Interleukin-2 improves amyloid pathology, synaptic failure and memory in Alzheimer’s disease mice

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Interleukin-2 (IL-2)-deficient mice have cytoarchitectural hippocampal modifications and impaired learning and memory ability reminiscent of Alzheimer’s disease. IL-2 stimulates regulatory T cells whose role is to control inflammation. As neuroinflammation contributes to neurodegeneration, we investigated IL-2 in Alzheimer’s disease. Therefore, we investigated IL-2 levels in hippocampal biopsies of patients with Alzheimer’s disease relative to age-matched control individuals. We then treated APP/PS1ΔE9 mice having established Alzheimer’s disease with IL-2 for 5 months using single administration of an AAV-IL-2 vector. We first found decreased IL-2 levels in hippocampal biopsies of patients with Alzheimer’s disease. In mice, IL-2-induced systemic and brain regulatory T cells expansion and activation. In the hippocampus, IL-2 induced astrocytic activation and recruitment of astrocytes around amyloid plaques, decreased amyloid-β42/40 ratio and amyloid plaque load, improved synaptic plasticity and significantly rescued spine density. Of note, this tissue remodelling was associated with recovery of memory deficits, as assessed in the Morris water maze task. Altogether, our data strongly suggest that IL-2 can alleviate Alzheimer’s disease hallmarks in APP/PS1ΔE9 mice with established pathology. Therefore, this should prompt the investigation of low-dose IL-2 in Alzheimer’s disease and other neuroinflammatory/neurodegenerative disorders.
Keywords: neurological diseases; immunomodulation; immunosuppression; AAV; gene therapy
Abbreviations: AAV = adeno-associated virus; fEPSP = field excitatory postsynaptic potential; Treg = regulatory T cell

Introduction

There are many interactions between the CNS and immune system. The immune privileged nature of the brain has been revisited and it is clear that cells from the adaptive as well as innate immune system circulate in the brain (Louveau et al., 2015). This has led to a renewed interest for investigating the role of the immune system in neurological diseases, beyond clearly immune-mediated pathologies such as multiple sclerosis. In this line, accumulating evidence suggests that inflammation plays an important role in the pathophysiology of neurodegenerative disorders, including Alzheimer’s disease (Skaper, 2007; Heneka et al., 2015; Heppner et al., 2015). In Alzheimer’s disease, amyloid-β peptide processed from the amyloid precursor protein (APP), accumulates and aggregates in senile plaques (Hardy and Higgins, 1992). Reactive astrocytes are observed in close association with senile plaques in Alzheimer’s disease brains (Shao et al., 1997), contain intracellular amyloid-β deposits (Pihlaja et al., 2008) and degrade amyloid-β contributing to amyloid-β clearance (Nicoll and Weller, 2003). Activated microglia also accumulates around senile plaques in patients with Alzheimer’s disease (McGeer et al., 1987) and may restrict amyloid plaque formation by phagocytosing amyloid-β (Simard et al., 2006). Early stages of the neurodegenerative process are associated with glial dysfunction that may result in reduced amyloid-β clearance and cause disruptions in synaptic connectivity (Heneka et al., 2015).

Cytokines, molecules involved in the cross-talk between cells of the immune and neuroendocrine systems, are secreted by microglia and astrocytes and regulate the intensity of the brain immune response (Serpente et al., 2014). Hence, administration of interleukin-4 (IL-4) and interleukin-10 (IL-10), cytokines stimulating anti-inflammatory cascades, has been reported to improve symptoms in Alzheimer’s disease mice (Kiyota et al., 2010, 2012).

Strikingly, the role of interleukin-2 (IL-2) has not been much investigated in neurodegenerative diseases although numerous works highlighted the role of IL-2 in the CNS and immune system. It has been described that IL-2 can contact the CNS through the blood–brain barrier (Waguespack et al., 1994). Despite this, it has also been reported that IL-2 access into the brain is limited given the absence of a transporter to the brain and further slowed by circulating elements (Banks et al., 2004). It has numerous effects on hippocampal neurons, where its receptors are enriched, thus improving cognitive performances in rodents (Hanisch et al., 1997; Lacosta et al., 1999; Dansokho et al., 2016). Furthermore, IL-2 can afford trophic support to neurons and glia (Awasuji et al., 1993), enhancing neurite branching (Sarder et al., 1996), dendritic development and spinogenesis (Shen et al., 2010), thus playing a role in neuronal development (Sarder et al., 1993). In line with these observations, IL-2 knockout mice display cytoarchitectural modifications in the dentate gyrus of the hippocampus and have impaired learning and memory ability (Petitto et al., 1999; Beck et al., 2005). Recently, it has been suggested that impact on learning and memory in IL-2 knockout mice mainly relies on indirect immune-related effects (Petitto et al., 2015). Besides these direct roles on brain cells and functions, IL-2 has pleiotropic immune functions (Klatzmann and Abbas, 2015). However, low dose IL-2 supports survival and function of regulatory T cells (Tregs), that control inflammation and autoimmunity (Klatzmann and Abbas, 2015). The anti-inflammatory effects of Tregs have been observed in various models of inflammatory diseases in mice (Sakaguchi et al., 2008; Wing and Sakaguchi, 2010) and in humans. The role of Tregs in neuroinflammation is controversial but Tregs are considered as inflammation-resolving immune cells, as well as monocyte-derived macrophages (Baruch et al., 2015).

Peripheral blood Tregs numbers have been described as the best outcome predictor in amyotrophic lateral sclerosis (ALS), with fewer peripheral Tregs correlating with worse clinical outcome (Henkel et al., 2013). Importantly, recent clinical trials showed that low-dose IL-2 is safe, selectively activates Tregs without activating effector T cells and improves autoimmune and alloimmune inflammatory conditions in humans (Klatzmann and Abbas, 2015). These trials also revealed the IL-2 anti-inflammatory effects (Saadoun et al., 2011). IL-2 has thus dual properties, improving memory formation and controlling inflammation, which warrants its investigation in Alzheimer’s disease. Here, we report that sustained IL-2 expression using single administration of an AAV induces brain tissue remodelling and recovery of memory deficits in APP/PS1ΔE9 mice.

