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

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Abstract

Interleukin-2 (IL-2) deficient mice have cytoarchitectural hippocampal modifications and impaired learning and memory ability reminiscent of Alzheimer's disease (AD). Interleukin-2 stimulates regulatory T cells (Tregs) which role is to control inflammation. As neuroinflammation contributes to neurodegeneration, we investigated Interleukin-2 in Alzheimer's disease. Therefore, we investigated Interleukin-2 levels in hippocampal biopsies of Alzheimer's disease patients relatively to age-matched control individuals. We then treated APP/PS1 Δ E9 mice having established Alzheimer's disease with Interleukin-2 for five months using single administration of an AAV-IL-2 vector.

We first found decreased Interleukin-2 levels in hippocampal biopsies of Alzheimer's disease patients. In mice, Interleukin-2 induced systemic and brain Treg expansion and activation. In the hippocampus, Interleukin-2 induced astrocytic activation and recruitment of astrocytes around amyloid plaques, decreased A β (42/40) ratio and amyloid plaques load, improved synaptic plasticity and significantly rescued spine density. Noteworthy, this tissue remodeling was associated with recovery of memory deficits, as assessed in the Morris water maze task. Altogether, our data strongly suggest that Interleukin-2 can alleviate Alzheimer's disease hallmarks in APP/PS1 Δ E9 mice with established pathology. Therefore, this should prompt the investigation of low-dose Interleukin-2 in Alzheimer's disease and other neuroinflammatory/neurodegenerative disorders.

INTRODUCTION

There are many interactions between the central nervous and immune systems. 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 (AD) (Heneka et al., 2015; Heppner et al., 2015; Skaper, 2007). In Alzheimer's disease, amyloid- β peptide ($A\beta$) processed from the β -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 $A\beta$ deposits (Pihlaja et al., 2008) and degrade $A\beta$ contributing to $A\beta$ clearance (Nicoll and Weller, 2003). Activated microglia also accumulates around senile plaques in Alzheimer's disease patients (McGeer et al., 1987) and may restrict amyloid plaque formation by phagocytosing $A\beta$ (Simard et al., 2006). Early stages of the neurodegenerative process are associated with glial dysfunction that may result in reduced $A\beta$ 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; Kiyota et al., 2012).

Strikingly, the role of Interleukin-2 (IL-2) has not been much investigated in

neurodegenerative diseases although numerous works highlighted the role of Interleukin-2 in the central nervous system (CNS) and immune systems. It has been described that Interleukin-2 can contact the CNS through the blood-brain-barrier (BBB) (Waguespack et al., 1994). Despite this, it has also been reported that Interleukin-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 (Dansokho et al., 2016; Hanisch et al., 1997; Lacosta et al., 1999). Furthermore, Interleukin-2 can afford trophic support to neurons and glia (Awatsuji 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, Interleukin-2 knockout (KO) mice display cytoarchitectural modifications in the dentate gyrus of the hippocampus and have impaired learning and memory ability (Beck et al., 2005; Petitto et al., 1999). Recently, it has been suggested that impact on learning and memory in IL-2 KO mice mainly rely on indirect immune-related effects (Petitto et al., 2015). Besides these direct roles on brain cells and function, Interleukin-2 has pleiotropic immune function (Klatzmann and Abbas, 2015). However, low dose Interleukin-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 monocytes-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 Interleukin-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 Interleukin-2 anti-inflammatory effects (Saadoun et al., 2011). Interleukin-2 has thus dual properties, improving memory formation and controlling inflammation, which warrants its investigation in Alzheimer's disease. Here, we report that sustained Interleukin-2 expression using single administration of an AAV induces brain tissue remodeling and recovery of memory deficits in APP/PS1 Δ E9 mice.

MATERIALS AND METHODS

Human AD 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 (PMI) of 30 to 59 h) and five hippocampal samples from five age-matched control subjects (male and female, aged between 69 and 92 years, PMI of 6 to 63 h) were used in this study.

