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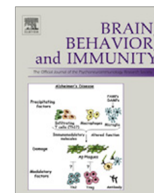

CNS response to a second pro-inflammatory event depends on whether the primary demyelinating lesion is active or resolved

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ABSTRACT

Interleukin-1β (IL-1β) is considered to be one of the most important mediators in the pathogenesis of inflammatory diseases, particularly in neurodegenerative diseases such as multiple sclerosis (MS). MS is a chronic inflammatory disease characterized by demyelination and remyelination events, with unpredictable relapsing and remitting episodes that seldom worsen MS lesions. We proposed to study the effect of a unique component of the inflammatory process, IL-1β, and evaluate its effect in repeated episodes, similar to the relapsing-remitting MS pathology. Using adenoviral vectors, we developed a model of focal demyelination/remyelination triggered by the chronic expression of IL-1β. The long-term expression of IL-1β in the striatum produced blood–brain barrier (BBB) breakdown, demyelination, microglial/macrophage activation, and neutrophil infiltration but no overt neuronal degeneration. This demyelinating process was followed by complete remyelination of the area. This simple model allows us to study demyelination and remyelination independently of the autoimmune and adaptive immune components. Re-exposure to this cytokine when the first inflammatory response was still unresolved generated a lesion with decreased neuroinflammation, demyelination, axonal injury and glial response. However, a second long-term expression of IL-1β when the first lesion was resolved could not be differentiated from the first event. In this study, we demonstrated that the response to a second inflammatory stimulus varies depending on whether the initial lesion is still active or has been resolved. Considering that anti-inflammatory treatments have shown little improvement in MS patients, studies about the behavior of specific components of the inflammatory process should be taken into account to develop new therapeutic tools.

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1. Background

Neuroinflammation is a hallmark of most neurodegenerative diseases. Central nervous system (CNS) inflammation has different features than peripheral inflammation: cytokines are involved in distinct leukocyte recruitment patterns in the brain compared to the periphery (Blond et al., 2002; Holman et al., 2011). Inflammatory cytokines such as interleukin-1β (IL-β), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) are usually described as noxious molecules to the CNS (Codarri et al., 2010; Merson et al., 2010; Perry et al., 2007). These and other cytokines could contribute to exacerbating the progression of neurodegenerative diseases or even be involved in their etiology. Although some pieces of the puzzle have been uncovered, some of the mechanisms and specific components of inflammation that have an impact on neurodegenerative diseases are still poorly understood.

In particular, IL-1β is considered to be one of the most important mediators in the pathogenesis of inflammatory diseases (Dinarello, 2009; Simi et al., 2007). This cytokine has been shown to play a pivotal role in the exacerbation of acute neurodegeneration caused by ischemia, head trauma, and stroke; it has also been implicated in the pathology of multiple sclerosis (MS), Alzheimer's disease, and other chronic diseases of the CNS. The IL-1β cytotoxic action may be related either to a direct effect on the CNS cells or to a secondary effect via recruitment of leukocytes (Simi et al., 2007). A single-bolus injection of IL-1 into the CNS parenchyma gave rise to delayed and localized polymorphonuclear neutrophil (PMN) recruitment but no overt damage to CNS integrity (Andersson et al., 1992; Anthony et al., 1997; Depino et al., 2003). However, the long-term expression of IL-1β induced neutrophil recruitment, reversible demyelination and blood–brain barrier (BBB) breakdown with no neurodegeneration (Ferrari et al., 2004; Shaftel et al., 2007). However, a dual role has been described for this cytokine because a protective effect can be exerted when it is administered prior to an injury (Saura et al., 2003), and it was reported to induce growth factors (e.g., Insulin growth factor 1) during the remyelination process both *in vitro* and *in vivo* (Mason et al.,

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2001; Watzlawik et al., 2010). Moreover, IL-1 priming markedly reduced the symptoms of experimental autoimmune encephalomyelitis (EAE) in an MS animal model (Huitinga et al., 2000).