Materials and methods

Human Alzheimer’s disease brain samples

Post-mortem samples were obtained from brains collected as part of the Brain Donation Program of the GIE-Neuro-CEB Brain Bank, Pitié-Salpêtrière Hospital (Paris). Autopsies were carried out by accredited pathologists, after informed consent had been obtained from the relatives, in accordance with French Bioethics laws. Five hippocampal samples from five patients with sporadic forms of Alzheimer’s disease (male and female; Braak 6 Thal 5; aged between 69 and 89 years, with a post-mortem interval of 30 to 59h) and five hippocampal samples from five age-matched control subjects (male and
female, aged between 69 and 92 years, post-mortem interval 6–63 h) were used in this study.

**Animals**

Eight-month-old APPswe/PS1ΔE9 male mice (n = 31) (hereafter referred as APP/PS1ΔE9) and the wild-type littersmates (n = 32) (Jackson Laboratories) were used in this study. APP/PS1ΔE9 mice overexpress the mutated human APP (Swedish mutation, K595N/M596L) gene as well as the human PSEN1 gene (PS1) deleted from its exon 9 (Jankowsky et al., 2004). APP/PS1ΔE9 mice and wild-type littersmates were bred and maintained in our animal facility under specific pathogen-free conditions. Mice were housed in a temperature-controlled room and maintained on a 12-h light/dark cycle. Food and water were available ad libitum. The experiments were carried out in accordance with the European Community Council directive (86/609/EEC) for the care and use of laboratory animals. All procedures were approved by the Regional Ethics Committee in Animal Experiment No. 5 of the Ile-de-France region (Ce5/2012/031).

**Recombinant adeno-associated virus generation and in vivo administration**

Recombinant rAAV8 vectors were generated by triple transfection of human embryonic kidney 293 T cells, as described previously (Churlaud et al., 2014). Transgenes used were luciferase (LUC) and murine IL-2 (IL-2) driven by the hybrid cytomegalovirus enhancer/chicken beta-actin constitutive promoter (CAG). Mice were injected once intraperitoneally with 10^10 viral genomes (vg) of rAAVs (AAV8-CAG-IL2 or AAV8-CAG-LUC) diluted in 100 μl of 0.1 M phosphate-buffered saline (PBS) (APP/PS1ΔE9 AAV8- CAG-IL2, n = 16; APP/PS1ΔE9 AAV8-CAG-LUC, n = 15; wild-type littersmates AAV8-CAG-IL2, n = 16; wild-type littersmates AAV8-CAG-LUC, n = 16).

**Detection of IL-2 in the serum**

Sera were collected, frozen and kept at –80°C until use. Levels of IL-2 were measured using a mouse IL-2 ELISA (eBioscience) according to the manufacturer’s recommendations.

**Analysis of cell surface markers and FOXP3 expression**

Direct ex vivo immunostaining was performed on 150 μl of heparinized fresh whole blood from mice after red blood cells lysis, as described (Churlaud et al., 2014). Briefly, blood was stained with the following monoclonal antibodies for 20 min at 4°C: CD3-PE, CD8-Alexa700, CD4-HorizonV500, CD25-PeCy7, NKP46-APC and B220-FITC (eBioscience). Intracellular detection of FOXP3 (Foxp3-E450, eBioscience) was performed on fixed and permeabilized cells using appropriate buffer (eBioscience). Cells were acquired on an LSR II (Becton Dickinson) and analysed with FlowJo (Tree Star, Inc.) software. Dead cells were excluded by forward/side scatter gating. Tregs were defined as CD25+ Foxp3+ cells among CD4+ cells, and activated effector T cells as CD25+ cells among CD4+ Foxp3+ cells. For the fluorescence-activated cell sorting (FACS) analysis of Tregs, CD25 mean fluorescence intensity (MFI) in Tregs and Foxp3 MFI in Tregs were assessed.

**Brain samples**

APP/PS1ΔE9 mice and wild-type littersmates were sacrificed 5 months post-injection (13-months-old). The animals, given an overdose of sodium pentobarbital, were perfused transcardially with ice-cold PBS 0.1 M before brain extraction. For flow cytometry analysis, freshly perfused brain was dissociated and digested in collagenase/DNase solution in RPMI medium (Churlaud et al., 2014). A Percoll® (Sigma-Aldrich) gradient was used to isolate brain-infiltrating lymphocytes. Cells were then stained as described earlier for blood.

For histological processing, the left cerebral hemisphere was dissected and post-fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS for 1 week. Brains were cryoprotected by incubation in a 30% sucrose/0.1 M PBS solution. Coronal brain sections (40 μm) were cut on a freezing microtome (Leica), collected serially, and stored at −20°C until additional analysis. The right hemisphere was dissected to extract the hippocampus, used biochemistry analysis. Samples were then homogenized in a lysis buffer (TBS, NaCl 150 mM and Triton® 1%) containing phosphatase (Pierce) and protease (Roche) inhibitors. After centrifugation (20 min, 13 000 rpm, 4°C), the supernatant was collected and the protein concentration was quantified (BCA Protein Assay, Thermo Fisher Scientific). Lysate aliquots (3 mg of protein/ml) were stored at −80°C until use. The same procedure was conducted for human samples (GIE NeuroCEB Brain Bank).

**Primary antibodies**

Details of antibodies used in western blot and immunohistochemical analyses can be found in Table 1.

**Western blot**

Total protein concentrations were determined using the BCA kit (Pierce). Equal amounts of total protein extract (30 μg) were electrophoretically separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 4–12% Bis–Tris gels (NuPAGE® Novex Bis-tris midi gel 15 or 26 wells, Life Technologies) and transferred to nitrocellulose membranes. Blocked membranes (5% non-fat dry milk in TBS–0.1% Tween-20) were incubated with primary antibodies overnight at 4°C, and washed three times with TBS–0.1% Tween-20 (T-BST) for 10 min. Membranes were then labelled with secondary IgG-HRP antibodies raised against each corresponding primary antibody. After three washes with T-BST, the membranes were incubated with ECL chemiluminescent reagent (Clarity Western ECL substrate; GE Healthcare) according to the instructions of the supplier. Peroxidase activity was detected with camera system Fusion TX7 (Fisher Scientific). Normalization was done by densitometry analysis with the Quantity One 1D image analysis software (version 4.4; Bio-Rad). The optical densities were normalized with respect to a standard protein (GAPDH). A partition ratio was calculated and normalized with respect to the sample with the highest value defined as 1.
Table 1 Primary antibody details

