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ORIGINAL ARTICLE

IL4 gene delivery to the CNS recruits regulatory T cells and induces clinical recovery in mouse models of multiple sclerosis

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Central nervous system (CNS) delivery of anti-inflammatory cytokines, such as interleukin 4 (IL4), holds promise as treatment for multiple sclerosis (MS). We have previously shown that short-term herpes simplex virus type 1-mediated IL4 gene therapy is able to inhibit experimental autoimmune encephalomyelitis (EAE), an animal model of MS, in mice and non-human primates. Here, we show that a single administration of an IL4-expressing helper-dependent adenoviral vector (HD-Ad) into the cerebrospinal fluid (CSF) circulation of immunocompetent mice allows persistent transduction of neuroepithelial cells and long-term (up to 5 months) CNS transgene expression without toxicity. Mice affected by chronic and relapsing EAE display clinical and neurophysiological recovery from the disease once injected with the IL4-expressing HD-Ad vector. The therapeutic effect is due to the ability of IL4 to increase, in inflamed CNS areas, chemokines (CCL1, CCL17 and CCL22) capable of recruiting regulatory T cells (CD4$^+$CD69$^-$CD25$^+$Foxp3$^+$) with suppressant functions. CSF delivery of HD-Ad vectors expressing anti-inflammatory molecules might represent a valuable therapeutic option for CNS inflammatory disorders.
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Introduction

Multifocal and chronic inflammatory disorders of the central nervous system (CNS), such as multiple sclerosis (MS), might benefit from gene therapy based on the use of viral vectors expressing anti-inflammatory molecules. However, gene therapy using viral vectors as carriers of therapeutic molecules is of limited therapeutic efficacy due to several constrains.[1] Viral vectors do not reach the CNS upon systemic injection owing to the presence of the blood–brain barrier.[2] The direct injection of viral vectors into the brain parenchyma allows only a restricted diffusion—few centimeters away from the injection site—of viral particles and large molecules (except for proteins actively transported by neurons).[3,4] Systemic as well as intraparenchymal delivery of viral vectors usually induces humoral and cell-mediated immune responses against the vectors and/or transgene products, leading to inflammation, short-term persistence of gene expression and elimination of transduced cells.[5–7]

We, and others, have previously shown that injection of viral vectors into the cisterna magna, or trough lumbar puncture, might represent an efficient CNS delivery system in both small (mice) and large (non-human primates) animals.[8–11] Administration of vector into the cerebrospinal fluid (CSF) circulation, while bypassing the blood–brain barrier, allows viral vector transduction of neuroepithelial cells and delivery of transgene products to the whole CNS through the ventricular circulation. However, viral vector delivery via the CSF circulation still does not represent a realistic gene therapy option for chronic CNS disorders because of the short-term expression and residual immunogenicity of the vectors so far tested (for example, first-generation herpes simplex virus type 1-derived vectors or first generation Ad vectors).[10,12]

We have now established a long-lasting and nontoxic cytokine delivery system, both in mice and monkeys, based on the intrathecal administration of an interleukin 4 (IL4)-producing helper-dependent adenoviral vector (HD-Ad). We have shown evidence of the clinical, neuropathological and neurophysiological efficacy of this therapy in mice affected with chronic or relapsing–remitting experimental autoimmune encephalomyelitis (EAE), modeling the most common clinical subtypes of MS. Finally, we demonstrate that the protective mechanism induced by IL4 gene therapy in EAE is mediated, at least partially, by an increased recruitment of CD25$^+$Foxp3$^+$ T regulatory (Treg) cells to the CNS.

Results

Whole-CNS distribution and long-term transgene expression following intracisternal administration of an HD-Ad vector in C57Bl/6 mice

CNS distribution and persistence of HD-Ad vectors injected into the CSF were analyzed in 25 C57Bl/6 mice. A single injection of 10^7 t.u. ($\sim 5 \times 10^8$ t.u. kg^{-1}) of an STK-120-derived HD-Ad vector—expressing green fluorescent protein (GFP) and murine IL4 under independent transcriptional control (hereafter referred as Ad-G/IL4 vector)—was administered into the cisterna magna of each mouse. The Ad-G/IL4 vector spread through the entire mouse brain ventricular system (Figures 1a and b), and efficiently transduced leptomeningeal cells on the external, especially basal, surface of the brain (Figure 1c), and cells from the ependyma and choroid plexuses along the inner ventricular spaces (Figure 1d). No GFP signal was detected within the CNS parenchyma in all mice evaluated (Figures 1c and d). Biodistribution of Ad-G/IL4 was further analyzed by PCR in the DNA obtained from different tissues and organs from five injected mice. PCR analysis showed that the number of vector genomes in the CNS declined steadily in the first month, and persisted at a stable level of approximately 5×10^3 copies µg^{-1} of DNA up to 5 months after injection (Figure 1h). Outside the CNS, approximately 10^3 copies µg^{-1} of DNA were detected, 1 month after injection, only in the kidney and intestine (Supplementary Figure 1). Transgenes expression was then analyzed. *In situ* hybridization showed that cells facing liquoral spaces were strongly positive for GFP mRNA (Figure 1e). To exclude possible transcription of endogenous IL4 mRNA, we set up an RT-PCR assay specific for the exogenous adenoviral IL4 expression cassette and confirmed that only IL4 gene therapy-treated mice were positive (Figure 1f). Finally, synthesis of up to 350 pg ml^{-1} of murine IL4 was detected in the CSF, by a fluorescent ELISA, throughout the entire observational period. Despite that fact that very low amounts of CSF available for analysis (1–3 µl per mouse) might lead to underestimation, detectable levels of IL4 persisted up to 5 months after injection (Figure 1g), further supporting long-term CNS persistence of the vector. The level of IL4 in the serum remained below 15 pg ml^{-1} in all mice throughout the analyzed period (data not shown).

Intracisternal delivery of Ad-G/IL4 induces clinical and functional recovery from chronic progressive EAE

We then studied the therapeutic potential of Ad-G/IL4-mediated gene therapy in a chronic-progressive model of EAE (C-EAE). C-EAE was induced in C57Bl/6 mice by immunization with MOG$_{35-55}$, as described previously.[13] The Ad-G/IL4 vector was injected intracisternally at a dose of 10^7 t.u. per mouse on the day of disease onset, occurring between days 10 and 19 post-immunization. Control mice were injected with an HD-Ad vector expressing only GFP (Ad-G vector). Administration of the Ad-G/IL4 vector significantly decreased the disease burden, as measured by the average (Figure 2a) and cumulative clinical disease scores (Table 1) between days 30 and 70. Treatment with Ad-G/IL4 significantly improved also the neurophysiological parameters. Motor-evoked potentials (MEP)—used as a functional measure of corticospinal tract integrity[14]—were measured in C-EAE mice at 75 days post-immunization. MEP was recordable in 10/11 (91%) of the Ad-G/IL4-treated mice but in only 5/10 (50%) of the control, Ad-G-treated mice ($P < 0.05$, χ^2). Central conduction time (CCT) averaged 3.0 ± 0.4 ms in Ad-G/IL4-treated mice (reference value in healthy mice: 2.8 ± 0.3) vs 3.3 ± 0.5 ms in control, Ad-G-treated mice ($P = 0.02$, Mann–Whitney) (Figure 2b).

