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Cerebrospinal fluid-targeted delivery of neutralizing anti-IFN γ antibody delays motor decline in an ALS mouse model

Belkacem Otsmane^a, Julianne Aebischer^{a,c}, Anice Moumen^a
and Cédric Raoul^{a,b}

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder characterized by the selective and gradual loss of motoneurons in the brain and spinal cord. A persistent inflammation, typified by the activation of astrocytes and microglia, accompanies the progressive degeneration of motoneurons. Interferon gamma (IFN γ), a potent proinflammatory cytokine that is aberrantly present in the spinal cord of ALS mice and patients, has been proposed to contribute to motoneuron death by eliciting the activation of the lymphotoxin- β receptor (LT- β R) through its ligand LIGHT. However, the implication of IFN γ in the pathogenic process remains elusive. Here, we show that an antagonistic anti-IFN γ antibody efficiently rescues motoneurons from IFN γ -induced death. When transiently delivered in the cerebrospinal fluid through a subcutaneously implanted osmotic minipump, the neutralizing anti-IFN γ antibody significantly retarded motor function decline in a mouse model of ALS. However,

this transient infusion of anti-IFN γ antibody did not increase the life expectancy of ALS mice. Our results suggest that IFN γ contributes to ALS pathogenesis and represents a potential therapeutic target for ALS. *NeuroReport* 00:000–000 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and incurable neurodegenerative disease that primarily affects upper and lower motoneurons. ALS initiates in mid-life in the form of progressive paralysis, evolving into a generalized muscle wasting that leads irrevocably to death within 2 or 5 years of clinical onset. In most cases, the causes of ALS remain largely unknown. About 10% of cases are inherited, and among them, about 20% are caused by dominant mutations in the superoxide dismutase-1 (*SOD1*) gene [1]. Mice expressing human *SOD1* mutations develop a motor syndrome with features of the human disease [2]. Accumulating evidence suggests that neighboring cells of motoneurons, including astrocytes and microglial cells, actively participate in ALS pathogenesis.

Interferon gamma (IFN γ), a potent immunomodulatory cytokine, is significantly increased in both spinal motoneurons and astrocytes of *SOD1*^{G93A} mice at disease onset and symptomatic stages [3]. Astrocytes expressing the ALS-associated *SOD1* mutant aberrantly produce IFN γ , which triggers motoneuron death through activation of the tumor necrosis factor receptor superfamily (TNFRSF) member lymphotoxin- β receptor (LT- β R) by its ligand LIGHT (TNFRSF14). The genetic deletion of *LIGHT* in *SOD1*^{G93A} mutant mice retards the progression, but not the onset, of disease and prolongs their life expectancy [3,4]. In addition, abnormal levels of IFN γ

have been found in the spinal cord, cerebrospinal fluid (CSF), and serum of sporadic ALS patients [5,6]. However, the contribution of IFN γ to the pathogenic process, as well as its therapeutic potential, remains to be investigated.

To address this question, we here used a monoclonal anti-IFN γ antibody that we showed to efficiently rescue motoneurons from the killing effect of IFN γ *in vitro*. To circumvent the barriers limiting central nervous system (CNS) penetration and to minimize systemic side effects of IFN γ inhibition, the antibody was delivered in the CSF by means of an osmotic pump. We show that transient intracerebroventricular delivery of the neutralizing anti-IFN γ antibody improves motor functions of treated ALS mice, for the period of time during which the antibody is present in the CNS. These results suggest that IFN γ participates in the pathogenic process and represents a potential therapeutic target for ALS. Further, they highlight the importance of defining the appropriate therapeutic approach to ameliorate the pharmacokinetics of the delivered IFN γ -neutralizing molecule.