| Primary antibodies | Source | Western blot | Immunohistochemistry |
|--------------------|--------|--------------|----------------------|
| Rabbit anti-IL2 (mouse/human) | Abcam | 1:500 | 1:500 |
| Mouse anti-beta amyloid 1-16 (clone 6E10) | Covance | 1:4000 | 1:2000 |
| Rabbit anti-APP/ beta amyloid (clone 22C11) | LifeSpan Biosciences | 1:2000 | - |
| Mouse anti-beta amyloid 17-24 (clone 4G8) | Covance | - | 1:2000 |
| Rabbit anti-glial fibrillary acidic protein (GFAP) | Dako | 1:5000 | 1:4000 |
| Rat monoclonal anti-mTREM-2B | R&D systems | 1:2000 | - |
| Rabbit anti-ionized calcium binding adapter molecule 1 (Iba1) | Wako | 1:500 | 1:3000 |
| Rabbit anti-insulin degrading enzyme (IDE) | Abcam | 1:1000 | - |
| Rabbit anti-arginase | Abcam | 1:2000 | - |
| Rabbit anti-TGF-β | Abcam | 1:2000 | - |
| Rabbit anti-pSTAT3 | Cell signalling | 1:1000 | - |
| Rabbit anti-STAT3α | Cell signalling | 1:1000 | - |
| Mouse anti-GAPDH | Abcam | 1:4000 | - |

ELISA assay

Amyloid-β38, amyloid-β40 and amyloid-β42 were measured using the MSD Human Aβ42 V-PLEX Kit and the triplex Aβ Peptide Panel 1 (6E10) V-PLEX Kit (Mesoscale Discovery). β-CTF was measured using the human APP β-CTF Assay Kit (IBL). IL-2 was measured using the MSD proinflammatory panel 1 (Mesoscale Discovery). ELISA assays were performed following supplier instructions.

Immunostaining

The immunohistochemical procedure was initiated, by incubating slices in 88% formic acid solution for 15 min (antigen retrieval) and then by quenching endogenous peroxidase by incubating free-floating sections in hydrogen peroxide for 30 min at room temperature. After three washes, slices were blocked in PBS/0.1% Triton X-100 containing 10% normal goat serum (NGS, Gibco) for 1 h at room temperature. The sections were then incubated with the primary antibody (4G8), overnight at 4°C. After three washings, the sections were incubated with the corresponding biotinylated secondary antibody (1:250; Vector Laboratories Inc.) diluted in PBS/0.1% Triton X-100 and 10% NGS for 2 h at room temperature. After three washes, bound antibodies were visualized by the ABC amplification system (Vectastain ABC kit, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (peroxidase substrate kit, DAB, Vector Laboratories) as the substrate. The sections were mounted, dehydrated by passing twice through ethanol and toluol solutions, and coverslipped with Eukitt (O. Kindler).

For immunofluorescence, slices were washed with PBS 0.1 M, permeabilized in PBS-Triton 0.1% before blocking in PBS-Triton 0.1% containing 5% NGS for 1 h. Sections were then incubated with the respective primary antibodies, overnight at 4°C. After three successive washes, brain slices were incubated for 2 h at room temperature with fluorescent secondary Alexa Fluor®-conjugated antibodies (Invitrogen). Slices were stained with DAPI (1:5000; Sigma), mounted in Vectashield fluorescent mounting media (Vector laboratories) and conserved at 4°C.

Quantification of the microglia and astrocytes around plaques

Plaques, GFAP and Iba1 immunoreactivity were quantified using ImageJ (NIH, Bethesda, USA) or Icy (Institut Pasteur, Paris, France). Laser power, numeric gain and magnification were kept constant between animals to avoid potential technical artefacts. Images were first converted to 8-bit grey scale and binary thresholded to highlight a positive staining. At least three sections per mouse (between –1.7 mm to –2.3 mm caudal to bregma) were quantified. The average value per structure was calculated for each mouse. For quantification of Iba1 and GFAP immunoreactivity around plaques, a region of interest was drawn around the center of the plaque. The diameter of the circular region of interest was set as three times the diameter of the plaque. Mean fluorescence intensity values were measured for either Iba1 or GFAP immunoreactivity and were processed via Icy software (Institut Pasteur). Analysis of data was blind with respect to treatments and genotypes.

Image acquisition

Images of immunostained sections were acquired with a Z6 APO macroscope (Leica) and LAS V3.8 (Leica) software, at room temperature, with a brightfield Leica DM 5000B microscope equipped with a Leica DFC310FX digital camera. Confocal images were acquired with a Leica SP8 confocal microscope. Photographs for comparison were taken under identical conditions of image acquisition, and all adjustments of brightness and contrast were applied uniformly to all images.

Morris water maze behavioural assessment

Experiments were performed in a 120-cm diameter, 50-cm deep tank filled with opacified water kept at 21°C and equipped with a 10-cm diameter platform submerged 1 cm under the water surface. Visual clues were disposed around the pool as spatial landmarks for the mouse and luminosity was kept at 430lx. Training consisted of daily sessions (three
trials per session) for five consecutive days. Start positions varied pseudorandomly among the four cardinal points. Mean intertrial interval was 15 min. Each trial ended when the animal reached the platform. A 60-s cut-off was used, after which mice were gently guided to the platform. Once on the platform, animals were given a 30-s rest before being returned to their cage. Four hours (short-term memory) and 72 h after the last training trial (Day 8), retention was assessed during probe trial in which the platform was no longer present. Animals were video tracked using Ethovision software (Noldus, Wageningen, Netherlands) and behavioural parameters (swim speed, travelled distance, latency, percentage of time in each quadrant) were automatically calculated. Experiments and statistical evaluation of data were performed by an experimenter blind to genotype and treatment group.

