa and *Comité Ético de Experimentación Animal del Hospital Nacional de Parapléjicos*). This was submitted to these committees for approval in accordance with the European directive n° 2013/63/UE (agreement number D14118001) and with the French and Spanish National and Regional Guidelines for Animal Experimentation and the Use of Genetically Modified Organisms (French Ministry of Research, project license number 02653.2, Decree 87/848; RD 53/2013 and 178/2004, Law 32/2007 and 9/2003, Decree 320/2010).

Reagents. Recombinant human tPA (Actilyse ®) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Several controls of tPA and other reagents were used along the study: dialyzed tPA, tPA GGACK, or corresponding vehicles (DMSO, Sigma-Aldrich). tPA dialysis was performed for 48 h at 4 °C against 0.3M HEPES to remove the arginine vehicle. tPA GGACK was obtained by incubating GGACK (1,5-dansyl-L-glutamyl-L-glycyl-L-arginine chloromethylketone, EMD) with Actilyse in a fourfold molar excess for 6 h at room temperature (RT), followed by dialysis for 48 h at 4 °C with 0.3M HEPES to remove unbound GGACK and the arginine vehicle. The loss of proteolytic activity of tPA GGACK was confirmed with spectrozyme assay (American Diagnostica) [4]. Aprotinin and epsilon-aminocaproic acid (εACA) were purchased from Sigma-Aldrich. AG1478 hydrochloride was purchased from Tocris.

EAE induction. Monophasic EAE was induced in 6-12-week-old C57BL6/J female and C57BL/129 $tPA^{-/-}$ male mice by active immunization with MOG_{35-55} peptide (Cambridge Research Biochemicals, Genscript). To this end, mice were injected subcutaneously with 200 µg of Complete Freund's adjuvant (Sigma-Aldrich) containing 400 µg of inactivated particles of *Mycobacterium tuberculosis* (BD Biosciences). The emulsion was administered to regions above the shoulders and the flanks into four sites (50 µL at each injection site). All animals were intraperitoneally injected at days 0 and 2 with 250 ng of pertussis toxin (Sigma-Aldrich) in 200 µL of saline. Mice were weighted and scored daily in a double blind manner for clinical signs of EAE as follows: 0: no disease; 1: limp tail; 2: hindlimb weakness: no hindlimb reflex; 3: hindlimb paresis; 4: hindlimb paralysis; 5: moribund or dead. In accordance with the ethical standards and regulations, the humane endpoint criteria were applied when an animal reached a clinical score ≥ 4 for more than 48 hours or presented signs of stress or pain (generation of sounds, stereotypic behaviour, lordokyphosis, hair loss or loss of weight superior to 2 g/day for more than 48 hours).

Isolation of leukocytes from spleens and spinal cords. Mice were deeply anesthetized with 5% isoflurane (Aerrane, Baxter) and transcardially perfused with 50 mL of cold phosphate buffered saline, pH 7.4 (PBS, Sigma-Aldrich). Spinal cords were harvested and homogenized in PBS. Leukocytes were recovered at the 30:70% Percoll (Fisher Scientific) interface after gradient centrifugation as described in the literature [22], and were then counted with Malassez chamber. Spleens were aseptically removed from naïve and MOG-immunized C57BL/6 mice at the peak of clinical score (≥ 3), as described previously [23], mechanically processed to obtain a splenocyte suspension by passing the cells through a 40 µm filter (Falcon). Erythrocytes were lysed in lysis buffer [0.15 M NH4Cl, 9 mM HKCO3, 0.5 M EDTA, pH 7.4 (Stemcell Technologies)] and the sterile splenocytes were resuspended in supplemented sterile PBS with 10%

filtered and inactivated fetal bovine serum (FBS, Stemcell Technologies), 1% Penicillin/Streptomycin (Gibco) and 2.5% (v/v) HEPES (Fisher).

Analysis of the effect of tPA on myeloid cell functions. Splenocytes (10^6 per well in 24 well plates) were cultured and maintained in RPMI (Gibco) supplemented with 10% FBS (Linus), 1% Penicillin/Streptomycin, 2.5% (v/v) HEPES, 2 mM L-Glutamine (Gibco), and 50 µM β-mercaptoethanol in the presence of the following molecules for 24 hours: tPA or its vehicle [34.84 mg/ml arginine (Sigma-Aldrich), 10.72 mg/ml phosphoric acid (Sigma -Aldrich) and 0.1 mg/ml Tween 80 (Sigma-Aldrich)] at different concentrations (0.2, 2 and 20 µg/ml), 10 µg/ml Glunomab® antibody or its isotypic control (Macrez et al., 2016), 2 µg/ml tPA-GGACK (with blocked serine protease activity), 5 µM of the EGFR inhibitor (AG1478 hydrochloride, additional 30 min preincubation).