IL-1β has been described in neurodegenerative diseases such as in MS demyelinated lesions (Argaw et al., 2006; Howe et al., 2006; Zeis et al., 2008). MS is a chronic inflammatory disease characterized by demyelination/remyelination events and axonal damage, which lead to a variable clinical course, including motor, sensory and cognitive deficits (Hagemeier et al., 2012; Lindquist et al., 2011; Lucchinetti et al., 2004; Popescu and Lucchinetti, 2012; Zivadinov and Pirko, 2012). Despite the heterogeneity of the disease, most patients present relapsing and remitting (RR) episodes from the onset. Its etiology is still unclear, and therapeutic interventions only reduce the frequency of new relapsing episodes and they do not influence the progressive phase of the disease. In particular, elevated IL-1β levels have been found in lesions, cerebrospinal fluid (CSF) and serum of MS patients, especially during relapses (Brosnan et al., 1995; Dujmovic et al., 2009; Hauser et al., 1990; Tsukada et al., 1991). Moreover, certain IL-1β genotypes and the balance between IL-1β and interleukin 1 receptor antagonist (IL-1ra) have been associated with disease severity, susceptibility, and/or progression (Aggelakis et al., 2010; Borzani et al., 2010; Dincic et al., 2006; Sarial et al., 2008). In MS animal models, it has been demonstrated that IL-1 is associated with an increase in the severity of the symptoms, and the administration of IL-1ra ameliorates the disease (Furlan et al., 2007; Moreno et al., 2011). In addition, interleukin 1 receptor type I (IL-1RI) is a mediator in EAE pathogenesis (Li et al., 2011; Matsuki et al., 2006; Ruggiero, 2011; Schiffenbauer et al., 2000; Sutton et al., 2006).

As noted previously, MS is characterized by relapsing and remitting episodes. These episodes are unpredictable and could potentially worsen MS lesions, leading to incomplete recovery and resulting in a progressive deterioration. The number of IL-1β, IL-6 and TNF-α secreting cells is higher in RRMS patients only during exacerbations (Ysrraelit et al., 2008). The CSF cytokine profile can change from remission to relapse stage, with up-regulation of IL-1β, TNF-α and transforming growth factor beta (TGF-β) (Edwards et al., 2011; Hauser et al., 1990).

The role of inflammation in MS is more complex than previously described because there is evidence that demonstrates a protective function of both the inflammatory environment and immune cells in demyelinating diseases (Hohlfeld et al., 2005; Watzlawik et al., 2010). However, most therapies include immunosuppressive and anti-inflammatory treatments (Comi and Filippi, 2005; Hartung et al., 2005; Kanwar, 2005; Kieseier et al., 2005). In particular, interferons, used as anti-inflammatories in MS treatment (Loma and Heyman, 2011), exert their action by reducing IL-1β production (Ludigs et al., 2012). However, it has not been demonstrated that anti-IL-1 treatments are effective in treating MS patients (Warabi, 2007). Even though immunomodulation may provide temporary relief, some patients have shown long-term adverse secondary effects or even worsening of the pathology (Watzlawik et al., 2010). To achieve more effective MS treatment, more information about the pathological process underlying the disease is needed. The development of appropriate experimental models is crucial in obtaining this knowledge.

Our laboratory has developed a model of focal demyelination/remyelination based on long-term IL-1β expression in the striatum according to Ferrari et al., 2004. To achieve the long-term expression of IL-1β, we injected a recombinant adenovirus expressing this cytokine. The chronic expression of IL-1β induced neutrophil recruitment, microglia/macrophage activation, reversible demyelination, and BBB breakdown with no neurodegeneration (Ferrari et al., 2004). This simple model allows us to study demyelination and remyelination independently of the autoimmune and adaptive immune components. The aim of this work was to study the response of the striatum to repeated challenges of long-lasting IL-1β expression. Special attention was given to the relapse event and the evaluation of its effect on CNS tissue in a RR episode similar to MS pathology. Analysis of inflammation, demyelination, axon integrity and glial behavior after the secondary event was emphasized. Interestingly, we found that the magnitude of the second lesion was milder if the relapse occurred while the first event was still in the recovery phase. On the other hand, if the second stimulus was applied once the lesion had completely recovered, there was no difference in the response of the CNS to the second insult. We believe that understanding the mechanisms behind this differential response to relapses in our model will provide valuable information to improve understanding of the role of inflammation in demyelination and relapses.