Materials and methods

Motoneuron culture

Motoneurons from the embryonic stage 12.5 (E12.5) spinal cord of *Hb9::GFP* embryos were isolated as

described previously [7], using iodixanol density gradient centrifugation. Motoneurons were plated onto polyornithine/laminin-treated wells in the presence of a cocktail of neurotrophic factors [0.1 ng/ml glial-derived neurotrophic factor, 1 ng/ml brain-derived neurotrophic factor, and 10 ng/ml ciliary neurotrophic factor in supplemented Neurobasal medium (Invitrogen, Carlsbad, California, USA)]. Motoneurons were seeded at a density of 1500 cells/cm² and maintained for 24 h at 37°C, 7.5% CO₂, before being treated or not with an indicated concentration of soluble mouse recombinant IFN γ (Calbiochem, San Diego, California, USA), antagonistic rat monoclonal anti-IFN γ (purified hybridoma products, R4-6A2), or IgG₁ isotype control antibody (provided by LASCCO SA, Geneva, Switzerland). The surviving neurons were directly counted under a fluorescence microscope. Between 60 and 130 motoneurons were counted in two diameters of each well, as we have described previously [3]. The number of motoneurons cultured in the presence of neurotrophic factors was taken as 100% survival.

Animals

Animal experiments were approved by the National Ethics Committee on Animal Experimentation and were performed in compliance with the European Community and National Directives for the Care and Use of Laboratory Animals. *Hb9::GFP* mice (TM Jessell's laboratory, Columbia University, New York, USA) were maintained on a CD1 background (Charles River laboratories, Wilmington, Massachusetts, USA).

Intracerebroventricular infusion of function-blocking anti-IFN γ antibody

Mice were deeply anesthetized by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (15 mg/kg). A 30-G stainless steel infusion cannula (Alzet Brain Infusion Kit 3, Charles River laboratories) was stereotaxically implanted into the lateral ventricle (0.3 mm anterior and 1 mm lateral relative to bregma; 2.6 mm below the surface of the skull) of 13-week-old mice. The cannula was secured to the skull using glue and dental cement. The Alzet osmotic pump (model 2004, Alzet; Charles River laboratories) was implanted into a subcutaneous pocket in the midscapular area of the mouse's back. Rat monoclonal antagonistic anti-IFN γ antibodies (R4-6A2) or irrelevant rat IgG₁ monoclonal antibodies were diluted in PBS at a concentration of 300 μ g/ml and infused intraventricularly at a continuous rate of 0.25 μ l/h.

Immunohistochemistry

Animals were deeply anesthetized and transcardially perfused with PBS (pH 7.4) for collecting fresh tissues, followed by 4% paraformaldehyde in PBS for immunohistochemical analysis. The brain and spinal cord were dissected out and incubated in the same fixative for 4 h. Tissues were immersed in 30% sucrose in PBS and

embedded in optimal cutting temperature compound (CML, Nemours, France).

Cryostat sections (18 μ m) of the brain and spinal cord regions were washed with PBS, incubated for 20 min in 100 mM sodium periodate, and washed again with PBS. Sections were blocked for 2 h with 4% bovine serum albumin and 4% heat-inactivated Donkey serum in PBS containing 0.1% Triton X-100; they were then incubated overnight at +4°C with biotinylated anti-rat antibodies (Invitrogen). Tissues were washed with PBS and 0.1% Triton X-100, and incubated in Vectastain peroxidase/DAB detection solution following the manufacturer's instruction (Dako, Glostrup, Denmark).

Footprinting analysis

Footprint analysis was carried out weekly for mice. The forepaws and the hindpaws were inked with water-based nontoxic paints of two different colors. The mice were placed on a sheet of paper and allowed to walk through a tunnel. Footprint patterns were scanned and the distance between forepaw and hindpaw prints was calculated using the NIH Image J software (National Institutes of Health, Bethesda, Maryland, USA). For each set of paw prints four measurements were taken. For statistical purposes, we set the maximum distance at 26 mm.