IL4 gene therapy ameliorates EAE neuropathology and promotes the CNS recruitment of CD4⁺CD69⁻CD25⁺Foxp3⁺-regulatory T cells

In C57Bl/6 mice affected by C-EAE, IL4 gene therapy significantly reduced demyelination and axonal loss (Table 1; Figure 3). Neuropathological features were accompanied by an increase of T-cells (CD3⁺) infiltrating the CNS (Table 1). This finding was not due to the alteration in peripheral antigen-driven T-cell expansion, as T-cell proliferation (Figure 4a), interferon (IFN)γ (Figure 4b) or IL4 (Figure 4c) release, and the percentage of IFNγ- or IL4-releasing CD4⁺ cells (Figures 4e–f) analyzed in T cells purified from spleen or draining lymph nodes—activated in culture in the presence of the nominal antigen MOG$_{35-55}$—were found to be comparable between treated and control mice. Similar to cellular immunity, humoral immunity—tested by measuring anti-MOG serum antibody (Ab) levels—was also similar in Ad-G/IL4-treated and control mice (Figure 4d). The phenotype of CNS-infiltrating cells and the kinetics of the different T-cell subpopulations in the CNS, blood and lymphoid organs were then analyzed by flow cytometry. During C-EAE, the CD4⁺CD69⁻CD25⁺-regulatory T-cell fraction—known to have suppressant activity during autoimmune reactions[15] decreased in the spleen and peripheral circulation, whereas increased in draining lymph nodes and in the CNS at the peak of disease, 14–21 days after immunization (Figure 5a). In all mice treated with the Ad-G/IL4 vector affected by either C-EAE, the percentage and absolute number of CD4⁺ CD25⁺ Foxp3⁺ T cells significantly increased in the CNS 21 days after immunization compared to mice treated with the control Ad-G vector (Figures 5b–c). In addition, mRNA levels of foxp-3, a transcription factor specifically expressed in CD25⁺-regulatory T cells,[15] were significantly increased in the brain and spinal cords of C-EAE mice treated with the Ad-G/IL4 vector as compared to control mice (Figure 5d). When we compared CD25⁺-regulatory T cells from IL4-treated mice and controls, purified from draining lymph nodes at the peak of disease, we found similar suppressive ability in an *in vitro* assay (Figure 5e). Intracellular cytokine staining analysis showed no difference in the percentage of double-positive IFNγ⁺IL2⁺ cells in both CD4⁺ and CD8⁺ T-cell fractions between Ad-G/IL4-treated and control animals (Supplementary Figure 2a). On the contrary, the percentage of Annexin V⁺, propidium iodide⁻ cells increased in both the CD4⁺ and CD8⁺ T-cell fraction in Ad-G/IL4-treated mice (Supplementary Figure 2b). Taken together, these results indicate that vector-mediated IL4 release within the CSF circulation promotes the recruitment within inflamed CNS areas of regulatory T cells, which, in turn, induce apoptosis rather than anergy of CNS-infiltrating encephalitogenic T cells.
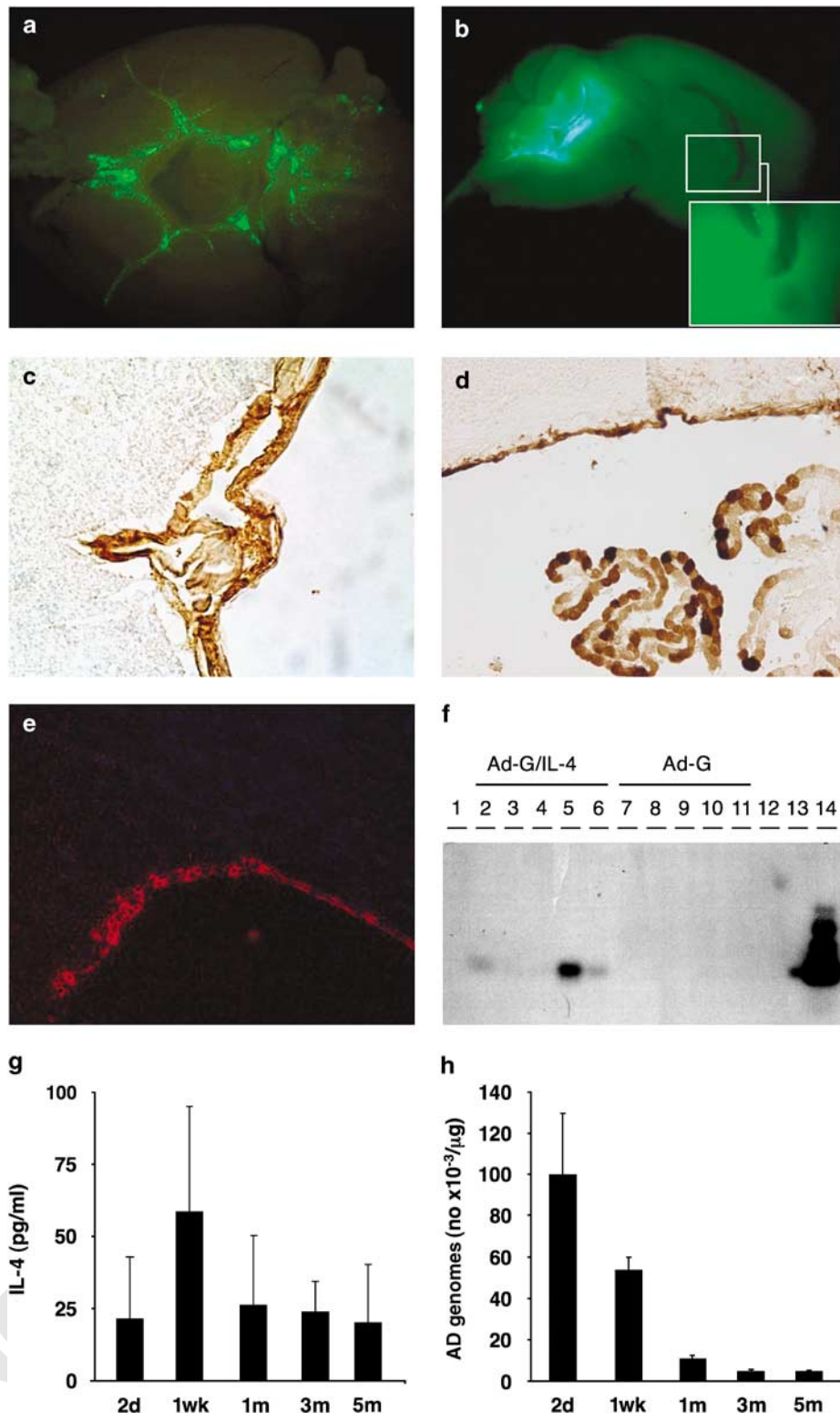
Figure 1 Ad-G/IL4 vector injected into the cisterna magna efficiently infects ependymal and leptomeningeal cells and expresses the transgene for up to 5 months. (**a** and **b**) In a representative C57Bl/6 mouse injected with the Ad-G/IL4 vector, GFP reactivity (green) is mainly located on the basal brain surface (**a**), or circumvents liquoral spaces, including the most cervical tract of the ependymal channel and lateral ventricles (inset) (**b**). (**c** and **d**) GFP+ cells (brown) are leptomeningeal, (**c**), ependymal (**d**) and choroidal cells (**d**). (**e** and **f**) IL4 mRNA is detected in ependymal cells by radioactive *in situ* hybridization (**e**), and by RT-PCR using primers specific for the IL4 encoded by the adenoviral expression cassette (**f**); lanes 2–6 Ad-G/IL4-treated mice; lanes 7–11, Ad-G-treated mice; lane 12, negative control; lane 14, positive control. (**g** and **h**) IL4 levels in the CSF (**e**) and adenoviral (AD) genome in the CNS (**f**) are found in C57Bl/6 mice intracisternally injected with 10^7 t.u. of Ad-G/IL4 for up to 5 months after treatment, as measured by real-time PCR and a fluorescent ELISA, respectively (d = days, wk = week, m = months). Magnification ×4, panel **c**; ×20 panel **d**. CSF, cerebrospinal fluid; GFP, green fluorescent protein.
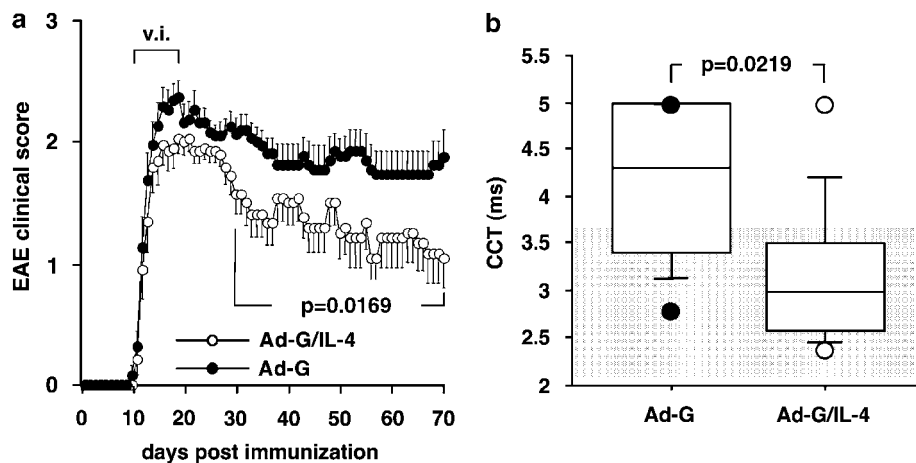
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Figure 2 Gene therapy by intracisternal injection of Ad-G/IL4 at the time of disease onset inhibits clinical and neurophysiological signs of C-EAE. (**a**) C-EAE mice exhibit a significantly milder disease course when intracisternally injected (v.i. = vector injection) with Ad-G/IL4 (open dots) as compared to control mice treated with the empty Ad-G vector (closed dots). (**b**) A significantly ($P \leqslant 0.05$, Mann–Whitney) restored central conduction time in IL4-treated C57BL/6 mice affected by C-EAE was observed. Box plots indicate 25th and 75th percentile, median value and standard deviation. The dashed area indicates the range of reference values for naive, healthy mice. C-EAE, chronic-progressive model of EAE; EAE, experimental autoimmune encephalomyelitis.