Animals

Eight-month old APP^{swe}/PS1 Δ E9 male mice (n=31) (hereafter referred as APP/PS1 Δ E9) and the wild-type littermates (n=32) (Jackson Laboratories, Bar Harbor, Maine, USA) were used in this study. APP/PS1 Δ E9 mice overexpress the mutated human APP (*Swedish* mutation, K595N/M596L) gene as well as the Human PS1 gene deleted from its exon 9 (Jankowsky et al., 2004). APP/PS1 Δ E9 mice and wild-type littermates 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).

rAAV generation and *in vivo* administration of AAVs

Recombinant AAV8 vectors were generated by triple transfection of human embryonic kidney 293T cells, as described previously (Churlaud et al., 2014). Transgenes used were luciferase (LUC) and murine Interleukin-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 μ L of 0.1M phosphate-buffered saline (PBS) (APP/PS1 Δ E9 AAV8-CAG-IL2, n=16; APP/PS1 Δ E9 AAV8-CAG-LUC, n=15; wild-type littermates AAV8-CAG-IL2, n=16; wild-type littermates AAV8-CAG-LUC, n=16).

Detection of Interleukin-2 in the serum

Sera were collected, frozen and kept at -80°C until use. Levels of Interleukin-2 were measured using a mouse Interleukin-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 μ L 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 analyzed 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 littermates were sacrificed 5 months post-injection (13 months old). The animals, given an overdose of sodium pentobarbital, were perfused transcardially with ice-cold PBS 0,1M 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 0.1M 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, Wetzlar, Germany), 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 150mM and Triton 1%) containing phosphatase (Pierce) and protease (Roche) inhibitors. After centrifugation (20min, 13 000 rpm, 4°C), the supernatant was collected and the protein concentration was quantified (BCA Protein Assay, Thermo Fisher Scientific, Waltham, USA). Lysate aliquots

(3mg of protein/ml) were stored at -80°C until use. The same procedure was conducted for human samples (GIE NeuroCEB Brain Bank).

Western Blot

Total protein concentrations were determined using the BCA kit (Pierce). Equal amounts of total protein extract (30 µg) were electrophoretically separated using SDS–PAGE in 4–12% Bis–Tris gels (NuPAGE® Novex Bis-tris midi gel 15 or 26 wells, Life Technologies, Carlsbad, USA) 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 labeled 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, Little Chalfont, UK) according to the instructions of the supplier. Peroxydase 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; Biorad, Hercules, CA, USA). 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.

Primary antibodies

Antibodies used in western-blot (WB) and immunohistochemical (IHC) analyses:

Primary antibodies	Source	WB	IHC
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-STAT3 α	Cell signalling	1:1000	-
mouse anti-GAPDH	Abcam	1:4000	-

ELISA assay

A β -38, A β -40 and A β -42 were measured using the MSD Human A β 42 V-PLEX Kit and the triplex A β Peptide Panel 1 (6E10) V-PLEX Kit (MesoScale Discovery, Rockville, MD, USA). β -CTF was measured using the Human APP β -CTF Assay Kit (IBL, Hamburg, Germany). Interleukin-2 was measured using the MSD proinflammatory panel 1 (MesoScale Discovery, Rockville, MD, USA). 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 (RT). After three washes, slices were blocked in PBS/0.1% Triton X-100 containing 10% Normal Goat Serum (NGS, Gibco) for 1h at RT. 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., CA, USA) diluted in PBS/0.1% Triton X-100 and 10% NGS for 2 h at RT. After three washes, bound antibodies were visualized by the ABC amplification system (Vectastain ABC kit, Vector Laboratories, West Grove, USA) and 3,3'-diaminobenzidine tetrahydrochloride (peroxidase substrate kit, DAB, Vector Laboratories, CA, USA) as the substrate. The sections were mounted, dehydrated by passing twice through ethanol and toluol solutions, and coverslipped with Eukitt® (O. Kindler GmbH & CO, Freiburg, Germany).