**Electrophysiology**

Female 13.5-month-old APP/PS1dE9 mice injected with the rAAV-IL-2 (n = 5) or rAAV8-LUC (n = 5) and their littermates administered with rAAV-IL-2 (n = 4) or rAAV8-LUC (n = 4) were used for electrophysiological examination of synaptic plasticity in hippocampus. Mice, deeply anaesthetized by inhalation of high concentration of CO2, were decapitated and brain was removed and divided into two parts. One hemisphere was transferred into the Golgi staining solution and processed accordingly to allow investigation of neuronal morphology of regions of interest. The second hemisphere was transferred into cold (4°C) artificial CSF, containing the following (in mM): 124 NaCl, 4.9 KCl, 1.2 KH2PO4, 2.0 MgSO4, 2.0 CaCl2, 24.6 NaHCO3, 10-d-glucose, equilibrated with 95% O2 and 5% CO2. The hippocampus was dissected from the second hemisphere and transverse hippocampal slices of 400μm were cut using a tissue chopper. Hippocampal slices were incubated at 32°C in an interface chamber with the constant flow of carbogenated (95% O2 and 5% CO2) artificial CSF for 2 h prior to recording. Field excitatory postsynaptic potentials (fEPSPs) were recorded in stratum radiatum of the CA1 region of hippocampus. Synaptic responses were evoked by stimulation of the Schaffer collaterals. An input/output curve (dependence of fEPSP slope on stimulation intensity) was plotted prior to each experiment.

Data of electrophysiological recordings were collected, stored and analysed with LABVIEW software (National Instruments, Austin, USA). The initial slope of fEPSPs elicited by stimulation of the Schaffer collaterals was measured over time, normalized to baseline and plotted as average ± standard error of the mean (SEM). Analysis of the paired-pulse facilitation data was performed by calculating the ratio of the slope of the second fEPSP divided by the slope of the first one and multiplied by 100.

**Dendrite and spine analysis in Golgi-Cox stained slices**

**Golgi-Cox staining**

Golgi staining was performed using the Golgi Staining Kit (FD NeuroTechnologies) according to the manufacturer’s instructions. All procedures were performed under dark conditions. Brains hemispheres used for Golgi cox staining were immersed in 2 ml mixtures of equal parts of kit solutions A and B and stored at room temperature for 2 weeks. Then, brain tissues were stored in solution C at 4°C for at least 48 h and up to 7 days before sectioning. Solutions A, B and C were renewed within the first 24 h. Coronal sections of 200μm were cut with a vibrating microtome (Leica, VT1200S) while embedded in 2% agar in 0.1 M PBS. Each section was mounted with solution C on an adhesive microscope slide precoated with 1% gelatin/0.1% chromalum on both slides and stained according to the manufacturer’s protocol with the exception that AppliClear (AppliChem) was used instead of xylene. Finally, slices were coveredslipped with PermounTM (Thermo Fisher Scientific).

**Imaging and analysis of spine density in Golgi-Cox stained slices**

Imaging of dendritic branches of hippocampal pyramidal neurons was done with an Axioplan2 imaging microscope (Zeiss) using a 63 × oil objective (NA 1.3) and a z-stack thickness of 0.5μm under reflected light. The number of spines was determined per micrometre of dendritic length (in total 100μm) at apical compartments using ImageJ (1.48v, National Instruments of Health, USA). Three animals per genotype and 8–10 neurons per animal were analysed blinded to genotype and injected AAV. Data were analysed using Graphpad Prism (Version 5.01) software. Spine density is expressed as mean ± SEM. Differences between genotypes were detected with one-way ANOVA followed by Bonferroni’s post hoc test using IBM SPSS Statistics 21.

**Statistical analysis**

Statistical analyses were defined regarding the experimental design used. All data are presented as the mean ± SEM. In most cases, data were analysed using Student’s t-test, the Mann-Whitney test or one-way ANOVA with experimental group as factor. One-way ANOVA with repeated measures were carried out when required by the experimental plan to assess statistical effects. Correlations were generated using non-parametric Spearman rank correlation coefficient. For all analysis statistical significance was set to a P-value < 0.05. All analyses were performed using Statistica (StatSoft Inc., Tulsa, USA) or GraphPad Prism (GraphPad Software, La Jolla, USA).

**Results**

**IL-2 expression is decreased in the hippocampus of patients with Alzheimer’s disease**

We first analysed IL-2 protein levels in frozen hippocampal biopsies from five severely affected patients with Alzheimer’s disease (Braak 6/Thal5) and five age-matched healthy control subjects. We observed a 2-fold decrease in IL-2 levels (P < 0.05) (Fig. 1A and B) paralleled by a ≈ 80% reduction in APP levels (P = 0.001) and synaptic protein PSD-95 levels (P = 0.004) (Fig. 1A, C and D) in the hippocampus of patients with Alzheimer’s disease relative to controls. The amyloid-β42/amyloid-β40 ratio, an outcome measure for Alzheimer’s disease severity (Ferrari et al., 2014), was found increased in Alzheimer’s disease patients (P = 0.07) (Fig. 1E). Remarkably, there is a significant positive Spearman correlation between IL-2 and APP levels.
Peripheral IL-2 delivery induces increased IL-2 and regulatory T cell levels in the brain of APP/PS1ΔE9 mice

As Alzheimer’s disease is a slow developing disease, we anticipated that long term Treg stimulation could be necessary for obtaining therapeutic benefit. As IL-2 has a short half-life in mice, maintaining an effect on Tregs would require frequent subcutaneous injections that could have an effect on mice behaviour. We thus delivered IL-2 by intraperitoneal injection of a recombinant AAV coding for murine IL-2, which allows sustained and stable release of IL-2 for at least 20 weeks (Wang et al., 2005; Churlaud et al., 2014). Eight-month-old APP/PS1ΔE9 mice and littermates were injected with rAAV8-IL2 or luciferase-expressing control vectors (rAAV8-LUC). Four months after rAAV8 injections, serum IL-2 was undetectable in mice receiving rAAV8-LUC, and was 25.2 ± 5.3 or 29.5 ± 4.8 pg/ml (mean ± SEM) in IL-2-treated littermates and APP/PS1ΔE9, respectively (Fig. 2A). These serum IL-2 levels are those necessary for expanding and activating Tregs without effects on effector T cells, as previously described (Churlaud et al., 2014). Indeed, peripheral IL-2 production expanded blood Tregs, significantly more in APP/PS1ΔE9 mice than in wild-type littermates (Fig. 2C). Tregs from IL-2-treated mice were also more activated as assessed by increased CD25 cell surface expression (Rosenzwajg et al., 2015) (Fig. 2E). Mice were sacrificed at 5.5 months post-injection (13.5 months of age). In hippocampal biopsies, there was a significant increase of IL-2 levels only in IL-2-treated APP/PS1ΔE9 mice (Fig. 2B). These levels were approximately doubled compared to IL-2-treated wild-type littermates and rAAV8-LUC controls, as detected both by western blot (Supplementary Fig. 1) and ELISA (Fig. 2B). There was a concomitant 3-fold increase of brain Tregs in IL-2-treated normal and APP/PS1ΔE9 mice (Fig. 2D). These Tregs also showed a higher activation status as attested by increased CD25 expression (Fig. 2F). To evaluate whether the increased IL-2 levels observed in APP/PS1ΔE9 transgenic mice could be due to the transduction of brain cells by AAV vectors, we analysed the biodistribution of AAV after injection of rAAV8-LUC in normal and in APP/PS1ΔE9 transgenic mice. We assessed luciferase bioluminescence in peripheral organs and also in the brain. As negative control, we used non-injected age-matched APP/PS1ΔE9 mice. Two
weeks post-injection, luciferase expression could be detected in most of peripheral organs (liver, heart, kidney and spleen) of rAAV8-LUC-injected APP/PS1ΔE9 mice. In accordance with the known tropism of AAV8, most of the expression was detected in the liver. In contrast, no expression could be detected in the brain (Supplementary Fig. 2A and B). To further substantiate these data, we also used a sensitive quantitative polymerase chain reaction targeting the inverted terminal repeat (ITR2) sequence from the AAV8 vector genome to probe its biodistribution. ITR2 sequences were readily detected in the liver, while no ITR2 sequences could be detected in the brain of APP/PS1ΔE9 mice injected with rAAV8-LUC (Supplementary Fig. 2C). Thus, the increased brain IL-2 levels in APP/PS1ΔE9 mice injected with rAAV8-IL-2 results from passage from the periphery and not from local production by rAAV8 transduced brain cells.