IL4 gene therapy increases the recruitment of Treg cells by increasing the synthesis of chemo-attractant cytokines in inflamed CNS areas

To understand the mechanism(s) underlying the increased recruitment of Treg cells in the CNS of Ad-G/IL4-treated mice, we measured mRNA levels of chemokines known to attract $CD4^+CD69^-CD25^+$-regulatory T cells in the CNS of treated and control mice, 24 days after immunization.[16] As shown in Figures 6a–c, CCL1, CCL17 and CCL22 mRNA levels were significantly increased ($P < 0.05$) in the brain and spinal cord of C57BL/6 mice affected by C-EAE and treated with Ad-G/IL4 compared to control mice. It has been previously shown that CCL1 and CCL22 are expressed in EAE lesions.[17] We then investigated which cell subpopulation is able to release these chemokines during CNS inflammation, and found that BV.2, a murine microglial cell line, secretes CCL22 when stimulated with pro-inflammatory cytokines (Figure 6d). The amount of CCL22 released by stimulated BV.2 was significantly increased, in a dose-dependent way, by the addition of IL4 (Figure 6d). By confocal microscopy, we were able to colocalize $CCL17^+$ cells and inflammatory cell (Figure 6e) and endothelial (Figure 6f) markers. We also measured the levels of three mast-cell-specific markers in the CNS of Ad-G/IL4- and Ad-G-treated mice, as there are data indicating that CCL1 is mostly released by mast cells[18] and that mast cells are involved in $CD4^+CD69^-CD25^+$-regulatory T cells function.[19] No significant difference in mRNA of mast cell markers were observed between treated and control mice (Supplementary Figure 3). Our data suggest that Ad-G/IL4 treatment increases the synthesis of chemo-attractant chemokines in the CNS, supporting, in turn, the increased recruitment of Treg cells to the neuroinflammatory lesions.

Intracisternal delivery of Ad-G/IL4 induces clinical and functional recovery from relapsing EAE

To verify whether IL4 gene therapy is able to interfere also with relapsing EAE (R-EAE), modeling the most common clinical form of human MS, using the same Treg-mediated mechanism, we immunized SJL mice with PLP139-151. Indeed, after intracisternal injection of Ad-G/IL4 on the day of disease onset, occurring between days 12 and 16 post-immunization, we again observed decreased average (Figure 7a) and cumulative clinical disease scores (Table 1) between days 40 and 102 post-immunization. Furthermore, a significant decrease in the rate and severity of relapses was observed in R-EAE mice as compared to Ad-G-treated control mice (Table 1). Accordingly, we found improved neurophysiological parameters in IL4-treated R-EAE mice: resting motor threshold (cMEP threshold) was restored after Ad-G/IL4 treatment (11.2 ± 5.6 vs 30.9 ± 11.7 mA in control mice, normal range: 3–15 mA) (Figure 7b). Finally, IL4-treated R-EAE mice exhibit increased percentage of CD4CD25+CD69− Treg cells and of foxp3 mRNA, compared to Ad-G-treated R-EAE mice, thus suggesting a similar effect of exogenous IL4 delivery in both EAE models tested (Figures 7c–d).

Discussion

To prove the therapeutic value of intra-CSF gene therapy, we administered intratechally an IL4-producing HD-Ad vector into mice affected by either chronic-progressive or relapsing-remitting EAE, two autoimmune demyelinating disorders reproducing some of the pathological features of human MS. Administration of the vector after disease onset induced significant clinical and functional recovery in both models. The neurophysiological parameters used as a measure of myelin and axonal integrity are similar to those used in human MS patients. Interestingly, IL4 reduced demyelination and axonal loss in EAE mice, while increasing rather than reducing the number of CNS-infiltrating T cells. A similar phenomenon was previously observed in relapsing-remitting EAE induced in Biozzi AB/H after treatment with an IL4-expressing herpes simplex virus type 1-derived vector.[20] We now show that the increased

Table 1 EAE clinical and pathological features in C57BL/6 and SJL mice treated from the day of disease onset with the Ad-G/IL-4 or Ad-G