Analysis of the effect of tPA on lymphoid cell proliferation. Naive splenocytes (1.5×10^6/mL in suspension) were incubated for 20 min at RT in PBS with 0.5 µM CFSE (Life technologies). Cells were then washed and suspended in complete DMEM containing: 1% penicillin-streptomycin (P/S, Sigma-Aldrich), 1% GlutaMax-I 100X (Gibco), 10% FBS and 0.1% β-mercaptoethanol. 96 well U bottom plates were coated with 1 µg/mL anti-mouse CD3 antibody (eBioscience) in PBS overnight. The plates were washed twice with 200 µL of PBS and 10^5cells were plated (P96 well) in 200 µl of complete DMEM and incubated for 96 hours with 1 µg/mL anti-mouse CD28 antibody (eBioscience) in presence or not of tPA at different concentrations (0.1, 1 and 10 µg/ml, Correa et al., 2011), 10 µg/ml Glunomab® antibody or its isotypic control (Macrez et al., 2016), εACA, an inhibitor of the protease function of tPA (200 mM), the plasmin inhibitor aprotinin (20 IU mL^{-1}) [24], or tPA-GGACK (10 µg/mL). Then, cells were harvested by centrifugation (2000 rpm, 10 min, RT), washed in PBS and stained for the flow cytometry assay. To study the effect of tPA during MOG-induced stimulation, splenocytes were obtained from MOG-immunized C57BL6/J mice at the peak of clinical score (≥ 3), as described previously [25, 26] and were plated in IMDM, (BioWest, Nuaillé, France) supplemented with 2 mM L-glutamine, 1% P/S, 10% FBS (Gibco) and 50 µM β-mercaptoethanol, in U-bottom 96-well plates at a density of 2×10^5 cells. Splenocytes were exposed to 2 µM Tag-it Violet™ Proliferation and Cell Tracking Dye (Biolegend) diluted in PBS supplemented with 0.1% BSA at 37ºC with shaking for 20 min, protected from light. After washing, splenocytes were stimulated for 72 hours with 5 µg/mL MOG and treated with 2 µg/mL tPA, 5 µM AG1478 or the combination of both. Cells were harvested by centrifugation at 2000 rpm at RT, washed in PBS and stained for the flow cytometry assay.

Flow cytometry. Cells were resuspended in 50 µl of staining buffer and Fc receptors were blocked for 15 min at 4 °C with 10 µg/mL anti-CD16/CD32 antibodies (BD Biosciences 553142). Then, cells were labelled for 20 min at 4 °C with the following fluorochrome-conjugated monoclonal antibodies BV510 or APC hamster anti-mouse CD3e (BD Biosciences 563024 or 553066), APC or PE rat anti-mouse CD4 (BD Biosciences 553051 or 553049), PE-Cy7 or FITC rat anti-mouse CD8a (BD Biosciences 552877 or 553031), BV421 rat anti-mouse CD25 (BD Biosciences 564571), PE rat anti-mouse FoxP3 (BD Biosciences 560408), BV421 or PerCP-Cy5.5 rat anti-mouse CD11b (BD Biosciences562605 or 561114),

APC hamster anti-mouse CD11c (eBioscience 17-0114-82), PE-Cy7 rat anti-mouse CD45 (BD Biosciences 561868), eFluor 450 rat anti-mouse F4/80 (Invitrogen 48-4801-82) and PE-Cy7 rat anti-mouse MHC-II (Invitrogen 25-5321-82). The BD Pharmingen transcription factor buffer set was used according to the manufacturer's protocol to detect expression of FoxP3. When necessary, cells were fixed in a final volume of 300 µl with 4% paraformaldehyde (PFA) for 10 minutes. Samples were acquired on a FACS Verse or a FACS Canto II cytometer (Beckton Dickinson) and data were analysed with the FlowJo 7.6.5 software (TreeStar Inc.).