2. Methods

2.1. Adenoviral vectors

Adenoviral vectors expressing the reporter gene β-galactosidase (Ad-βgal) or the human interleukin 1β gene (AdIL-1β) were generated as described previously (Ferrari et al., 2004). Stocks were obtained by large-scale amplification in HEK293 cells, purified in double cesium chloride gradients and quantified by plaque assay (final titers: Ad-βgal = 8.41×10^{11} infective particles/ml.; AdIL-1β = 8.41×10^{11} infective particles/ml.). Stocks had less than 1 ng/ml of endotoxin assayed with E-TOXATE reagents (Sigma, St. Louis, MO). Viral stocks were free of autoreplicative particles as assessed by PCR. Ad-βgal was kindly provided by Dr. J. Mallet (Hospital Pitié Salpetrière, Paris, France).

2.2. Animals and injections

Adult male Wistar rats (Jackson Laboratory, Bar Harbor, Maine, USA, bred for several generations in the Instituto Leloir's animal facility) aged 8–10 weeks were used in all experiments. Animals were housed under standard conditions in groups of four in ventilated racks under controlled temperature conditions (22 °C ± 2 °C) with food and water *ad libitum* and a 12:12 dark:light cycle with lights on at 08:00 h. All surgical procedures and euthanasia were performed in full compliance with NIH and internal Institute Foundation Leloir guidelines.

For stereotaxic injections, animals were anesthetized with ketamine chlorhydrate (80 mg/kg) and xylazine (8 mg/kg). Adenoviral vectors were delivered in the left striatum (bregma, +1 mm; lateral, +2.5 mm; ventral, – 4 mm) (Paxinos and Watson, 1986) with a finely drawn graduated capillary. The volume of the intrastriatal injection was 1 μl, infused over a 4-min time period, with the capillary left in place for another minute to minimize reflux. Human IL-1β and β-galactosidase (βGal)-expressing vectors were diluted in sterile Tris–Cl buffer (Tris–Cl 10 mM, $MgCl_2$ 1 mM, pH 7.8) to a concentration of 1×10^6 infective particles/μl. Previous work published by the lab (Ferrari et al., 2004) and pilot studies demonstrated that this was the minimum dose of both AdIL-1β and Ad-βgal that provided long-term expression of the transgenes with minimum inflammatory response to the control vector (Ad-βGal). Animals from the single-injection groups were sacrificed 21, 30 or 51 days after a single stereotaxic injection, double injection animals were sacrificed 21 days after the second injection, and their brains were processed for histological or molecular analysis. All surgical procedures were performed in the morning to avoid possible circadian variation in cytokine expression.

2.3. Experimental groups

All animals were first injected with AdIL-1β or Ad-βGal in the striatum and then re-injected at a time when the lesion was unresolved (30 days) or resolved (50 days). According to the first or second stimulus received four experimental groups were generated: IL-1β /IL-1β; IL-1β/βGal; βGal/IL-1β; and βGal/βGal. These four groups were present in each experiment (depending on the characteristics of the lesion at the time of adenovector re-administration). Finally, because different time points were studied, we generated several single-injection groups to fully understand the response of the striatum at any studied time point (see timeline in Fig. 1A). Single-injection groups 21, 30 and 50 days post injection (dpi), showed the tissue response to a single injection of AdIL-1β at those time points after treatment. We will refer to these experimental groups as single-injection group 21, 30 or 50 dpi.