Mouse strain	Vector	No. of mice	EAE maximum score (range)	EAE cumulative score (range)[a]	No. of relapses (range)	Relapse maximum score (range)	No. of infiltrates/mm²	No. of CD3 per mm²	Macrophages per mm²	% Demyelination	% Axonal loss
C57BL/6	Ad-G	19	2.7±0.1 (1.5–4)	55.6±8.5 (66–153)	NA	NA	1.5±0.5	4.2±0.9	72.1±23.4	1.5±0.2	1.7±0.1
C57BL/6	Ad-G/IL-4	19	2.4±0.1[b] (1.5–3.5)	36.8±6.8[c] (39.5–129.5)	NA	NA	1.4±0.7	20.1±12.0	84.9±1.6	0.5±0.1[d]	0.8±0.1[e]
SJL	Ad-G	9	2.5±0.2 (2–3)	83.7±16.4 (23–153.5)	2.3±0.2 (1–3)	2.2±0.1 (1–3)	5.1±0.6	116.2±19.1	58.7±7.7	1.9±0.2	1.2±0.2
SJL	Ad-G/IL-4	8	2.3±0.1 (2–3)	40.7±13.6[f] (0–106.5)	1.4±0.2[g] (1–2)	1.8±0.1[h] (1–2.5)	4.7±0.9	99.0±21.6	52.4±5.0	1.6±0.3	1.1±0.2

Abbreviations: EAE, experimental autoimmune encephalomyelitis; NA, not applicable.
[a]Cumulative scores have been calculated from day 30 to day 70 in C57BL/6 mice and from day 40 to day 102 in SJL mice.
[b]AD-G/IL-4 vs. AD-G, $P = 0.09$ Mann–Whitney nonparametric test.
[c]AD-G/IL-4 vs. AD-G, $P = 0.0237$ Mann–Whitney nonparametric test.
[d]AD-G/IL-4 vs. AD-G, $P < 0.0001$ Student's t-test.
[e]AD-G/IL-4 vs. AD-G, $P = 0.004$ Student's t-test.
[f]AD-G/IL-4 vs. AD-G, $P = 0.05$ Mann–Whitney nonparametric test.
[g]AD-G/IL-4 vs. AD-G, $P = 0.0118$ Mann–Whitney nonparametric test.
[h]AD-G/IL-4 vs. AD-G, $P = 0.0251$ Mann–Whitney nonparametric test.

T-cell infiltrate is associated with an increased number of CD4+CD69−CD25+foxp3+ Treg cells in the CNS. This finding may be explained by in situ proliferation, increased recruitment or decreased clearance of CD4+CD69−CD25+Foxp3+ Treg cells. Indeed, it has been shown that IL4 induces proliferation of CD4+CD25+ Treg cells, in the presence of APCs, without interfering with their suppressive activity.[21] We have gradient purified the infiltrating cells from the CNS of EAE mice and cultivated them in vitro in the presence of IL4, without evidence of CD4+CD25+ Treg expansion (data not shown), but we cannot exclude that this may happen in vivo. On the other hand, recruitment of Treg cells is known to occur at sites of autoimmunity, and strategies aimed at increasing this pathway are under investigation as a therapy for several autoimmune disorders.[22] How may IL4 gene therapy increase CD4+CD69−CD25+ T cell recruitment to the CNS? It has previously been reported that CD25+ Treg cells express preferentially CCR4 and CCR8 chemokine receptors, and migrate in response to chemokines CCL1, CCL17, CCL22,[16,23,24] and more recently it has been shown that CCR4 plays a pivotal role in tissue-specific homing of Treg cells.[25] We found that synthesis of CCL1, CCL17 and CCL22 is increased in the CNS by IL4 gene therapy. It is known that IL4 is able to induce CCL17 and CCL22 in mononuclear cells.[18] Furthermore, it had previously shown that microglial cells express CCL22 during EAE.[26] Here, we show that microglial cells are stimulated by IL4 to significantly increase the release of CCL22 during inflammation, thus supporting the idea that IL4 increases CD25+ Treg cells recruitment. We also show that CCL17 is expressed by endothelial cells and infiltrating T cells during EAE, although we have no evidence, other than the increase of CCL17 mRNA, of a direct or indirect effect of IL4 on these cells. Thus, engagement by CCL17 and CCL22 of the CCR4 receptor expressed on CD4+CD69−CD25+Foxp3+ T cells may explain their increased recruitment and migration to the CNS. Sustained synthesis of IL4- and CSF-mediated diffusion to the whole CNS is most likely crucial to allow widespread recruitment of Treg cells to a large number of autoimmune lesions. Are additional Treg cells recruited by the IL4 gene therapy beneficial to EAE mice? We, and others, have previously shown that CD4+CD69−CD25+ T cells are protective when passively transferred to EAE mice,[27,28] and we show here that CD4+ CD25+ T cells from IL4-treated mice retain their suppressive ability. IL4 is a pleiotropic cytokine and we have described, in the past, additional mechanisms mediating the beneficial effect of IL4 gene therapy.[10] As, however, Treg cell-based therapy is difficult to conceive in practical terms, due to difficulties in ex vivo cell manipulation and delivery, induction of CD4+CD69−CD25+ T cell migration to the CNS by administration of a cytokine such as IL4 may be a feasible and promising therapeutic option. We show that the combination of an HD-Ad vector to deliver IL4 and a delivery confined to the CSF induces a sustained and effective recruitment of Treg cells with little side effects.