Statistics

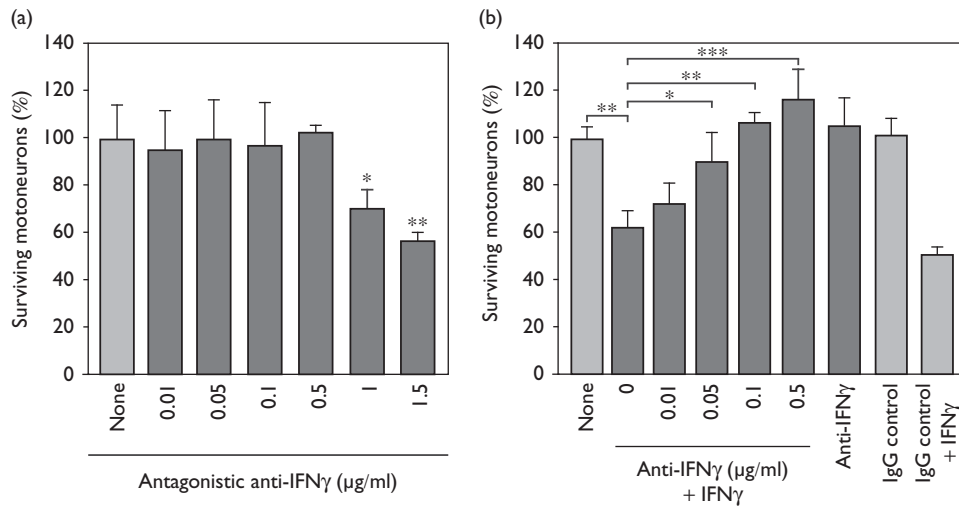
For motoneuron survival, statistical analyses were carried out by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls multiple comparisons post-hoc test, using the GraphPad Prism 5 software (GraphPad Software, La Jolla, California, USA). For footprinting analysis, data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni's post-hoc test, using StatSoft Statistica software (StatSoft, Tulsa, Oklahoma, USA). The level of significance was set at a *P*-value less than 0.05 (*n* represents the number of independent observations).

Results

Neutralizing anti-IFN γ antibodies protect motoneurons from IFN γ -induced death

We selected a rat anti-IFN γ monoclonal IgG₁ antibody (clone R4-6A2), which specifically neutralizes mouse IFN γ [8]. We first evaluated the inherent toxicity of purified antagonistic anti-IFN γ antibodies toward motoneurons. Motoneurons were isolated from the spinal cord of *Hb9::GFP* transgenic mouse embryos, which express the green fluorescent protein (GFP) under the control of the motoneuron-selective promoter Hb9 [9], facilitating identification of motoneurons. We found that below the concentration of 1 μ g/ml, anti-IFN γ monoclonal antibodies did not show any cytotoxicity (Fig. 1a). We next evaluated the capacity of rat anti-IFN γ antibodies to rescue cultured motoneurons from death induced by recombinant mouse IFN γ . Motoneurons were exposed

Fig. 1



(a) Mouse motoneurons were cultured for 24 h and then incubated with increasing concentrations of monoclonal rat anti-IFN γ antibodies (clone R4-6A2). The survival of *Hb9::GFP* motoneurons was determined 48 h later and expressed relative to that of nontreated neurons (none). (b) Mouse motoneurons were cultured for 24 h before being treated, or not, with recombinant mouse IFN γ (250 ng/ml) alone or in combination with indicated concentrations of anti-IFN γ antibodies. IFN γ (250 ng/ml) in combination with irrelevant rat IgG₁ antibodies (0.5 $\mu\text{g/ml}$) or anti-IFN γ antibodies (0.5 $\mu\text{g/ml}$) alone were used as control. The survival of EGFP-positive motoneurons was determined 48 h later and expressed as a percentage of surviving motoneurons in the absence of treatment (none). All values are expressed as means \pm SD of three independent experiments, each performed in triplicate (ANOVA with the Student–Newman–Keuls post-hoc test, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). ANOVA, analysis of variance; EGFP, enhanced green fluorescent protein; IFN γ , interferon gamma.

for 48 h to mouse recombinant IFN γ and to increasing concentrations of anti-IFN γ antibodies in the range that did not cause toxicity (Fig. 1a). Neuron survival was assessed by counting the number of GFP-positive neurons isolated from *Hb9::GFP* embryos. Although IFN γ triggered death of about 50% of the isolated motoneurons, R4-6A2 monoclonal antibodies rescued motoneurons from the killing effect of IFN γ in a dose-dependent manner. The isotope-matched control antibody did not show any effect on motoneuron survival and inhibition of IFN γ -induced death (Fig. 1b).