**IL-2 treatment rescues memory impairment in APP/PS1ΔE9 mice**

To evaluate the therapeutic effects of increasing brain IL-2 on spatial learning and memory, mice were tested in the Morris water-maze place navigation task (Fig. 3). Treated APP/PS1ΔE9 mice or wild-type littermates were tested 5 months post-injection. All mice learned platform position across time during learning session, as demonstrated by decreased latency (Fig. 3A) or path length (Fig. 3B) to reach the platform over the 5 days of training. Noteworthy, an overall significantly improved learning was detected in rAAV-IL-2-treated littermates ($P < 0.05$). Average swimming speed was comparable in all groups ruling out potential motor abilities differences (Fig. 3C). The 4-h probe trial, which evaluates spatial reference
Figure 3 IL-2 treatment rescues spatial memory impairment in APP/PS1ΔE9 mice. Transgenic APP/PS1ΔE9 mice (n = 7/8 per group) or littermate controls (n = 8 per group) were intraperitoneally injected with either rAAV8-IL-2 or rAAV8-LUC vectors at 8 months of age and tested 5 months later, i.e. at 13 months of age. Training phase consisted of daily sessions with three trials/day during five consecutive days. Four hours after the last training trial (Day 5), the platform was removed and memory retention was assessed during a probe test. (A) Escape latency (B) path length and (C) swim speed of APP/PS1ΔE9 mice or age-matched littermates injected with rAAV8-IL-2 or rAAV8-LUC during learning session. Despite the absence of IL-2 effect in APP/PS1ΔE9 mice, IIL-2 treatment enhanced littermate memory abilities in comparison to the three other groups (Two-way ANOVA followed by Tukey post hoc test: *P < 0.05). (D) Probe trial performance at 4h (short-term memory). rAAV8-LUC treated APP/PS1ΔE9 mice were impaired compared to rAAV8-LUC treated littermates, confirmed by no preference for the trained target quadrant (TQ) (% of time and % of distance). Strikingly, by contrast to APP/PS1ΔE9 mice injected with rAAV8-LUC, APP/PS1ΔE9 mice...
memory (short-term memory) after the last training trial, revealed strong memory impairment in APP/PS1ΔE9 mice injected with rAAV-LUC compared to rAAV-LUC littermates (P = 0.0002 and P = 0.0002 using %time and %distance as readouts; Fig. 3D, F and H); wild-type littermates treated with either rAAV-LUC or rAAV-IL-2, showed similar preference. Strikingly, by contrast to APP/PS1ΔE9 mice injected with rAAV-LUC, IL-2-treated APP/PS1ΔE9 mice showed a clear preference for the target quadrant (rAAV-LUC-treated vs IL-2-treated APP/PS1ΔE9 mice: % of time in target quadrant; P = 0.0034 and % of distance in target quadrant; P < 0.0001), and were statistically indistinguishable from control littermates. These results were confirmed at 72 h (long-term memory; rAAV-LUC-treated versus IL-2-treated APP/PS1ΔE9 mice: % time in target quadrant; P = 0.0199 and % of distance in target quadrant; P = 0.0092) (Fig. 3E, G and I) suggesting a beneficial effect of IL-2 during memory consolidation phase. These data clearly demonstrate that IL-2 rescued impairments in memory retention observed in APP/PS1ΔE9 mice.