In conclusion, we provide preclinical evidence of the safety and efficacy of intrathecal HD-Ad-vector-mediated gene therapy in mice modeling the two most common clinical forms of human MS. Furthermore, we describe that IL4-mediated recruitment of Treg cells is
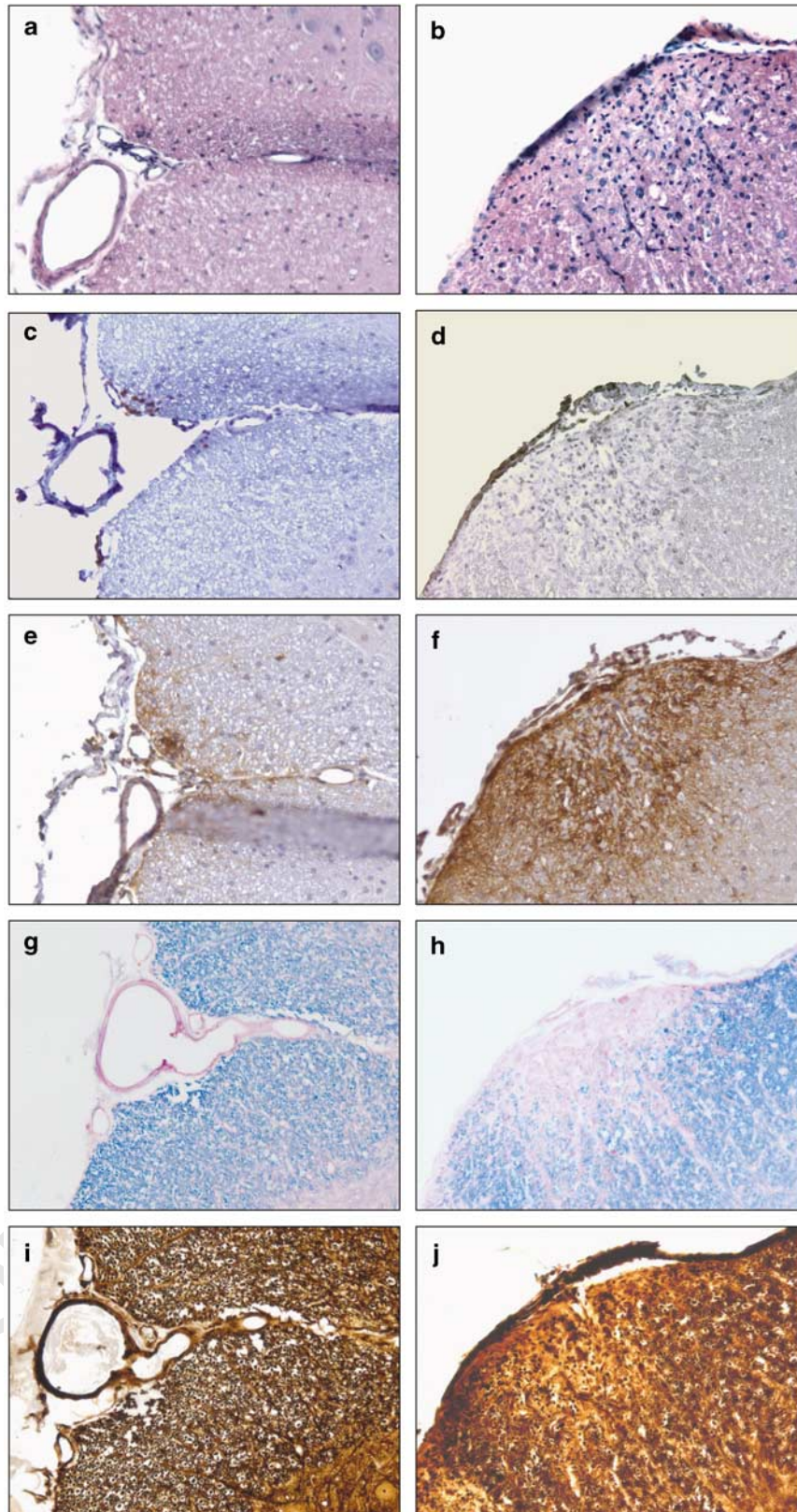
Figure 3 Neuropathological analysis. (**a–f**) Inflammation in the spinal cord of two representative C57BL/6 mice affected by EAE and treated, at EAE onset, with 10^7 t.u. of the Ad-G/IL4 (**a**, **c** and **e**) or with the control vector Ad-G (**b**, **d** and **f**). Adjacent sections were stained for inflammatory infiltrates (hematoxylin and eosin) (**a** and **b**), CD3$^+$ cells (**c** and **d**), and macrophages (**e** and **f**). (**g–j**) Demyelination (Kluver–Barrera) (**g** and **h**) and axonal loss (Bielshowsky) (**i** and **j**) are predominant in the control mouse (**h** and **j**), as compared to the IL4-treated mouse (**g** and **i**). All panels shown are ×20. EAE, experimental autoimmune encephalomyelitis.
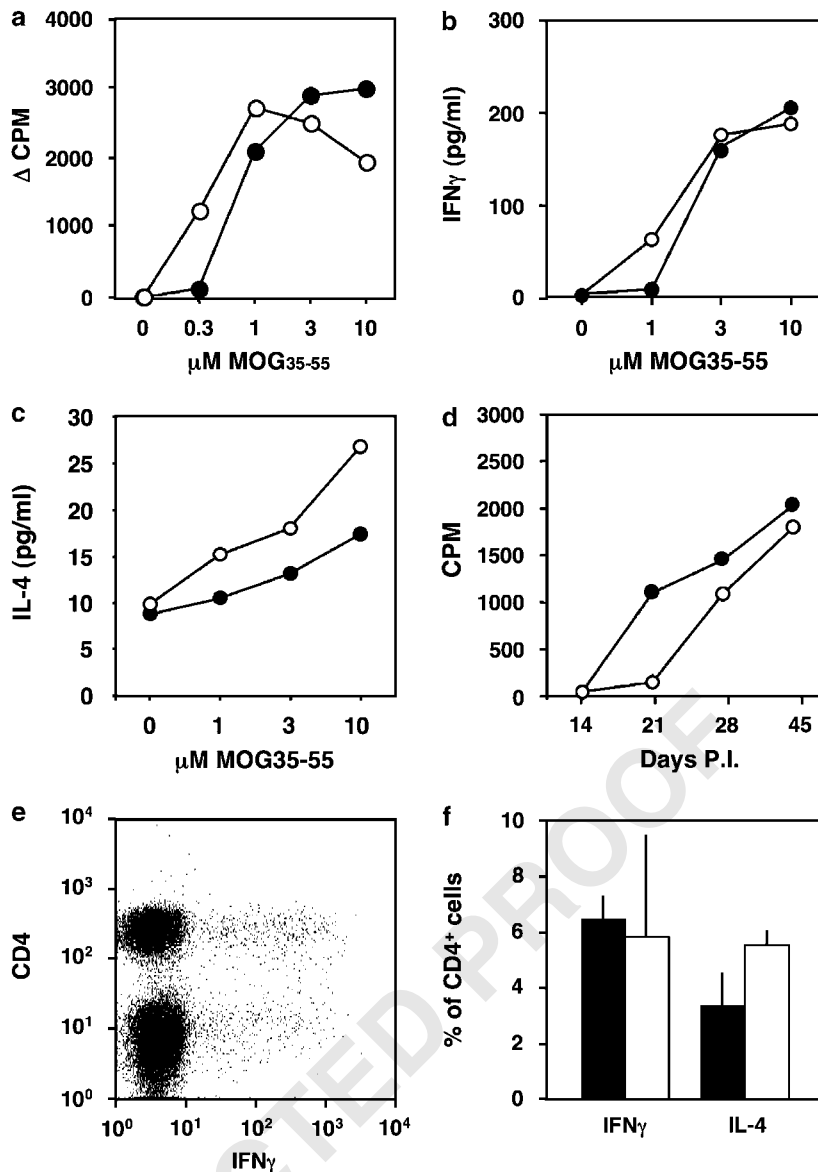
Figure 4 Gene therapy by intracisternal injection of Ad-G/IL4 at the time of disease onset does not influence peripheral immune responses towards the encephalitogenic antigen. (**a**) Spleen cells from IL4-treated mice (open dots) and controls (closed dots), *in vitro* re-stimulated with increasing MOG_{35-55} concentrations, display similar proliferative responses as shown by H^3-thymidine incorporation. (**b** and **c**) Spleen cells from IL4-treated mice (open dots) and controls (closed dots), *in vitro* re-stimulated with increasing MOG_{35-55} concentrations release overimposable amounts of IFNγ (**b**) and IL4 (**c**) as measured by ELISA. (**e** and **f**) *In vitro* re-stimulation with MOG_{35-55} induces comparable percentages of IFNγ or IL4-positive CD4 cells in spleen cells from IL4-treated mice (open bars) and controls (closed bars) as analyzed by intracellular cytokine staining. An example of the latter is shown in (**e**). (**d**) MOG_{35-55} immunized EAE mice injected with Ad-G/IL4 (open dots) or Ad-G (closed dots) have similar levels of circulating anti-MOG Abs, as measured by radio-immuno-binding assay. EAE, experimental autoimmune encephalomyelitis; IFN, interferon.

the major mechanism suppressing CNS autoimmunity, providing a rationale to further explore the feasibility of this gene therapy approach to human autoimmune demyelinating disorders, such as MS and acute disseminated encephalomyelitis.