2.4. Histology

Animals were deeply anesthetized and transcardially perfused with heparinized saline followed by ice cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2. Brains were dissected and placed in the same fixative overnight at 4 °C. After cryoprotection in a 30% sucrose, 0.1 M PB solution, brains were frozen in isopentane, and 40 μm serial coronal sections throughout the striatum were cut in a cryostat. Serial sections were mounted on gelatin-coated slides and stained with Cresyl-Violet for general nervous tissue integrity and inflammation assessment or Red Oil-O for myelin evaluation. For immunohistochemistry, sections were stored in a cryoprotective solution at −20 °C until needed.

2.5. Volume quantification

For the calculation of lesion volume, every sixth 40-μm-thick serial section of the entire ipsilateral striatum was used, based on anatomical landmarks defined in Paxinos and Watson (1986). All of the sections were photographed using a Nikon Eclipse E600 microscope and a CX900 camera (MicrobrightField Inc., USA) with 2x magnification, and Image J software (Media Cybernetics, Silver Spring, MD) was used to measure the inflammatory/demyelinated area in each section. The area of each observed inflammatory infiltrate or demyelinated tissue was delineated. One set of serial sections stained with Cresyl-Violet was used for inflammation analysis, and the adjacent group of serial sections stained with Red Oil-O was used for demyelinating volume. All of the measurements were performed in a blind manner. An inflammatory/demyelinating average (IA and DA) was calculated for each animal, and the average volume (IV and DV) was estimated by multiplying each average area by the section's width (0.04 mm) and the total number of sections obtained for the whole striatum. IV and DV are expressed as the percentage of the entire ipsilateral striatum.

2.6. Immunohistochemistry

For immunofluorescence, free-floating sections were incubated in blocking buffer (1% donkey serum, 0.1% Triton in 0.1 mol/L PB) for 45 min, rinsed in 0.1% Triton in 0.1 mol/L PB and incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The antibodies used were anti-glial fibrillary acidic protein (GFAP) (1:700; Dako, Carpinteria, CA) and anti- major histocompatibility complex class II (MHC II) (1:200, Serotec, Oxford, UK). After three 5-min washes with 0.1 mol/L PB, the sections were incubated with indocarbocyanine Cy3 (Cy3)-conjugated donkey anti-mouse antibody (1:250; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 2 h at room temperature, rinsed in 0.1 mol/L PB, and mounted in Mowiol (Calbiochem, San Diego, CA). Digital images were collected on a Zeiss LSM 510 laser scanning confocal microscope equipped with a krypton-argon laser.

MHC II-positive cells were quantified using a 20x objective, and one picture of the ipsilateral striatum was taken from each section. Laser power, gain and offset conditions were kept constant for the acquisition of all images. Approximately 12 fields were quantified for each animal using the Zeiss LSM Image Browser. The total number of positive cells was normalized to the total area counted for each animal.

For the immunohistochemical identification of injured axons, the sections received acid citric antigen retrieval treatment and were incubated with anti β-amyloid precursor protein (APP) primary antibody (1:100, Zymed, San Francisco, CA). After rinsing, the sections were incubated with donkey anti-rabbit biotin conjugated antibody (Jackson, ImmunoResearch Laboratories Inc., West Grove, PA), followed by the Vectastain standard ABC kit (Vector Laboratories, Burlingame, CA), and developed with 3,3′-diaminobenzidine (Sigma, Saint Louis, Missouri). We used a 40× objective to quantify APP-positive axons. The total number of APP-positive axons was counted in one out of every sixth 40 μm serial section of the entire striatum (6–8 sections per animal).