Cytokine assay. Levels of cytokines in supernatants of anti-CD3/CD28 stimulated splenocytes were determined using the cytometric bead array (CBA) mouse Th1/Th2/Th17 cytokine kit (BD Biosciences) according to manufacturer's protocol. Measurements were performed using FACS Verse flow cytometer and data were analysed with the FCAP Array^{TM}software (version 3.0).

Immunofluorescence. Mice at the EAE plateau phase (d20 ± 3) and with matching mean scores were deeply anesthetized and transcardially perfused with 50 mL of cold 1X PBS. Tissue samples were postfixed for 24 h at 4 °C with 4% PFA and then cryoprotected for 24 h at 4 °C with 20% sucrose solution, before freezing process into Tissue-Tek (Miles Scientific). Samples were then cut with a cryomicrotome (Leica) to obtain 10 µm sections which were thaw-mounted on poly-lysine coated slides and stored at −80 °C before experiment. After warming, sections were rehydrated three times in PBS during 15 min and incubated overnight at RT with the following antibodies: rabbit anti-CD3 (1:200, ab5690 Abcam), rat anti-CD4 (1:25, eBiosciences14-0042-82, clone RM4-5), goat anti-collV (1/1000, Southern Biotech 1340). Primary antibodies were revealed by using affinipure F(ab')2 fragments of donkey IgGs conjugated to Alexa 647, Alexa 488 or Cy3 (1:1000, Jackson Immuno Research). Then, sections were washed in PBS and coversliped with antifading medium containing DAPI. Images were digitally captured on Leica DM6000 microscope-coupled coolsnap camera, visualized with Metavue 5.0 software (Molecular Devices, USA) and further processed using ImageJ 1.49e software (NIH). For cell counting, images of three WT and three tPA$^{-/-}$ hemi-spinal cord sections from cervical, upper thoracic, lower thoracic and lumbar/sacral regions were analysed with the Fiji (ImageJ) software: an image-by-image threshold was applied to obtain a binary image for automatic particle counting (cells/mm^2) using a minimal particle size assigned to overcome the background noise of the image.

Statistical Analysis. Results are presented as the mean + SD and were analysed with the GraphPad Prism and SigmaPlot 11.0 softwares. Normality tests were performed on all samples (Agostino-Pearson omnibus and Shapiro–Wilk tests). Nonparametric Kruskal–Wallis for multiple comparisons were used, followed by two-by-two comparisons by Mann–Whitney's U tests when relevant. For multiple comparisons, an ANOVA test or its corresponding ANOVA on RANKS was performed followed by the Tukey or Dunn post-hoc tests (compared with the control condition or all pairwise), respectively. The minimum value of statistical significance considered was $P < 0.05$; for comparison of incidence curves, log-rank (Mantel-cox test) was used.

Results

EAE is less severe in tPA deficient mice.

$tPA^{-/-}$ mice developed less severe EAE disease than WT mice, with lower clinical scores during the plateau phase between days 17 and 23 (d20 ± 3) (Fig. 1A). Although no significant difference was observed between $tPA^{-/-}$ and WT mice in the incidence of the disease ($P = 0.1194$, Fig. 1B) nor for the day of disease onset ($tPA^{-/-}$: 15.13 ± 3.54 *vs* WT: 14.6 ± 2.01, P = 0.8159, data not shown), peak score and cumulative clinical score were significantly lower in $tPA^{-/-}$ than in WT mice ($tPA^{-/-}$ peak score: 1.16 ± 1.38 *vs* WT: 2.07 ± 1.56, $P = 0.0164$; cumulative clinical score: 10.17 ± 17.12 *vs* 20.38 ± 20.23, $P = 0.0163$, Fig. 1C and D). In addition, severity index ([25]), was significantly lower in $tPA^{-/-}$ than in WT mice ($tPA^{-/-}$ severity index: 0.3440 ± 0.4986 *vs* WT: 0.6652 ± 0.5522, $P = 0.0075$, Fig. 1E).

tPA enhances T-cell response by a proteolytic mechanism.