**IL-2 rescues impaired synaptic plasticity and restores decreased spine density in APP/PS1ΔE9 mice**

Amyloid-β-induced damage of synaptic transmission is one probable mechanism inducing memory impairments in APPPS1 mice (Snyder et al., 2005). We evaluated whether IL-2-based memory restoration in APP/PS1ΔE9 mice was reflected at the functional neuronal network level. We investigated synaptic plasticity at 13.5 months of age, which is considered to represent the basis of newly shaped declarative memory. Long-term potentiation (LTP) was induced at the Schaffer collateral to hippocampal CA1 pathway by theta-burst stimulation after baseline recording (Fig. 4A). As expected, slices from transgenic APP/PS1ΔE9 mice exhibited significantly lower induction and maintenance of LTP compared with littermates after rAAV8-LUC administration [1.15 ± 0.001 (n; number of slices = 12, P < 0.05, F-value 6.01, ANOVA) compared with 1.51 ± 0.003 (n = 9)]. IL-2-treated APP/PS1ΔE9 mice showed significantly improved LTP as evidenced by statistically significant increase of average potentiation (1.23 ± 0.01, n = 10, P < 0.05, F-value 5.92, ANOVA) compared with APP/PS1ΔE9 mice receiving rAAV8-LUC. However, LTP magnitude was lower relative to IL-2-treated littermates (1.45 ± 0.001, n = 8, P < 0.05, F-value 6.01) (Fig. 4A and B). The fEPSP slope was not different between different groups (Fig. 4C), suggesting that basal synaptic transmission in all groups was not affected. In addition, we analysed paired-pulse facilitation of fEPSP to afferent stimulation, a form of short-term synaptic plasticity. Analysis of the EPSP2/EPSP1 ratio revealed significant (P < 0.05, ANOVA) facilitation of second response in all interpulse intervals in APP/PS1ΔE9 mice administered with rAAV-IL-2 compared with their littermates. Paired-pulse facilitation in APP/PS1ΔE9 mice administered with rAAV-LUC was also significantly (P < 0.05, ANOVA) higher compared with littermates. Paired-pulse facilitation was not different between APP/PS1ΔE9 mice administered with rAAV-IL-2 or rAAV-LUC (Fig. 4D). Spine density was analysed as a correlate of excitatory synapses in the same animals used for electrophysiological recordings. Spine density of mid-apical dendritic segments (between 100 and 400 μm from soma) of the hippocampal CA1 pyramidal layer was analysed by the Golgi cox method (Fig. 4E). We found an overall decrease in spine density in APP/PS1ΔE9 mice treated with control rAAV-LUC at the CA1 apical dendritic compartment relative to littermates injected with either rAAV-LUC or rAAV-IL-2 (P < 0.001, one-way ANOVA followed by Bonferroni’s post hoc test). Importantly, rAAV-IL-2-treated APP/PS1ΔE9 mice revealed a complete restoration of the spine deficit in apical dendrites of the CA1 layer (P > 0.1) (Fig. 4F). Altogether, these data indicate that IL-2 strikingly ameliorates both structural and functional synaptic impairments in Alzheimer’s disease mice.

**IL-2 peripheral administration alleviates hippocampal amyloid pathology in APP/PS1ΔE9 mice**

APP/PS1ΔE9 mice show highly abundant plaques from 6 months (Jankowsky et al., 2004). IL-2 treatment was started at 8 months of age, a time at which we already evidence increased levels of amyloid-β peptides (amyloid-β38, amyloid-β40 and amyloid-β42) and β-CTF in hippocampus from APP/PS1ΔE9 mice (Supplementary Fig. 3). Mice were sacrificed at 13.5 months of age. Hippocampal APP levels were slightly increased in mice receiving rAAV8-IL-2 compared to rAAV8-LUC injected mice (Fig. 5A–C). The production of amyloid-β peptides and β-CTF, known to induce hippocampal neurophysiological impairments, was quantified in the hippocampus of injected mice by...
Figure 4  IL-2 expression rescues structural and functional synaptic deficits in APP/PS1ΔE9 mice. (A) Long-term potentiation (LTP) was induced by delivering the theta-burst stimulation (TBS; indicated as an arrow). Slices from transgenic APP/PS1ΔE9 mice injected with rAAV8-LUC exhibited significant deficit in LTP expression when compared with littermates injected with rAAV8-LUC. LTP magnitude observed in IL-2-treated APP/PS1ΔE9 mice was significantly lower than that in IL-2-treated littermates; however, it was significantly improved in comparison to APP/PS1ΔE9 injected with the control rAAV8-LUC. Insets are sample illustrations of the fEPSPs recorded before and after theta-burst stimulation. (B) Summary bar-graphs showing differences in mean values of LTP magnitude between genotypes. (C) The hippocampal slices obtained from APP/PS1ΔE9 mice injected with the rAAV-IL-2 or rAAV8-LUC and their littermates administered with rAAV-IL-2 or rAAV8-LUC showed no difference in input-output relations, suggesting no differences in basal synaptic transmission. (D) Statistically significant facilitation of second response in all interpulse intervals was revealed by paired-pulse stimulation (PPF) analysis in APP/PS1ΔE9 administered with rAAV-IL-2 compared with their littermates. Paired-pulse stimulation in transgenic APP/PS1ΔE9 mice administered with rAAV-LUC was also significantly higher at intervals of 20, 40, 60 and 80 ms, relative to their littermates. Paired-pulse stimulation was not significantly different between APP/PS1ΔE9 mice injected with rAAV-IL-2 or rAAV-LUC. *Significant differences at P < 0.05. (E) Representative images of Golgi-Cox stained apical dendrites of littermates as well as APP/PS1ΔE9 mice injected with rAAV-IL-2 or the control vector. Scale bar = 10 μm. (F) Spine number was reduced in APP/PS1ΔE9 (1.12 ± 0.03) compared to littermate controls injected with the control vector (1.42 ± 0.05) or rAAV-IL-2 (1.35 ± 0.04), a phenotype which could be rescued by injection with rAAV-IL-2 (1.29 ± 0.04). n = 3 animals per genotype, 8–10 dendrites per animal. Values represent mean ± SEM.
ELISA. No differences in β-CTF levels were detectable (Fig. 5D). There was a trend towards increased amounts of amyloid-β₃₈ and amyloid-β₄₀ (Fig. 5E and F) and a trend to decrease in soluble amyloid-β₄₂ levels (Fig. 5G). This translated into a significant reduction in the amyloid-β₄₂/amyloid-β₄₀ ratio (P = 0.0013) (Fig. 5H). This correlated with a decrease in the surface covered by plaques in APP/PS1ΔE9 mice (P = 0.023) (Fig. 5I and J). Altogether,
these data indicate that IL-2 reduces amyloid load and plaque deposition in the hippocampus of APP/PS1ΔE9 mice with established pathology.

**IL-2 administration induces widespread recruitment of astrocytes in the vicinity of amyloid plaques**

Amyloid plaques in APP/PS1ΔE9 mice were surrounded by microglia and astrocytes at the time of the treatment (8 months) (Supplementary Fig. 4).

The protein levels of microglial Iba1 were not statistically different in the hippocampus of IL-2-treated APP/PS1ΔE9 mice compared to APP/PS1ΔE9 mice receiving rAAV8-LUC (Fig. 6A and B); no differences were detected in littermates receiving rAAV8-LUC or rAAV8-IL-2. No differences were observed in the expression of microglia markers, cytokine transforming growth factor-beta (TGF-β), arginase-1 and triggering receptor expressed on myeloid cells (TREM2) or insulin-degrading enzyme (IDE), which contributes to amyloid-β clearance (Leissring et al., 2003) (Supplementary Fig. 5). No major difference in Iba1 immunoreactivity around hippocampal amyloid plaques was observed (Fig. 6D and E).