2.7. RNA isolation, reverse transcription and real-time PCR

Animals were decapitated, and their brains were quickly removed. The ipsi- and contralateral striatum were dissected, snap frozen in liquid nitrogen, and stored at −80 °C. For total RNA extraction, tissues were homogenized in 1 ml. of Tri Reagent (Sigma Aldrich Co., St. Louis, MO, USA), incubated 5 min at room temperature and then stored in ice. After adding 200 μl of chloroform, each sample was mixed by inversion and stored in ice for 15 min. Following a 15-min centrifugation at 12000 rpm and 4 °C, the aqueous phase was carefully removed and mixed with 500 μl of cold isopropanol. After precipitation for 45 min at −20 °C, samples were centrifuged at 12000 rpm and 4 °C for 10 min. After desalting with 70% alcohol, RNA was resuspended in 20 μl of RNAse free water. RNA was quantified with nanodrop (Nanodrop Technologies, Wilmington, Delaware, USA), and 10 μg of total RNA were reverse transcribed according to the manufacturer's protocol (Superscript II, Invitrogen, Life Technologies, Carlsbad, CA, USA) using oligo-dT primers. As a control for genomic contamination, a sample without reverse transcriptase was added to each PCR analysis.

Comparative quantification was performed by real-time PCR using the SYBR-green I fluorescence method and ROX as a passive reference dye. Stratagene MxPro TM QPCR Software and Stratagene Mx3005P equipment were used (Agilent Technologies, Santa Clara, CA, USA). For each molecule, samples were run in parallel to a standard curve that was used to transform Ct values to cDNA dilution values. TATA Box binding protein (TBP) and Cyclophilin A (Cyc A) were used as housekeeping genes because their expression was not altered by the treatment. When possible, primers were designed to anneal with two different exons to avoid genomic amplification; their sequences and amplification product sizes are specified in supplemental online digital content (Suppl. Fig. S1). All samples were run in triplicate. Melting curves controlled specificity.

2.8. Measurement of IL-1β

Animals were decapitated, and the injected and non-injected striatum were quickly dissected, snap frozen in liquid nitrogen, and stored in −80 °C until needed. Tissues were homogenized on ice in 400 μl of Tris–HCl buffer (pH 7.3) containing protease