Given that immune cell infiltration is a cardinal feature of EAE, we analysed T cell subpopulations in the spinal cords of EAE mice during the plateau phase of the disease (d20 ± 3) by flow cytometry and immunofluorescence. We found that the absolute number of $CD4^+$ T cells was lower in $tPA^{-/-}$ than in WT mice (6940 ± 2110 *vs* 27110 ± 8920 cells; $P = 0.0238$, Fig. 2A), an observation consistent with the clinical scores observed in $tPA^{-/-}$ EAE mice (Fig. 1). Neither $CD8^+$ (WT: 61291 ± 27112 cells *vs* $tPA^{-/-}$: 42721 ± 8640 cells, $P = 0.5476$, Fig. 2B) nor regulatory $FoxP3^+$ T cell (Treg) (WT: 2396 ± 2322 cells *vs* $tPA^{-/-}$: 394.6 ± 444 cells; $P = 0.1667$, Fig. 2C) were significantly altered. In accordance with the above data, $CD4^+$ T cells were the only $CD3^+$ T cell subset that showed changes in the spinal cord of $tPA^{-/-}$ mice ($tPA^{-/-}$: 13.87%±4.22 *vs* WT: 29.85%±9.83, $P = 0.0476$, Fig. 2D). Concordantly, the histological analysis of the spinal cords (Fig. 2E) further showed that the average density of $CD4^+$ T cells within infiltrated area was lower in $tPA^{-/-}$ than in WT EAE mice (absolute number of WT: 514.4 ± 235.1 *vs* $tPA^{-/-}$ 245.3 ± 87.95, $P = 0.0495$, Fig. 2E-G and Suppl. Figure 1A-C).

To determine the mechanisms involved in the decrease of $CD4^+$ T cell number in $tPA^{-/-}$EAE mice, we assessed their functional response *in vitro* after activation with anti-CD3ε/CD28. $CD4^+$ T cells from $tPA^{-/-}$ naive mice proliferated less than CD4 + T cells from WT mice (proliferation index: $tPA^{-/-}$: 2.03 ± 0.95 *vs* WT: 6.18 ± 3.08; $P = 0.0317$; Fig. 3A and B). Activation level measured by mean fluorescence intensity (MFI) of CD25 was also reduced in $CD4^+$ T cells from $tPA^{-/-}$ as compared with WT $CD4^+$ T cells (51.42% ±21.91 of WT; $P = 0.0286$, Fig. 3D). Importantly, the addition of exogenous tPA rescued the proliferation and activation of $tPA^{-/-}$ CD4 + T cells at the same level as WT CD4 + T cells ($P = 0.8413$ and $P = 0.3143$, respectively; Fig. 3A, B and D). As in the case of the *in vivo* analysis, $CD8^+$ T cell proliferation was not affected in $tPA^{-/-}$ (proliferation index WT: 11.65 ± 11.01 *vs* $tPA^{-/-}$: 6.57 ± 7.48, $P = 0.3095$) although their activation was reduced (59.34%±28.96 of WT, $P = 0.0286$; Fig. 3A, C and E).

We next examined the effect of tPA on T cell response *in vitro*. tPA significantly increased proliferation and activation of anti-CD3ε/CD28 activated $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner (Fig. 4A-E). At the dose of 10 µg/mL of tPA, proliferation of $CD4^+$ and $CD8^+$ T cells were increased to reach

163.40%±33.85 (P = 0.0004) and 170.90%±35.60 (P = 0.0012), respectively. No effect of tPA was observed on Treg cells (P = 0.9999, Fig. 4F).

Next, we investigated the mechanisms underlying tPA actions on T cells. Since the primary action of tPA is to activate plasminogen into plasmin, we interrogated the different elements of the tPA/plasminogen/plasmin cascade. Plasminogen treatment neither increased $CD4^+$ T cell proliferation nor potentiated the proliferative action of tPA on $CD4^+$ T cells (P = 0.400 and P = 0.200; Fig. 4J). However, aprotinin, a specific inhibitor of plasmin, did reverse tPA-mediated activation (for $CD4^+$ T cells, P = 0.0190; for $CD8^+$, P = 0.0109; Fig. 4H-I). In addition, the inactivation of the catalytic activity of tPA (tPA-GGACK) abolished tPA stimulatory effect on $CD4^+$ and $CD8^+$ T cell proliferation (P = 0.0252 and 0.0162, respectively; Fig. 4H-I). In addition, ε-ACA, an inhibitor of plasmin generation from plasminogen inhibited the stimulatory effect of tPA on $CD4^+$ and $CD8^+$ T cell proliferation (P = 0.0056 and P = 0.0040, respectively; Fig. 4H, I). This indicates that tPA increases T cell proliferation *via* the proteolytic activation of plasminogen into plasmin.