Efficient delivery of anti-IFN γ antibodies in the CNS

We next examined the parenchymal diffusion of rat anti-IFN γ monoclonal antibodies following intracerebroventricular delivery. A cannula, implanted into the lateral ventricle of 13-week-old mice, was connected to a subcutaneously implanted osmotic pump with the capacity to continuously deliver the antibodies. The miniature osmotic systems were filled with antibody solution and enabled the constant delivery of 1.8 μg of monoclonal antibody per day for up to 4 weeks. Three weeks after implantation, we observed widespread diffusion of rat anti-IFN γ antibodies into punctuate structures in different brain regions including the cortex, corpus callosum, midbrain, and hippocampus, as well as the cervical, thoracic, and lumbar spinal cord (Fig. 2a and b, and not shown). This punctuated accumulation is reminiscent of what has been previously observed

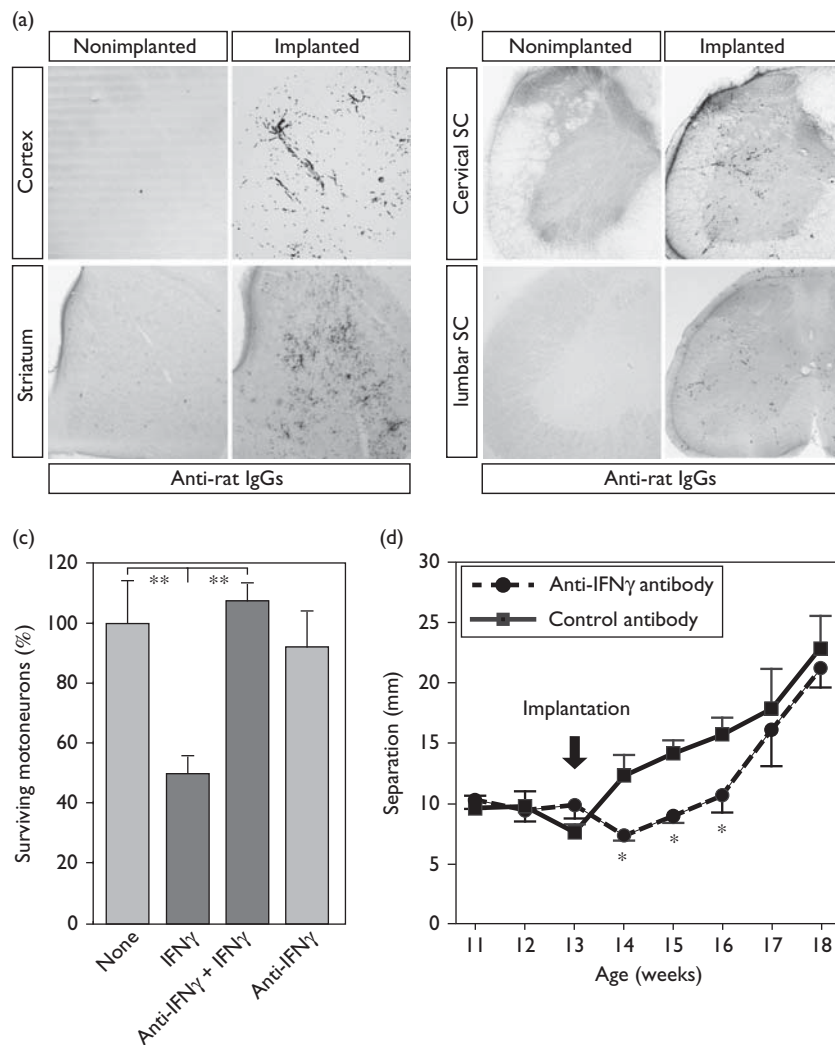
following CSF delivery of anti-Nogo-A, despite the precise nature of these structures being still undetermined [10]. The specificity of rat IgG immunodetection was confirmed using brain and spinal cord sections from age-matched nonimplanted mice. In contrast, 4 weeks after subcutaneous implantation with the osmotic pump, the antibody was no longer detected in the brain or spinal cord of implanted mice, despite the proper implantation of the cannula and depletion of antibody solution from the pump ($n = 4$, not shown).