Materials and methods

Animals

Q1 Six- to eight-week-old C57Bl/6 and SJL female mice were obtained from Charles River (Calco, Italy), and housed in specific pathogen-free conditions. All procedures invol-

ving animals were performed according to the guidelines of the Institutional Animal Care and Use Committee of the San Raffaele Scientific Institute.

Vector construction and production

Two helper-dependent adenoviral vectors were generated in the context of the STK-120 vector backbone:[29] the Ad-G/IL4 vector carried the expression cassettes for murine IL4 and the GFP cDNAs, respectively, driven by the phosphoglycerokinase and the cytomegalovirus immediate-early promoter; the Ad-G control vector contained only the cytomegalovirus-GFP expression cassette. Viral stocks were obtained and titrated as
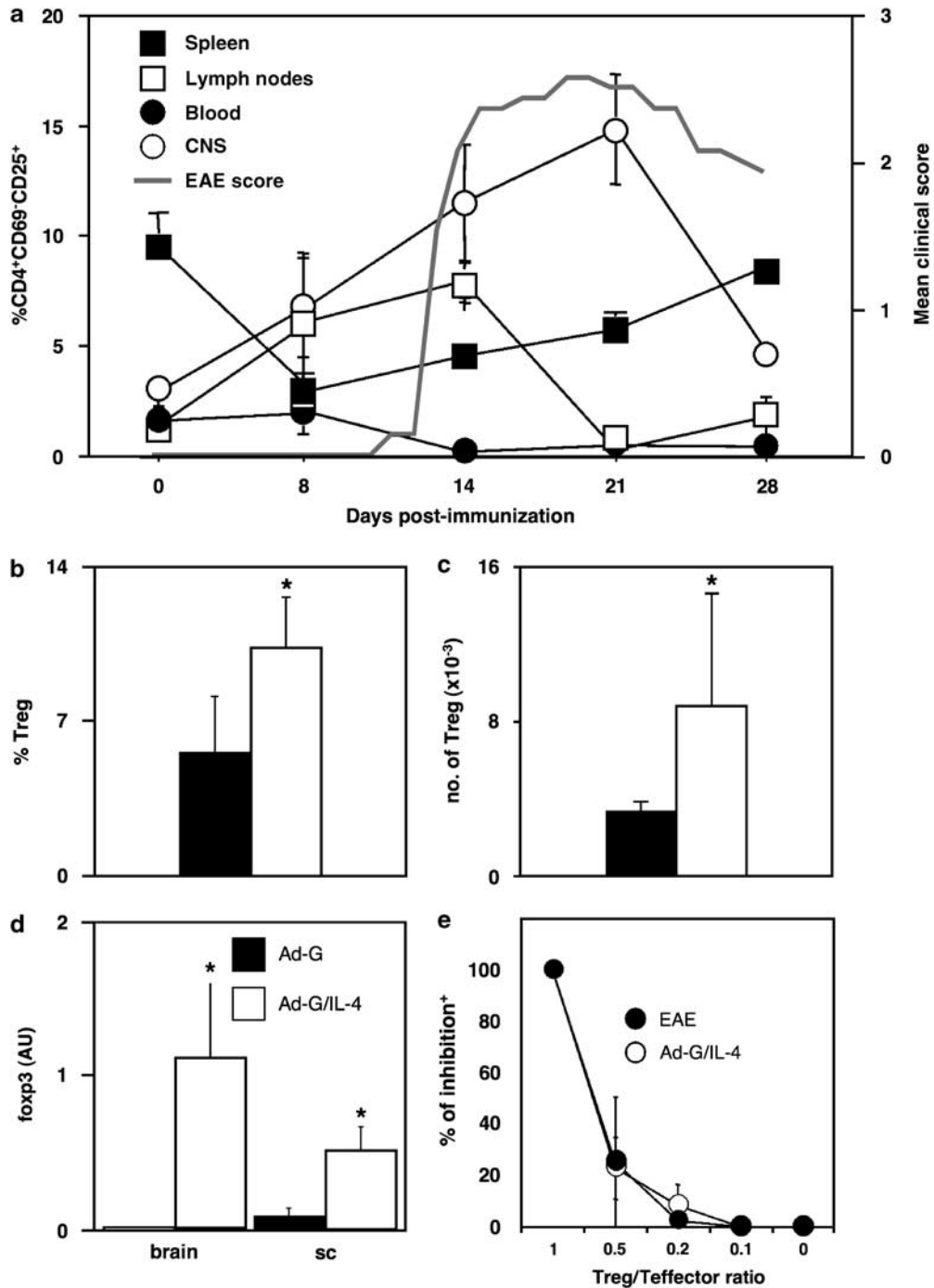
Figure 5 IL4 gene therapy recruits Treg cells to the brain. (**a**) Kinetic of regulatory CD4+CD69−CD25+ T cells during the course of C-EAE in the spleen (closed squares), inguinal lymph nodes draining the immunization site (open squares), blood (closed dots) and brain (open dots) from C57BL/6 mice—sacrificed at different time points after immunization. CD4+CD69−CD25+ cells increase in the CNS in coincidence with the peak of disease severity (mean clinical score is represented by the gray line), putatively migrating from spleen and lymph nodes. These fluctuations in the target and lymphoid organs are not reflected in the blood. Data are the average ± s.e. of four mice per time point. (**b–c**) CD4+Foxp3+ T cells are significantly ($P < 0.05$, Mann–Whitney) increased both in percentage over CD4+ cells (**b**) and in absolute numbers (**c**) in Ad-G/IL4-treated mice (open bars) as compared to Ad-G-treated control mice (closed bars) 24 days after C-EAE induction. (**d**) Twenty-four days after immunization, foxp3 mRNA level is increased in the brain and spinal cord (sc) of mice affected by C-EAE and injected with Ad-G/IL4 (open bars) compared to mice injected with Ad-G (closed bars). *$P \leqslant 0.05$, *t*-test. (**e**) CD4+CD25+ T cells from IL4-treated EAE mice (open dots), purified from draining lymph nodes at the peak of disease, are equally able to suppress proliferation of effector T cells as those taken from control EAE mice (closed dots). EAE, experimental autoimmune encephalomyelitis.
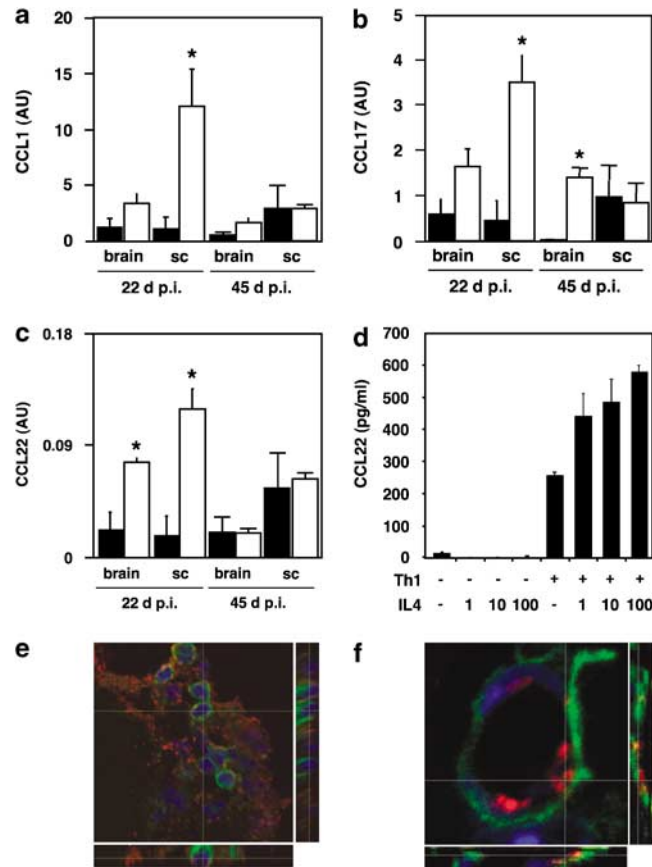
Figure 6 Regulatory T cells are recruited into the brain through the increase of chemoattractant chemokines. (**a**–**c**) Twenty-four days after immunization, CCL1 (**a**), CCL17 (**b**) and CCL22 (**c**) mRNA levels are increased in the brain and spinal cord (sc) of mice affected by C-EAE and injected with Ad-G/IL4 (open bars) compared to mice injected with Ad-G (closed bars). *$P \leq 0.05$, t-test. (**d**) The murine (C57Bl/6) microglial cell line BV.2 releases CCL22 when stimulated with IFNγ, IL1β, TNFα (Th1 mix), but not with IL4 alone. IL4, however, induces a dose-dependent increase of CCL22 release in TH1 mix-treated cells. Data are average ± s.d. of triplicate wells. Shown is one representative of two independent experiments. (**e** and **f**) Confocal microscopy shows that CCL17 (red) is expressed by CNS infiltrating CD45-positive cells of hematogenous origin (green) (**e**) and laminin-positive endothelial cells (green) (**f**). Nuclei are stained with DAPI and are shown in blue. No colocalization of CCL17 was found with GFAP and Neu-N neuronal differentiation markers (data not shown). Panel **f** is shown in pseudo-colors to use the same color code as in (**e**). EAE, experimental autoimmune encephalomyelitis.