We next explored if tPA-mediated effects on T cell proliferation was related to its previously demonstrated proteolytic action on NMDAR [27], as this receptor was previously reported to be expressed on T cells [28, 29] Glunomab®, a monoclonal antibody that blocks the interaction between tPA and NMDAR [18], did not alter the proliferative effect of tPA on $CD4^+$ T cells (Supplementary Fig. 2). This result excluded that tPA may act on T cell proliferation by acting on NMDAR.

Furthermore, since cytokines are key mediators of T cell-driven autoimmunity, we analysed the impact of tPA on the late cytokine pattern of activated T cells. tPA induced an increase of IL-6 and IL-10 secretion by activated splenocytes at four days of culture (respectively 233.9%±125.5, P = 0.0289 and 499.4%±487.6, P = 0.0029, Fig. 4G). In addition, concerning IL6, this effect was not observed with tPA-GGACK and was inhibited in presence of ε-ACA or aprotinin (P = 0.0167, 0.0167 and 0.0333 respectively, Fig. 4K). ε-ACA also reverted the activation of IL-10 secretion by tPA (P = 0.0167, Fig. 4L). Together, these data indicate that tPA increases T cell proliferation *via* the generation of plasmin to increase their proliferation, activation, and secretion of cytokines.

tPA enhances myeloid cell maturation by a mechanism dependent of proteolysis.

Our next step was to analyse whether the distribution of myeloid cells is altered in the spinal cord of $tPA^{-/-}$ EAE mice. We found that absolute numbers of $CD11c^+/CD11b^+$ (dendritic cells, DCs) and $CD45^{high}/CD11c^-/CD11b^{high}$ (activated microglia and infiltrated macrophages, M□) were lower in the spinal cords of $tPA^{-/-}$ EAE mice as compared to WT EAE mice, (DCs: 15474 ± 5169 *vs* 72283 ± 20405; P = 0.0238; microglia/M□: 14980 ± 9426 *vs* 119228 ± 14980, P = 0.0238; Fig. 5A and B).

We then analysed whether tPA may affect the proportion and the phenotype of DCs and M□. First, splenocytes extracted from EAE mice at the peak of the clinical course were treated with different concentrations of exogenous tPA (0.2, 2 and 20 µg/ml) during 24 hours. None of the tPA concentrations

modified the percentage of antigen presenting DCs ($CD11c^+$ $MHC\text{-}II^+$) and M□ ($F4/80^+$ $MHC\text{-}II^+$) populations (Fig. 6A and B). However, tPA (2 µg/mL) induced a significant increase of $MHC\text{-}II^+$ MFI in both cell populations compared to control conditions (DCs: 120.75%±17.10; M□: 121.69%±21.12, P< 0.001, Fig. 6C and D). Interestingly, the same tPA dose promoted the polarization on the $MHC\text{-}II^+$ expressing antigen presenting cell (APC) subsets towards a more pro-inflammatory and immunogenic phenotype, with a significant increase in the percentage of $MHC\text{-}II^+$ $CD80^+$ $CD86^+$-APCs (DCs: 122.45 ± 27.79, P = 0.002; M□: 128.04 ± 25.49; P = 0.004, Fig. 6E and F) and a decrease in the percentage of $MHC\text{-}II^+$ $CD80^-$ $CD86^-$ tolerogenic APCs (DCs: 87.09%±14.29 M□: 86.71%±12.08; P = 0.005 and P< 0.001, respectively; Fig. 6G and H).

Then, we aimed at analysing the receptors and/or tPA functional domains enrolled in APC maturation. The inactivation of the catalytic activity of tPA (tPA-GGACK) partially reversed $MHCII^+$ upregulation on APCs (tPA: DCs: 121.12%±14.59 M□: 113.96%±15.26 with respectively P = 0.040 and P = 0,042 *versus* control condition; tPA GGACK: DCs: 108.30%±14.43; M□: 105.21%±16.10, with respectively P = 0.156 and P = 0.440 *versus* control condition and P = 0.072; P = 0.186 versus tPA; Fig. 7A and B). Nonetheless, none of the tPA-mediated effects on APC polarization were modified by the addition of Glunomab® (Suppl. Figure 3). These data indicate that the action of tPA on APCs is partly due to its proteolytic effect and is not mediated *via* interaction with NMDAR.