As the functional efficacy of a chronically delivered antibody relies on its stability over the therapeutic time window, we verified whether the blocking function of the anti-IFN γ antibody was still preserved when it was maintained at physiological temperature over a period of 4 weeks. We found that even after being stored at 37°C for such a long period, anti-IFN γ antibodies saved motoneurons from IFN γ -induced death *in vitro* (Fig. 2c). Therefore, these data indicate that the neutralizing anti-IFN γ antibodies can be efficiently delivered to the brain and spinal cord through intraventricular infusion for a limited period of 3 weeks.

Immunotherapy against IFN γ retards motor decline of SOD1 mutant mice

We used intracerebroventricular delivery of neutralizing anti-IFN γ antibodies to evaluate the functional contribution of IFN γ to the motoneuron disease. Toward this aim, we used *SOD1*^{G93A} mutant mice that develop motor

Fig. 2



(a, b) Parenchymal diffusion of anti-IFN γ antibodies. Mice at 13 weeks of age were intracerebroventricularly infused with rat monoclonal anti-IFN γ antibodies for 3 weeks using an Alzet osmotic pump. Brain and spinal cord sections were immunostained with anti-rat IgG antibodies ($n=3$). As examples, (a) cortex and striatum and (b) cervical and lumbar spinal cord (SC) sections of control (nonimplanted) and implanted mice are shown. Scale bar, 50 μ m. (c) Anti-IFN γ antibodies were incubated at 37°C for 4 weeks before being added to the motoneuron culture (at 0.5 μ g/ml) in the presence (or not) of recombinant IFN γ (250 ng/ml). Motoneuron survival was determined 48 h later. Data represent the mean values \pm SD of three independent experiments, each performed in triplicate (ANOVA with the Student–Newman–Keuls post-hoc test; statistical attributes: * $P<0.05$, and ** $P<0.01$). (d) Anti-IFN γ antibody infusion transiently ameliorates motor deficit in $SOD1^{G93A}$ mice. $SOD1^{G93A}$ mice were implanted subcutaneously at 13 weeks of age with osmotic pumps continuously delivering either anti-IFN γ antibodies ($n=8$) or IgG₁ isotype control antibodies ($n=6$) in the left lateral ventricle. Footprint analysis was carried out weekly and results are expressed as distance in mm between the forepaw and hindpaw (values are expressed as mean \pm SEM, ANOVA with Bonferroni’s post-hoc test). ANOVA, analysis of variance; IFN γ , interferon gamma.

deficits recapitulating many clinical features of ALS. We implanted osmotic pumps delivering either anti-IFN γ or irrelevant IgG₁ rat monoclonal antibodies in $SOD1^{G93A}$ mice at early onset of disease, when IFN γ starts to be detected in the spinal cord of ALS mice [3]. We found that passive immunization with anti-IFN γ significantly delayed the motor decline of $SOD1^{G93A}$ mice (Fig. 2d). This therapeutic benefit was however transient, lasting for the period of time during which the neutralizing antibody was efficiently delivered, and did not result in a prolongation of the life expectancy (median survival after

anti-IFN γ antibody infusion, 20.4 weeks, $n=8$; after control IgG₁ antibody infusion, 20.8 weeks, $n=6$; nonsignificant, log-rank test). The results support the hypothesis that IFN γ takes part in the pathogenic process and suggest the therapeutic potential of using IFN γ function-blocking molecules in this motoneuron disease.