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% normal goat serum (NGS) for 1 hour. Sections were then incubated with the respective primary antibodies, overnight at 4°C. After three successive washes, brain slices were incubated for 2 hours 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 Image J (NIH, Bethesda, USA) or Icy (Institut Pasteur, Paris, France). Laserpower, numeric gain and magnification were kept constant between animals to avoid potential technical artefacts. Images were first

converted to 8-bit gray scale and binary thresholded to highlight a positive staining. At least 3 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 (ROI) was drawn around the center of the plaque. The diameter of the circular ROI was set as 3 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, Paris, France). 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.

Behavioral assessment-Morris Water Maze

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 430 lux. Training consisted of daily sessions (three trials per session) during 5 consecutive days. Start positions varied pseudo-randomly among the four cardinal points. Mean inter-trial interval was 15 min. Each trial ended when the animal reached the platform. A 60-second cut-off was used, after which mice were gently guided to the platform. Once on the platform, animals were given a 30-second rest before being

returned to their cage. Four hours (short-term memory) and 72 hours 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 behavioral 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 months-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 anesthetized 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 cerebrospinal fluid (ACSF), 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 400 µm were cut using tissue chopper. Hippocampal slices were incubated at 32 °C in an interface chamber with the constant flow of carbogenated (95% O₂ and 5% CO₂) ACSF for 2 hours prior 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 analyzed 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 \pm SEM. Analysis of the Paired-pulse facilitation (PPF) 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, Columbia, USA) 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 RT for 2 weeks. Then, brain tissues were stored in solution C at 4°C for at least 48h and up to 7 days before sectioning. Solutions A, B and C were renewed within the first 24h. Coronal sections of 200 μ m were cut with a vibrating microtome (Leica, VT1200S) while embedded in 2% Agar in 0.1M PBS. Each section was mounted with Solution C on an adhesive microscope slide pre-coated with 1% gelatin/0.1% chromalaun 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 coverslipped with Permount (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 Axioplan 2 imaging microscope (Zeiss) using a 63x oil objective (NA 1.3) and a z-stack thickness of 0.5 μ m under reflected light. The number of spines was determined per micrometer of dendritic length (in total 100 μ m) at apical compartments using ImageJ (1.48v, National Instruments of Health, USA). Three animals per genotype and 8-10 neurons per animal were

analyzed blinded to genotype and injected AAV. Data were analyzed using Graphpad Prism (Version 5.01) software. Spine density is expressed as mean \pm 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 \pm SEM. In most cases, data were analyzed 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

Interleukin-2 expression is decreased in the hippocampus of AD patients

We first analyzed Interleukin-2 protein levels in frozen hippocampal biopsies from five severely affected Alzheimer's disease patients (Braak 6/Thal5) and five age-matched healthy controls. We observed a 2-fold decrease in Interleukin-2 levels (p <0.05) (Fig. 1A and B) paralleled by a \approx 80% reduction in APP levels (p=0.001) and synaptic protein PSD-95 levels (p=0.004) (Fig. 1A,C & D) in the hippocampus of AD patients relatively to controls. The A β 42/A β 40 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 Interleukin-2 and APP levels

(correlation coefficient 0.72, $p = 0.0017$) and between Interleukin-2 and PSD-95 levels (correlation coefficient 0.68, $p = 0.003$) (Fig. 1F and G).

Peripheral Interleukin-2 delivery induces increased Interleukin-2 and Tregs levels in the brain of APP/PS1 Δ E9 mice