On the other hand, it has been shown that tPA can act via non-proteolytic “growth factor-like” effects [30], some of them mediated by its EGF-like domain [4, 14] The addition of the EGFR blocking agent AG1478 decreased $MHC\text{-}II^+$ expression on $MHC\text{-}II^+$ APCs as compared to the condition with tPA (DCs: 55.49% ±20.64; M□: 77.76%±18.82; P< 0.001, Fig. 7C and D). These data indicate that tPA promotes APC maturation partly by the activation of EGFR.

tPA-mediated APC polarization is accompanied by a higher MOG-specific T cell response .

In order to determine whether the stimulatory effect of tPA on APC maturation may modify T cell functions, splenocytes extracted from EAE mice at the peak of the clinical course were cultured in presence/absence of tPA, with or without *ex-vivo* reactivation with MOG. In absence of MOG reactivation, tPA by itself did not induce $CD4^+$ or $CD8^+$ T cell proliferation (CD4+: 107.22%±29.02; CD8+: 124.73% ±66.40 data not shown). Condition with MOG reactivation showed a higher percentage of proliferation than control conditions for both $CD4^+$ and $CD8^+$ T cells (CD4+: 852.79%±525.09; CD8+: 1525.90% ±1096.05, both P< 0.05, data not shown). Interestingly, tPA potentiated MOGinduced $CD4^+$ T cell proliferation (132.43%±17.77; P< 0.05, Fig. 7E). In line with the previous results about APC maturation, the stimulatory effect of tPA on MOG-activated $CD4^+$T cell proliferation was abrogated when EGFR activity was blocked by AG1478 (81.88%±40.36; P< 0.05 Fig. 7E), while AG1478 did not modify MOG-induced APC proliferation in the absence of tPA (Fig. 7E). Remarkably, the stimulatory effect of tPA was not observed in MOG-activated $CD8^+$ T cells (108.26%±15.42; P> 0.05, Fig. 7F), in accordance with what

observed in the context of CD3/CD28 polyclonal activation of $CD8^+$ T cells extracted from $tPA^{-/-}$ mice (Fig. 3C).

In sum, our data indicated that the effect of tPA on APC maturation and pro-inflammatory polarization resulted in amplified MOG-induced $CD4^+$ T cell response. This effect may explain the deleterious role of tPA in EAE.

Discussion

Our study reports a deleterious role of tPA in EAE by an action on lymphoid and myeloid cell subsets by two complementary mechanisms: on the one hand, tPA directly increases T cell activation, proliferation and IL-6 secretion in a plasmin-dependent manner. On the other hand, tPA indirectly increases T cell proliferation by increasing the expression of MHC-II and costimulatory molecules in DCs and M, by both proteolytic and growth factor-like effects. These elements may bring an explanation to the deleterious effect of endogenous tPA observed here in EAE and put forward complementary mechanisms of tPA in immune response.

Previous data from the literature are contradictory about the role of tPA in neuroinflammation in EAE. Some reports indicate that $tPA^{-/-}$ mice present earlier and more severe disease that their WT counterparts, suggesting a protective role of tPA [12, 31, 32]. Other studies, including the present one, argue for a deleterious effect of tPA [11]. These discrepancies could be explained by different experimental conditions, including different MOG doses, the use of MOG re-boost or differences in the age of mice.

The fact that tPA promotes T cell activation and proliferation is in agreement with recently published data [32]. The present study provides further elements by demonstrating that this effect is dependent of plasminogen activation into plasmin. However, the potential mechanisms downstream of plasmin activation that may act on T cells in the context of EAE remain elusive. At least three potential candidates can be cited. First, tPA induces the release of the pro-form of matrix-metalloproteinase 9 (pro-MMP9) from endothelial cells by activating the lipoprotein receptor-related protein-1 (LRP) receptor [33], and activates pro-MMP9 into its active form, MMP9 *via* plasminogen activation [34]. Remarkably, MMP9 is involved in T cell proliferation, as MMP9-deficient T cells display altered proliferative ability [35]. Second, tPA activates heparin-bound EGF into free EGF, *via* the action of plasmin and matrix MMP9, leading to EGFR signalling in different cell types [36]. Third, plasmin is able to raise the bioavailability of the chemokine CCL21, known to regulate the homing of T cells and DCs towards lymphoid organs [37] and to act as a costimulatory molecule to promote T cell expansion and Th1 differentiation [38]. Interestingly, EGFR inhibition induces $CD4^+$ T cell anergy *in vitro* and *in vivo* [39]. Further experiments may clarify whether MMP9, EGFR and/or CCL21 participate in the mechanisms by which tPA promotes T cell proliferation and activation.