Discussion

Mutant $SOD1$ -expressing astrocytes produce IFN γ , which triggers a motoneuron-selective death pathway

through the activation of LT- β R by LIGHT. The genetic ablation of *LIGHT* in *SOD1^{G93A}* mice delays disease progression and increases the lifespan of these ALS mice [3]. Therefore, the motoneuron-selective IFN γ -induced LIGHT-mediated death pathway might represent a pathogenic mechanism underlying motoneuron loss in ALS. However, the role of IFN γ during the disease course remains to be addressed. Here, we report that a late therapeutic intervention consisting of intracerebroventricular delivery of a neutralizing anti-IFN γ antibody retards the progression of the disease, suggesting that IFN γ might contribute to ALS pathogenesis.

IFN γ is a potent immunomodulatory cytokine primarily produced by activated T and B lymphocytes, natural killer cells, and monocytes/macrophages. IFN γ plays a crucial role in both the innate and adaptive immunity by mediating protection against viral and microbial infections, as well as tumor cells [11]. IFN γ primes macrophages for activation, enhances expression of class II major histocompatibility complex molecules, potentiates the inflammatory T helper 1-driven immune response, and promotes T-cell growth or lymphocyte adhesion to endothelium. Accumulating evidence underscores the potential role of IFN γ in the motoneuron disease. Elevated levels of IFN γ were found in the spinal cord of ALS mice and patients [3,12], as well as in the CSF and serum of ALS patients as the disease progresses [13,14]. In the spinal cord of ALS mice, mainly motoneurons and astrocytes express IFN γ at the onset and the symptomatic stage of the disease [3]. In the spinal cord of sporadic ALS patients, IFN γ expression is observed in large ventral horn neurons and non-neuronal cells [5]. Signs of activation of signal transducer and activator of transcription 4 (STAT4), which acts as a transcription factor to elicit IFN γ expression, have also been reported in *SOD1* mutant spinal cords [15]. Further, a significant increase in the IFN γ -inducible protein, IP-30, and the IFN-stimulated gene 15, *ISG15*, is observed in the spinal cord of humans with ALS [16,17]. Finally, a mathematical model of a cell–cytokine communication network in ALS also predicted IFN γ as an effective therapeutic target and proposed that a late inhibition of IFN γ activity may moderate the inflammatory process, slow down disease progression, and prolong life expectancy [18].

The pleiotropic features of biological functions of IFN γ , however, suggest that a therapeutic intervention restricted to the target site of injury is of a consequential concern for therapeutic efficacy and safety. Although intracerebral passive immunization has been widely used to specifically inhibit the biological activity of target molecules in the CNS, the instability and/or rapid turn-over of antibodies in the CSF may explain the transitory effect we observed [19,20]. Indeed, intracerebral injection of an IgG_{2a} isotype antibody in mice

showed that half of the antibody was excluded from the CNS after 48 min. This rapid elimination of brain-injected antibodies is attributed to active efflux from the brain parenchyma through reverse transcytosis across the blood–brain barrier, and is proposed to be mediated by the neonatal Fc receptor, which is highly expressed in brain endothelial cells [21]. This underlines the need for neutralizing molecules and/or delivery systems that would circumvent the therapeutic limitations of antibodies in CNS applications. In addition, with the aim of preventing premature efflux from the CSF and ameliorating the pharmacokinetics of delivered antibodies, the use of F(ab')₂ modified fragments that efficiently impede interactions with the neonatal Fc receptor could also be envisaged [20]. The present results warrant further studies to improve stability, antigen recognition and neutralization, and parenchymal penetration of antibodies. The gene therapy-based approach, which has successfully been applied for long-lasting delivery of molecules to the CSF [22], could also be considered for the delivery of the IFN γ dominant negative inhibitor.

IFN γ has been proposed to participate in several inflammatory pathologies including Crohn's disease, rheumatoid arthritis, asthma, and multiple sclerosis [23–26]. Recently, a phase II randomized, double-blind, placebo-controlled study on intravenous administration of humanized anti-IFN γ antibodies has demonstrated tolerability and safety in patients with Crohn's disease [27]. Although further studies are required to explore the pathogenic mechanisms governed by IFN γ in ALS and to define the *modus operandi* of an IFN γ -based therapy, the neutralization of IFN γ activity could offer novel therapeutic perspectives for ALS.

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Conflicts of interest

There are no conflicts of interest.

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