As Alzheimer's disease is a slow developing disease, we anticipated that long term Tregs stimulation could be necessary for obtaining therapeutic benefit. As Interleukin-2 has a short half-life in mice, maintaining an effect on Tregs would require frequent sub-cutaneous (sc) injections that could have an effect on mice behavior. We thus delivered Interleukin-2 by intraperitoneal (ip) injection of a recombinant adeno-associated virus (AAV) coding for murine Interleukin-2, which allows sustained and stable release of Interleukin-2 for at least 20 weeks (Churlaud et al., 2014; Wang et al., 2005). Eight-month old APP/PS1 Δ E9 mice and littermates was injected with rAAV8-IL2 or luciferase-expressing control vectors (rAAV8-LUC). Four months after rAAV8 injections, serum Interleukin-2 was undetectable in mice receiving rAAV8-LUC, and was of 25.2 ± 5.3 or 29.5 ± 4.8 pg/mL (mean \pm SEM) in Interleukin-2 treated littermates and APP/PS1 Δ E9, respectively (Fig.2A). These serum Interleukin-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 Interleukin-2 production expanded blood Tregs, significantly more in the APP/PS1 Δ E9 mice than wild type littermates (Fig 2C). Tregs from Interleukin-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 Interleukin-2 levels only in Interleukin-2 treated APP/PS1 Δ E9 mice (Figure 2B). These levels were approximately doubled compare to Interleukin-2 treated wild-type littermates and rAAV8-LUC controls, as detected both by western blot (Supplementary Figure 1) and ELISA (Figure 2B). There was a

concomitant 3-fold increase of brain Tregs in Interleukin-2 treated normal and APP/PS1 Δ E9 mice (Fig 2D). These Tregs also showed a higher activation status as attested by an increased CD25 expression (Fig 2F). In order to evaluate whether the increased Interleukin-2 levels observed in APP/PS1 Δ E9 transgenic mice could be due to the transduction of brain cells by AAV vectors, we analyzed the biodistribution of AAV after injection of rAAV8-LUC in normal and in APP/PS1 Δ E9 transgenic mice. We assessed luciferase bioluminescence in peripheral organs including the brain. As negative control, we used non-injected age-matched APP/PS1 Δ E9 mice. Two weeks post-injection, luciferase expression could be detected in most of peripheral organs (liver, heart, kidney and spleen). 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 Figure 2A and B). To further substantiate these data, we also used a sensitive qPCR 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 expression could be detected in the brain of APP/PS1 Δ E9 mice (Supplementary Figure 2C). Thus, the increased brain Interleukin-2 levels in APP/PS1 Δ E9 mice results from passage from the periphery and not from local production by rAAV8 transduced brain cells.

Interleukin-2 treatment rescues memory impairment in APP/PS1 Δ E9 mice

To evaluate the therapeutic effects of increasing brain Interleukin-2 on spatial learning and memory, mice were tested in the Morris water-maze place navigation task (Fig. 3). Treated APP/PS1 Δ E9 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 hours probe trial that evaluates spatial reference 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 % of time and % of 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, Interleukin-2 -treated APP/PS1ΔE9 mice showed a clear preference for the target quadrant (rAAV-LUC-treated vs Interleukin-2 -treated APP/PS1ΔE9 mice: % of time in TQ; $p=0.0034$ and % of distance in TQ; $p<0.0001$), and were statistically indistinguishable from control littermates. These results were confirmed at 72 hours (long-term memory; rAAV-LUC-treated vs IL-2-treated APP/PS1ΔE9 mice: % of time in TQ; $p=0.0199$ and % of distance in TQ: $p=0.0092$) (Fig. 3E, G and I) suggesting a beneficial effect of Interleukin-2 during memory consolidation phase. These data clearly demonstrate that Interleukin-2 rescued impairments in memory retention observed in APP/PS1ΔE9 mice.

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

Aβ-induced damage of synaptic transmission is one probable mechanism inducing memory impairments in APP/PS1 mice (Snyder et al., 2005). We evaluated whether Interleukin-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)). Interleukin-2 treated APP/PS1 Δ E9 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/PS1 Δ E9 mice receiving rAAV8-LUC. However, LTP magnitude was lower relatively to Interleukin-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 analyzed paired-pulse facilitation (PPF) 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/PS1 Δ E9 mice administered with rAAV-IL-2 compared with their littermates. Paired-pulse facilitation in APP/PS1 Δ E9 mice administered with rAAV-LUC was also significantly ($p < 0.05$, ANOVA) higher compared with littermates. Paired-pulse facilitation was not different between APP/PS1 Δ E9 mice administered with rAAV-IL-2 or rAAV-LUC (Fig. 4D). Spine density was analyzed 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 μ m from soma) of the hippocampal CA1 pyramidal layer was analyzed by the Golgi cox method (Fig. 4E). We found an overall decrease in spine density in APP/PS1 Δ E9 mice treated with control rAAV-LUC at the CA1 apical dendritic compartment relatively to littermates injected with either rAAV-LUC or rAAV-IL-2 ($p < 0.001$, 1-way ANOVA followed by Bonferroni's post-hoc test). Importantly, rAAV-IL-2-treated APP/PS1 Δ E9 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 Interleukin-2 strikingly ameliorates both structural and functional synaptic impairments in Alzheimer's disease mice.