Our data emphasize plasmin-dependent immune functions of tPA in EAE. In accordance with our results, plasminogen deficiency in mice delays the onset and protects from demyelination in EAE [40]. Furthermore, plasmin is able to activate microglia [41], suggesting that tPA/plasmin axis is an important component to consider in future studies on the biological mechanisms underlying EAE.

Our data show that tPA acts also on myeloid cells by a distinct mechanism involving proteolytic effects and EGFR activation. By this action, tPA increases the expression of MHC-II and the co-stimulatory molecules CD80 and CD86 by DCs and M□, leading to a polarization from a tolerogenic to an immunogenic activity state. These results contrast with a previous study reporting that tPA reduces macrophage response to LPS by inhibiting ERK pathway and cytokine production, an effect mediated *via* the combined action of NMDAR and LRP pathways [19]. The mechanisms implied are likely to be different from what described in the present work: the increase in antigen presenting capacity of APCs, induced by tPA, is not prevented by Glunomab®, which points to the involvement of another pathway. In fact, in our co-culture system, the CD4 T cell proliferative effect of tPA on APC is reversed by EGFR inhibition, which indicates that tPA-mediated effects on antigen presentation are exerted *via* EGFR activation. Our study is the first that correlates the expression of MHC-II molecules with the activation of EGFR by tPA. However, the downregulation of MHC-II expression by AG1478 in absence of tPA may indicate that EGFR is intrinsically involved in antigen presentation. Interestingly, EGFR downstream signalling inhibition is a promising strategy in diverse tumour types by inducing MHC-II in APCs and breaking down tolerance [42]. Our data reinforces the idea of targeting EGFR activity as an interesting target to modulate MHC-II expression, either to potentiate it, as in the case of cancer, or to reduce it, as in the case of MS or other autoimmune diseases in which tolerance induction is one of the golden aims in cell-therapy-based strategies [43].

These data show that tPA can act on the myeloid population by mechanisms independent of plasmin generation and complete previous reports showing that tPA can activate microglial cells *via* its finger domain through annexin II [30].

tPA-induced enhancement of IL-6 cytokine production by activated splenocytes is also in favour of a proinflammatory role of tPA in EAE. Indeed, this cytokine is crucial for neuroinflammation as illustrated by the fact that IL-6 deficient mice are resistant to EAE [44]. However, tPA also intriguingly increases IL-10 production, a cytokine with immunomodulatory functions. So further works are needed to explore the net effect of tPA on the cytokine network and neuroinflammation in the course of EAE.

Conclusion

Our study opens new clues into the mechanisms by which tPA/plasmin axis participates in the pathogenesis of MS and its animal models. These mechanisms could be involved also in other neurological diseases such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease in which T cells are also involved [45]. Our study paves the way for further studies investigating the downstream targets involved in the proinflammatory effects of tPA in T cells and myeloid cells.

Declarations

Ethics approval

Animal experiments were performed following European, national, and institutional guidelines for the care and use of animals. All procedures were performed according to the guidelines of the institutional ethics committee (*Comité Normand d'éthique en matière d'expérimentation animale*, CeNomExa and *Comité Ético de Experimentación Animal del Hospital Nacional de Parapléjicos*). Projects were submitted to and approved by these committees in accordance with the European directive n° 2013/63/UE (agreement number D14118001) and with the French and Spanish National and Regional Guidelines for Animal Experimentation and the Use of Genetically Modified Organisms (French Ministry of Research, project license number 02653.2, Decree 87/848; RD 53/2013 and 178/2004, Law 32/2007 and 9/2003, Decree 320/2010).

Consent for Publication

Not applicable.

Availability of data and materials

The data generated during this study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interest.

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Author's contributions

PH performed the experiments and wrote the article; CCT, AJM, VVdS and LL performed experiments; MCO performed experiments and drafted the paper; DV and BLM drafted the manuscript; DC, FD and OT designed the experiments and wrote the article.

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