Astrocytic markers analysis demonstrated a 3–4-fold increase of GFAP expression (P < 0.0001) in APP/PS1ΔE9 mice injected with rAAV8-IL-2 compared to APP/PS1ΔE9 mice treated with rAAV8-LUC (Fig. 6A and C). Immunostaining clearly showed that these GFAP immunoreactive astrocytes were hypertrophic, indicating their activation (Fig. 6F). Moreover, a statistically significant increase of GFAP immunoreactivity around amyloid plaques was found in rAAV-IL-2-treated APP/PS1ΔE9 mice (Fig. 6G). In addition, in regions surrounding plaques, astrocytes were hypertrophic with thick proximal processes overlapping with the plaque, suggesting process invasion within plaques in AAV-IL2-treated APP/PS1ΔE9 mice. In contrast, astrocytes from APP/PS1ΔE9 mice treated with the control vector exhibited lower hypertrophic processes (Fig. 6F). Littermates treated with IL-2 or luciferase vectors did not exhibit hypertrophic process.

**IL-2 administration activates the JAK/STAT3 pathway in the hippocampus of APP/PS1ΔE9 mice**

We next assessed whether this IL-2 mediated astrocytic activation was correlated with stimulation of the JAK/STAT3 pathway. STAT3 is an important signalling molecule for many cytokines and growth factor receptors that prompts astrocyte reactivity (Heim, 1999; Chiba et al., 2009). Western blot analysis demonstrated a 2-fold increase in the levels of Stat3-α in APP/PS1ΔE9 mice receiving rAAV8-IL-2 compared to APP/PS1ΔE9 mice treated with the control vector (Fig. 6H and J) (P < 0.0001). The phosphorylated form of STAT3 [phospho-STAT3 (Tyr705)] was increased in APP/PS1ΔE9 mice treated with the control rAAV8-LUC, relative to littermates treated with rAAV8-IL-2 or rAAV8-LUC (Fig. 6H and J). Remarkably, we found a 2.2-fold increase of phospho-STAT3 in IL-2-treated APP/PS1ΔE9 mice as compared to APP/PS1ΔE9 mice that received the control vector (Luciferase) (Fig. 6H and J) (P < 0.0001). Taken together, these results reveal an increased recruitment of astrocytes around amyloid plaques and the activation of the JAK/STAT3 pathway in APP/PS1ΔE9 mice treated with IL-2.

**Discussion**

Gliosis and inflammation are hallmarks of Alzheimer’s disease (Schwab and McGeer, 2008). It is still not clear whether inflammation has a direct or indirect influence on the build-up of amyloid-β pathology. It has long been considered that the increase of pro-inflammatory mediators would contribute to Alzheimer’s disease progression, thereby implying potential benefit of anti-inflammatory immunotherapies (Birch et al., 2014). Likewise, inhibiting the signalling of the pro-inflammatory cytokines IL-12/IL-23 in APP/PS1 mice decreased glial activation, amyloid load and cognitive decline (Vom Berg et al., 2012). The hippocampal AAV-mediated overexpression of the anti-inflammatory cytokines IL-10 or IL-4 in Alzheimer’s disease mice enhanced neurogenesis and improved spatial learning and amyloid-β deposition in APP/PS1 mice (Kiyota et al., 2010, 2012; Latta et al., 2015). Despite this, two recent studies support a detrimental impact of IL-10 in Alzheimer’s disease pathology (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). Interestingly, hippocampal expression of IL-1β in Alzheimer’s disease mice did not result in the expected exacerbation of the amyloid plaque deposition, but instead in plaque improvement (Shaftel et al., 2007). AAV-mediated expression of IL-6 (Chakrabarty et al., 2010) and TNF-α (Chakrabarty et al., 2011) induced massive gliosis that suppressed amyloid-β deposition. Recently, immune checkpoint blockade directed against the programmed death-1 (PD-1) pathway evoked an interferon (IFN)-γ-dependent systemic immune response leading to clearance of plaques and improved cognitive performance (Baruch et al., 2016). Thus, and surprisingly, it appears that modulation of the immune system towards both effector and regulatory functions may counteract Alzheimer’s disease.

In this context, there is a strong rationale to investigate the therapeutic effects of IL-2 in Alzheimer’s disease *in vivo*: (i) IL-2 knockout mice exhibit impaired learning and memory formation and altered hippocampal development (Petitto et al., 1999); (ii) serum IL-2 levels are low in patients with Alzheimer’s disease, compared with both elderly and middle-aged subjects (Beloosesky et al., 2002); and (iii) IL-2, at low dose, has an anti-inflammatory effect (Saadoun et al., 2011). In this report, we show that IL-2 prompts Treg expansion and activation in the brain of
APP/PS1ΔE9 mice and improves Alzheimer’s disease pathology. Increased IL-2 concentrations were observed in the hippocampus of IL-2-treated APP/PS1ΔE9 mice but not in IL-2-treated littermates. As AAV vectors do not transduce brain cells and IL-2 is produced in the periphery, we may speculate that CNS penetration of peripherally produced IL-2 could be favoured in APP/PS1ΔE9 mice probably due to blood–brain barrier leakage. Indeed, the blood–brain barrier is relatively impermeable in healthy subjects, however compromised in Alzheimer’s disease given disruption of the tightly packed endothelial cells that support brain vasculature. Importantly, blood–brain barrier permeability has been reported in Alzheimer’s disease mouse models (Tanifum et al., 2014) and was shown to increase before plaque formation in Alzheimer’s disease mice (Ujiie et al., 2003). This has important implications for the route of administration of therapeutic molecules such as IL-2 (Waguespack et al., 1994).