Interleukin-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). Interleukin-2 treatment was started at 8 months of age, a time at which we already evidence increased levels of Aβ peptides (Aβ38, Aβ40 and Aβ42) and β-CTF in hippocampus from APP/PS1ΔE9 mice (Supplementary Figure 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 Aβ peptides and β-CTF, known to induce hippocampal neurophysiological impairments, was quantified in the hippocampus of injected mice by ELISA. No differences in β-CTF levels were detectable (Fig. 5D). There was a trend towards increased amounts of Aβ38 and Aβ40 (Fig. 5E-F) and a trend to decrease in soluble Aβ42 levels (Fig. 5G). This translated into a significant reduction in the Aβ42/Aβ40 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,J). Altogether, these data indicate that Interleukin-2 reduces amyloid load and plaque deposition in the hippocampus of APP/PS1ΔE9 mice with established pathology.

Interleukin-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 Figure 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 A β clearance (Leissring et al., 2003) (Supplementary Figure 5). No major difference in Iba1 immunoreactivity around hippocampal amyloid plaques was observed (Fig. 6D and E).

Astrocytic markers analysis demonstrated a 3 to 4-fold increase of GFAP expression ($p < 0.0001$) in APP/PS1 Δ E9 mice injected with rAAV8-IL-2 compared to APP/PS1 Δ E9 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 rAAV8-IL-2 treated APP/PS1 Δ E9 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/PS1 Δ E9 mice. In contrast, astrocytes from APP/PS1 Δ E9 mice treated with the control vector exhibited lower hypertrophic processes (Fig. 6F). Littermates treated with Interleukin-2 or Luciferase vectors did not exhibit hypertrophic process.

Interleukin-2 administration activates the JAK/STAT3 pathway in the hippocampus of APP/PS1 Δ E9 mice