described previously.[30] Primers specific for the HD-Ad backbone were designed to detect viral genomes in the animal tissues.

EAE induction and vector administration
Chronic progressive EAE (C-EAE) was induced in C57Bl/6 mice by immunization with 50 μg per mouse of MOG$_{35-55}$ (Espikem, Florence, Italy), and two injections of 500 ng pertussis toxin the day of immunization and 48 h later. Relapsing-remitting EAE (R-EAE) was induced in SJL mice by two immunizations, 7 days apart, with 200 μg per mouse of PLP$_{139-151}$ (Espikem, Florence, Italy), and four injections of 500 ng pertussis toxin on the day of immunization and 48 h later. Weight and clinical score (0 = healthy, 1 = flaccid tail, 2 = paresis of hindlimbs, 3 = paralysis of hindlimbs and/or paresis of forelimbs, 4 = tetraparalysis, 5 = moribund or dead) were recorded daily. HD-Ad vectors were administered by injecting a single dose of 10^7 t.u in 10 μl of PBS in the cisterna magna the day of clinical onset of EAE, as described previously.[20]

Pathology, immunohistochemistry and immunofluorescence
Mice were transcardially perfused with saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cords and brains were removed and either frozen or embedded in paraffin. To quantitate neurological damage in EAE mice, paraffin-embedded tissue sections were cut at 10 μm and stained with hematoxylin and eosin, Luxol fast Blue and Bielshowsky stain to detect inflammatory infiltrates, demyelination and axonal loss, respectively. T cells were stained using a rat anti-CD3 (pan-T-cell marker; Serotec Ltd, Oxford, UK) revealed with a biotin-labeled secondary anti-rat Ab (Amersham, UK). Macrophages were stained with BS-I isolectin B4 (Sigma, St Louis, MO, USA) revealed with a biotin-labeled secondary anti-rat Ab (Amersham). Neuropathological findings were quantified on an average of 10 complete cross-sections of spinal cord per mouse. The number of perivascular inflammatory infiltrates was calculated and expressed as the numbers of inflammatory infiltrates per mm², demyelinated areas and axonal
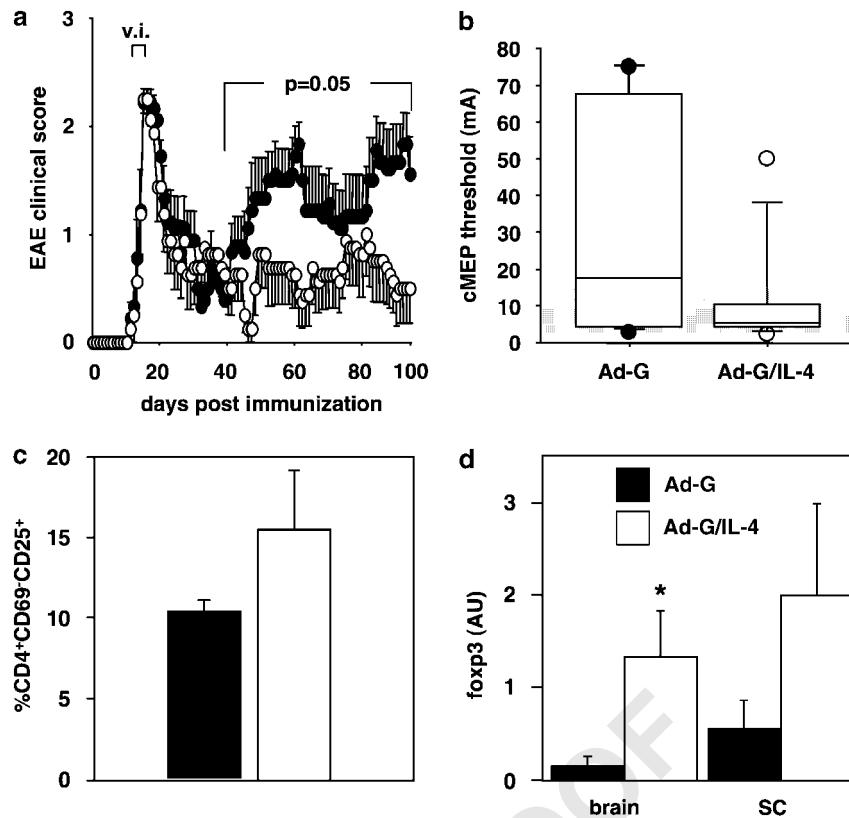
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Figure 7 IL4 gene therapy is efficient also in a relapsing model of EAE (R-EAE). (**a**) R-EAE mice exhibit a significantly milder disease course when intracisternally injected (v.i. = vector injection) with Ad-G/IL4 (open dots) as compared to control mice treated with the empty Ad-G vector (closed dots). (**b**) Conserved cMEP threshold in IL4-treated SJL mice affected by R-EAE was measured as a sign of corticospinal tract integrity. Box plots indicate 25th and 75th percentile, median value and standard deviation. Dashed area indicates the range of reference values for naive, healthy mice. (**c** and **d**) CD4+CD25+CD69− T cells are increased (**c**), as well as foxp3 mRNA levels (**d**) in Ad-G/IL4-treated mice (open bars) as compared to Ad-G-treated control mice (closed bars) 24 days after R-EAE induction. *$P \leqslant 0.05$, *t*-test. EAE, experimental autoimmune encephalomyelitis.

loss were expressed as percentage of damaged area per mm². T cells and macrophages were counted and expressed as number of cells per mm². For immunohistochemistry, 10-µm-thick frozen sections were stained with rabbit polyclonal Abs specific for GFP (1:1500; Molecular Probes), goat polyclonal Abs specific for CCL17 (1:100; Santa Cruz), rabbit polyclonal Abs specific for GFAP (1:700; Dako), mouse monoclonal Abs specific for NeuN (1:1000; Chemicon), rat monoclonal Abs specific for CD11b (1:100; Abcam), rabbit polyclonal Abs specific for laminin (1:500; Sigma) and rat monoclonal Abs specific for CD45.1 (1:100; Pharmingen). In all experiments, appropriate secondary Abs were used and nuclei stained with DAPI (1:25 000; Roche). Staining omitting the primary Abs were used as negative control.