Tregs have a controversial role in controlling or worsening Alzheimer’s disease. Treg depletion was reported to improve Alzheimer’s disease in 5xFAD mice (Baruch et al., 2015) or to accelerate the onset of cognitive deficits in APP/PS1 mice (Dansokho et al., 2016). In any case, how transient Treg ablation (observed in the periphery but not
asserted in the brain) can impact a disease that develops along months is unexplained. Actually, the brief and transient Tregs depletion reported as improving Alzheimer’s disease (Baruch et al., 2015) resulted in a marked enrichment of Tregs in the brain 3 weeks after the last Treg depletion modality (53.4% versus 18.1%) (Baruch et al., 2015). The authors concluded that Tregs recruitment to cerebral sites of Alzheimer’s disease pathology may have led to reduction of gliosis and amyloid-β plaque, with improvement of cognitive functions (Baruch et al., 2015). As efficient Treg depletion in mice leads to rapid (3 to 6 weeks) catastrophic autoimmunity, inflammation and death (Fontenot et al., 2003), this precludes the evaluation of the effects of long-term Treg ablation that might be important for slow-developing diseases. Thus, Treg depletion might not be an optimal method to assess Treg role in Alzheimer’s disease. Other investigators (Dansokho et al., 2016) used an anti-CD25 monoclonal antibody to deplete Tregs in vivo in APP/PS1 mice. We considered that this strategy was not suitable in our study, as Treg depletion is only transient and CD25 is also expressed by other immune cells such as natural killer cells, activated B cells, activated effector T cells, myeloid cells, which could induce non.expected effects by depleting these populations (Baeyens et al., 2013). In this study, we used the reverse setting and evaluated whether Treg activation and expansion could improve Alzheimer’s disease. We administered IL-2 over 5 months and observed increased Treg numbers and activation in the brain, correlating with histological and clinical Alzheimer’s disease improvement. Similar observations were also recently reported, although the authors did not assess Tregs in the brain (Dansokho et al., 2016). Thus, altogether, indirect evidences suggest a beneficial role of activated Tregs in Alzheimer’s disease. We show that IL-2-induced Alzheimer’s disease improvement is linked to an astrocytic activation. We observed a marked astrocytic activation that is considered as reflecting an attempt to recover from CNS injury (Wegiel et al., 2001). Indeed, astrocytes were proposed to protect neurons by forming a physical barrier around plaques (Wegiel et al., 2001). Interestingly, the astrocytic phenotypic activation was correlated with stimulation of the JAK/STAT3 pathway that was shown to activate astrocytes in models of acute brain injury and is involved in cell growth, neuronal survival and differentiation (Bareyre et al., 2011; Lang et al., 2013). Astrocytes have been described as a potential source of brain IL-2 (Eizenberg et al., 1995). Astrocytes make part of the blood–brain barrier and may well be the primary brain parenchymal cell type encountered by peripheral Tregs. Indeed, previous reports have described astrocyte–T cell interactions (Barcia et al., 2013). More recently, a close interaction between Tregs and astrocytes has been reported, in which the activation of an IL-2/STAT5 signalling pathway is implicated in an astrocyte-mediated maintenance of Tregs (Xie et al., 2015). Further studies are still needed to better understand the sequential cross-talk between astrocytes and Tregs during IL-2-based therapy.

Increased astrocytic reactivity around amyloid plaques suggests that astrocytes may influence Alzheimer’s disease-like pathogenesis through invasion of plaques as an attempt to clear amyloid-β and limit its extracellular deposition. In line with this finding, mouse astrocytes were reported to degrade amyloid-β in vitro and in situ (Wyss-Coray et al., 2003), and exogenous astrocytes transplanted into the brain of plaque-bearing Alzheimer’s disease mice, were shown to migrate towards amyloid-β deposits, internalizing them (Pihlaja et al., 2008). Furthermore, it was shown that attenuating astrocyte activation accelerates plaque deposition in Alzheimer’s disease mice (Kraft et al., 2013).

Consistent with these properties of astrocytes, IL-2 treatment and consequent astrocytic activation were accompanied by a reduction of amyloid plaques and a decrease in the amyloid-β42/amyloid-β40 ratio (Murray et al., 2012). The strong amyloid-β40 increase in IL-2-treated APP/PS1ΔE9 mice is in line with in vitro and in vivo data showing that amyloid-β40 protects neurons from amyloid-β2 induced damage in culture and in rat brain (Zou et al., 2003) and inhibits amyloid deposition in Alzheimer’s disease mice, protecting them from premature death (Kim et al., 2007). In a different mouse model of Alzheimer’s disease (APP/PS1 mice), IL-2 was proposed to work by a microglial activation (Dansokho et al., 2016). In our APP/PS1ΔE9 mice we did not observe such activation. Iba1, arginase-1, TGF-β, IDE and Trem2B detection by western blot were unchanged in the hippocampus. There was no increase of Iba1-positive cells by immunofluorescence around plaques.

We further report that the recovery of memory deficits observed in APP/PS1ΔE9 mice was supported by a remarkable IL-2-mediated tissue remodelling in the brain characterized by increased synaptic plasticity and restoration of spine density. To our knowledge, this is the first demonstration that in vivo immunomodulatory treatment can actually induce such brain tissue remodelling. These findings are in accordance with previous reports showing that IL-2 promotes survival and neurite extension of cultured neurons as well as enhances dendritic development and spino genesis (Awatsuji et al., 1993; Sarder et al., 1993; Shen et al., 2010).

IL-2 is an approved drug used for the stimulation of effector cells for the treatment of metastatic melanoma and renal cell carcinoma. In these indications it is given at very high doses (up to 160 MIU per day) and actually poorly used because of severe side effects (Klatzmann and Abbas, 2015). The demonstration that low-dose IL-2 is safe and selectively activates and expands Tregs without activating effector T cells in humans has changed the paradigm for IL-2 therapeutic use. IL-2 is now intensively developed as a stimulant of Tregs at daily dose around 1 to 3 MIU. At these low doses, IL-2 is well tolerated in humans with autoimmune diseases (Saadoun et al., 2011; Hartemann et al., 2013; Castela et al., 2014; He et al., 2016). Noteworthy, these doses lead to increased serum concentration of IL-2 that are in the range of the long-term elevated
concentrations observed during pregnancy (Curry et al., 2008). Finally, a preclinical study of the long-term effects of IL-2 in mice showed that a year-long treatment is well tolerated (Churlaud et al., 2014). Thus, long-term treatment with low-dose IL-2 in patients with Alzheimer’s disease can be envisioned. In summary, our results demonstrate the therapeutic effects of IL-2 in Alzheimer’s disease mice with established pathology. Although it remains to elucidate the direct and Treg-mediated contribution of IL-2 to Alzheimer’s disease improvements, these results warrant investigating low dose IL-2 for neuroinflammatory diseases.

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Conflict of interest

The authors declare financial interest. S.A., G.C., D.K. and N.C. are inventors of patent applications claiming the use of low-dose IL-2 in Alzheimer’s disease. G.C. and D.K. are shareholders of ILTOO pharma.

Supplementary material

Supplementary material is available at Brain online.

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