We next assessed whether this Interleukin-2 mediated astrocytic activation was correlated with stimulation of the JAK/STAT3 pathway. STAT3 is an important signaling molecule for many cytokines and growth factor receptors that prompts astrocyte reactivity (Chiba et al., 2009; Heim, 1999). Western-blot analysis demonstrated a 2-fold increase in the levels of Stat3- α in APP/PS1 Δ E9 mice receiving rAAV8-IL-2 compared to APP/PS1 Δ E9 mice treated with the control vector (Fig. 6H and I) ($p < 0.0001$). The phosphorylated form of STAT3 [Phospho-STAT3 (Tyr705)] was increased in APP/PS1 Δ E9 mice treated with the control rAAV8-LUC, relatively 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 Interleukin-2 treated APP/PS1 Δ E9 mice as compared to APP/PS1 Δ E9 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/PS1 Δ E9 mice treated with Interleukin-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 A 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 signaling of the pro-inflammatory cytokines IL-12/IL-23 in APPPS1 mice decreased glial activation, amyloid load and cognitive decline (Vom Berg et al., 2012). The hippocampal AAV-mediated overexpression of the anti-inflammatory cytokines Interleukin-10 or Interleukin-4 in AD mice enhanced neurogenesis and improved spatial learning and A β deposition in APP/PS1 mice (Kiyota et al., 2010; Kiyota et al., 2012; Latta et al., 2015). Despite this, two recent studies support a detrimental impact of Interleukin-10 in Alzheimer's disease pathology (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). Interestingly, hippocampal expression of IL-1 β 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 Interleukin-6 (Chakrabarty et al., 2010) and TNF- α (Chakrabarty et al., 2011) induced massive gliosis that suppressed A 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 Interleukin-2 in Alzheimer's disease *in vivo*: (i) Interleukin-2 KO mice exhibit impaired learning and memory formation and altered hippocampal development (Petitto et al., 1999); (ii) serum Interleukin-2 levels are low in Alzheimer's disease patients, compared with both elderly and middle-aged subjects (Beloosesky et al., 2002); (iii) Interleukin-2, at low dose, has an anti-inflammatory effect (Saadoun et al., 2011). In this report, we show that Interleukin-2 prompts Treg expansion and activation in the brain of APP/PS1 Δ E9 mice and improves Alzheimer's disease pathology. Increased Interleukin-2 concentrations were observed in the hippocampus of Interleukin-2 treated APP/PS1 Δ E9 mice but not in Interleukin-2 treated littermates. Since AAV vectors do not transduce brain cells and Interleukin-2 is produced in the periphery, we may speculate that CNS penetration of peripherally produced Interleukin-2 could be favored in APP/PS1 Δ E9 mice due to BBB leakage. Indeed, the BBB 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, BBB 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 Interleukin-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 APPPS1 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 three weeks after the last Treg depletion modality (53.4% vs 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 A 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 APPPS1 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 NK 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 Interleukin-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 Interleukin-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 Interleukin-2 (Eizenberg et al., 1995). Astrocytes make part of the BBB and may well be the primary brain parenchymal cell type encountered by peripheral Tregs. Indeed, previous reports have described astrocyte/T cells interactions (Barcia et al., 2013). More recently, a close interaction between Tregs and astrocytes has been reported, in which the activation of an Interleukin-2/STAT5 signaling 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 Interleukin-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 A β and limit its extracellular deposition. In line with this finding, mouse astrocytes were reported to degrade amyloid-beta *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 A 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, Interleukin-2 treatment and consequent astrocytic activation were accompanied by a reduction of amyloid plaques and a decrease in the A β 42/A β 40 ratio (Murray et al., 2012). The strong A β 40 increase in Interleukin-2 treated APP/PS1 Δ E9 mice is in line with *in vitro* and *in vivo* data showing that A β 40 protects neurons from A β 42 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 (APPPS1 mice), Interleukin-

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 Interleukin-2-mediated tissue remodeling 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 remodeling. These findings are in accordance with previous reports showing that Interleukin-2 promotes survival and neurite extension of cultured neurons as well as enhances dendritic development and spinogenesis (Awatsuji et al., 1993; Sarder et al., 1993; Shen et al., 2010).

Interleukin-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 Interleukin-2 is safe and selectively activates and expands Tregs without activating effector T cells in humans has changed the paradigm for Interleukin-2 therapeutic use. Interleukin-2 is now intensively developed as a stimulant of Tregs at daily dose around 1 to 3 MIU. At these low doses, Interleukin-2 is well tolerated in humans with autoimmune diseases (Castela et al., 2014; Hartemann et al., 2013; He et al., 2016; Saadoun et al., 2011). Noteworthy, these doses lead to increased serum concentration of Interleukin-2 that are in the range of the long-term elevated concentrations observed during pregnancy (Curry et al., 2008). Finally, a pre-clinical study of the long-term effects of Interleukin-2 in mice showed that a yearlong treatment is well tolerated (Churlaud et al., 2014). Thus, long-term treatment with low-dose Interleukin-2 of

Alzheimer's disease patients can be envisioned. In summary, our results demonstrate the therapeutic effects of Interleukin-2 in AD mice with established pathology. Although it remains to elucidate the direct and Treg-mediated contribution of Interleukin-2 to Alzheimer's disease improvements, these results warrant investigating the low dose Interleukin-2 for neuroinflammatory diseases.

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Author's contribution

Overall Study concept: DK

Study concepts, design and supervision: NC and DK

Acquisition of data: SA, GC, MA, KMP, RF, BS, JB, MK

Analysis and interpretation of data: SA, GC, MK, NC and DK

Drafting of the manuscript: SA, GC, MK, NC and DK

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