Neurophysiology
Cortical motor-evoked potentials (cMEP) were recorded as described previously (Amadio *et al.*, 2005). Anesthetized mice were placed under a heating lamp to avoid hypothermia. Cortical motor-evoked potentials (cMEP) were elicited by using a pair of monopolar needle electrodes placed over the intact scalp, with the tip of the stimulating cathode placed above the interaural line and the anode over the temporal bone. Muscle responses evoked by descending volleys through transsynaptic depolarization of spinal α-motoneurons were recorded

with a bipolar 'belly-tendon' montage: the active needle electrodes were inserted into hindlimb footpad muscles, while the references were placed under the skin of second digit. The electromyographic signal was band-passed with 50 Hz–5 KHz filters and recorded with a Myohandy electromyographer (Micromed, Mogliano Veneto, Italy), with the sweep velocity settled at 20 ms and sensitivity at 200 µV. Spinal MEP was recorded at the lumbar level inserting a pair of stimulating needle electrodes closely to spinal emergency of sciatic nerve roots. cMEP latency—measured the time of electrical impulse propagation from motor cortex to hindlimb muscle—and spinal MEP latency—measured the time of electrical impulse propagation from the origin of motor roots to the muscle—were obtained. Difference between the above-described latencies is an index of propagation between motor cortex and spinal cord, namely CCT.

Exogenous IL4 PCR
Total brain RNA of control and IL4-treated mice was extracted and reverse transcribed using the Thermo-Script RT-PCR System Kit (Invitrogen) and primers specific for 3H Histone, as house-keeping gene and specific for exogenous IL4 (5′gatgctctttaggctttcca3′). PCR amplification for exogenous IL4 was then performed (forw, 5′gtggtgcaaatcaaagaactgctcc3′, rev, 5′agaagagcagga tgacaactagctg3′). Amplified PCR products were run on

agarose (2%) gel and blotted onto nitrocellulose filter. A synthetic IL4 oligo (5′aagtgttacttctgctctaaaagct3′) has been labeled using T4 Kinase and gamma ^{32}P dATP, hybridized in 50% formamide and autoradiography exposed 1 week.

Semiquantitative real-time PCR

RNA samples from the whole brain parenchyma and spinal cord of mice were prepared by the RNAfast Kit (Molecular System). After retro-transcription by a commercially available kit (Ready-to-go, Pharmacia, Uppsala, Sweden), cDNAs and DNAs were amplified using specific commercially available primers (Pre-developed Taqman assays reagents, PDAR) on a 7700 Perkin Elmer thermocycler using the Taqman Kit (Applied Biosystems, Foster City, MA, USA). A threshold cycle was calculated and relative quantitation was obtained by comparison with the threshold cycle obtained by amplifying samples with glyceraldehyde-3-phosphate dehydrogenase-specific primers.

In situ *hybridization for GFP*

Sections were post-fixed 15 min in 4% paraformaldehyde, then incubated in 0.5 µg ml^{-1} of proteinase K in 100 mM Tris-HCl, pH8, 50 mM EDTA for 10 min at 30 °C. Sections were incubated in 0.1 M triethanolamine (pH 8) for 5 min and then 400 µl of acetic anhydride were added two times for 5 min each. Hybridizations were done using áP33 riboprobes at the concentration ranging from 10^6 to 10^7 cpm per slides, overnight at 60 °C. The following day sections were incubated in ribonuclease-A (Roche) 20 µg ml^{-1} in 0.5 M NaCl, 10 mM Tris-HCl pH 8, 5 mM EDTA; 30 min at 37 °C. Sections were washed in formamide 50% SSC 2 × for 30 min at 60 °C and then slides were rinsed two times in SSC 2 ×. Finally, slides were dried by using ethanol series. A 1 ml (Amersham) emulsion was applied in dark room, according to manufacturer's instructions. After 3 weeks, sections were developed in dark room, counterstained with Hoecsht; coverslipped with DPX (BDH) mounting solution. We used a GFP probe containing a 0.8 kb cDNA fragment.

Flow cytometry

Mononuclear cells purified on Percoll gradient from homogenized mouse brains and spinal cords were stained with CD4-APC, CD69-FITC and CD25-PerCP (Pharmingen), as described previously.[27] FITC- or PE-labeled anti-Foxp3 (Pharmingen) was used according to manufacturer's instructions. Cytofluorimetric analysis was performed with a FACScanR flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with CellQuest software. Events acquired are measured 50 000 per sample.

Proliferation assays

Spleen cells (4×10^5 cells per well) were cultured in 96-well culture plates (Costar, Cambridge, MA, USA) in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine (Sigma), and serial concentrations (1, 3, 10 µM) of the MOG$_{35–55}$ peptide or 0.1 µg ml^{-1} of anti-CD3 (for inhibition assays). Cultures were incubated for 3 days in 5% CO$_2$ in air and labeled 16 h before harvesting with 1 µCi [^{3}H]TdR (40 Ci mmol^{-1}, Amersham Pharmacia Biotech). Incorporation of [^{3}H]TdR was measured by

liquid scintillation spectrometry. Incorporation of [^{3}H]thymidine was measured in a β-counter (9600; Packard, Meriden, CT, USA). For inhibition assays, CD4$^+$CD25$^+$ cells were purified from lymph nodes using a MACS Cell Separation Kit (Miltenyi), following manufacturer's instructions.

ELISA assays

mIL4 and mIFNγ were measured in CSF, serum samples from mice injected with the HD-Ad vectors, or cell culture supernatants, using commercially available sandwich ELISA (R&D Systems, Minneapolis, MN, USA). The resulting signals were, or not, amplified with 10-acethyl-3,7-dihydroxyphenazine (Molecular Probes), read on Victor3 Perkin Elmer, detection limit ≈1 pg ml^{-1}. For *in vitro* assays, lymphocytes were grown as above, while BV.2 cells (kind gift from Francesca Aloisi) were grown in DMEM 10% FBS, supplemented with 2 mM glutamin and 100 U ml^{-1} penicillin/streptomycin, with or without 100 U ml^{-1} IL1β (Euroclone, Pero, Italy), 200 U ml^{-1} TNFα (Peprotech, Rocky Hill, NJ, USA), 500 U ml^{-1} IFNγ (Becton Dickinson) (Th1 mix) and with the indicated amount of IL4 (R&D Systems). Supernatants were assayed after 24 h using a commercial ELISA kit from R&D systems for CCL22, following manufacturer's instructions.

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