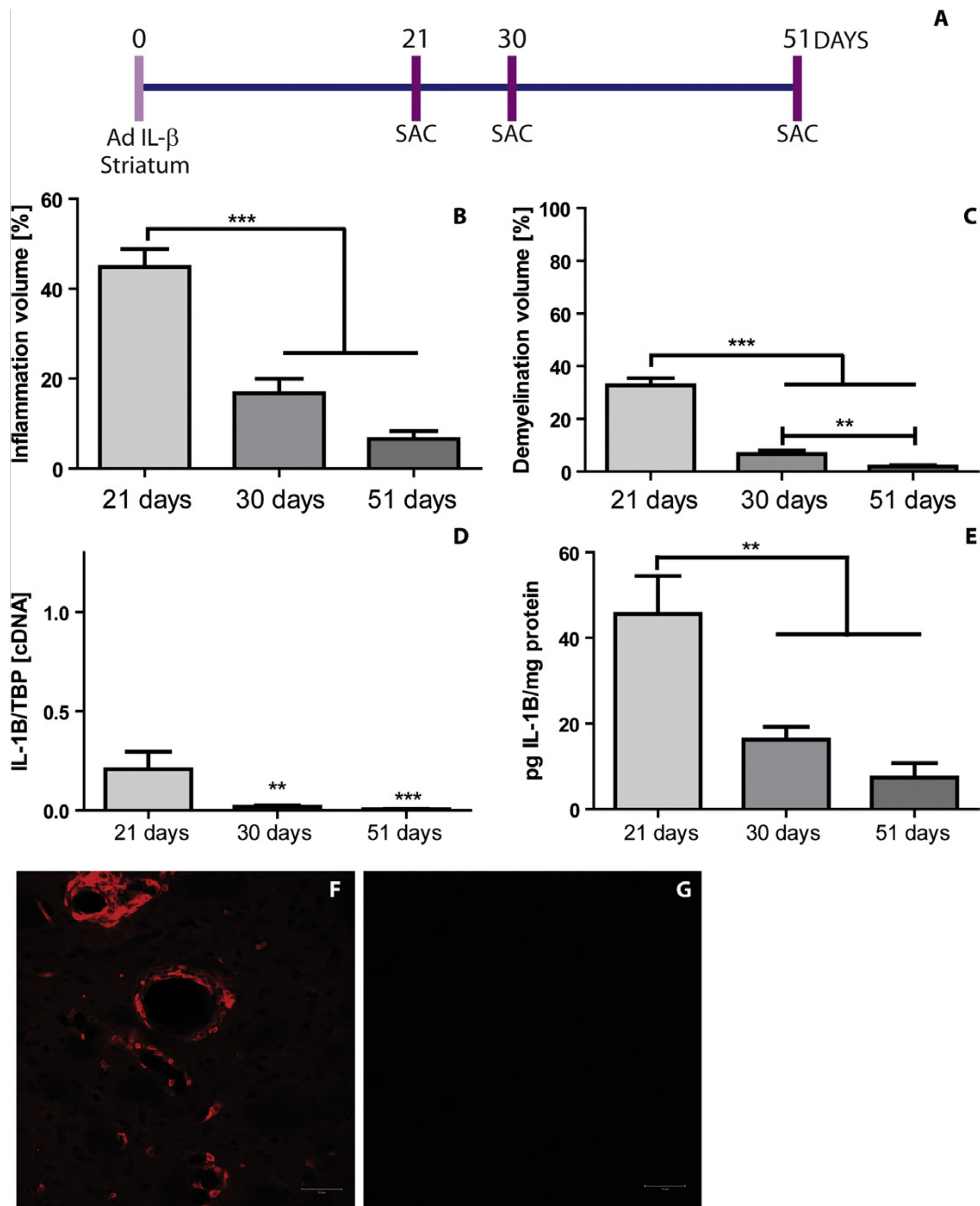

Fig. 1. Characterization of the single-injection groups: 21, 30 and 50 dpi. (A) Timeline indicating the injection day (AdIL-1β Striatum) and corresponding sacrifice day (SAC) for each of the single-injection groups. (B) Inflammation volume for each of the groups. One-way ANOVA with Bonferroni's multiple comparisons post hoc test. (C) Demyelination volume of the-single injection groups. One-way ANOVA of transformed values (squared root of proportions) with Bonferroni's multiple comparisons post hoc test. (D) Interleukin-1β (IL-1β) relative mRNA expression in the ipsilateral striatum of single-injection groups measured by real-time PCR. One-way ANOVA of Ln transformed values. (E) IL-1β protein quantification in the ipsilateral striatum of the single-injection groups. One-way ANOVA of Ln transformed values. (F) Representative photograph of MHC II immunofluorescence from the ipsilateral striatum of an animal from the single-injection 30 dpi group: MHC-II-positive cells indicate an active lesion. (G) The tissue fully recovered in the single-injection 50 dpi group, where no MHC-II signal was observed in the lesion. $^{**}p < 0.01$; $^{***}p < 0.001$. $N = 8–10$/group for histology; $N = 5–6$/group for real-time PCR and ELISA.

inhibitors (10 μg/ml aproteinin, 5 μg/ml peptastin, 5 μg/ml leupeptin, 1 mmol/L PMSF, Sigma). Homogenates were centrifuged at 10000*g* at 4 °C for 10 min, and the supernatants were then ultra-centrifuged at 40,000 rpm for 2 h. The supernatants were stored at −80 °C. The Bradford method was used to determine the total protein in each sample, and a commercial rat IL-1β ELISA kit (R&D, Minneapolis, MN) was used according to the manufacturer's instructions.

2.9. Statistical analysis

Each experiment was conducted in at least two replicates on two independent occasions. Results are expressed as means ± SEM in the different treatment groups. Comparisons between single-injection groups were performed with one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. For comparisons between double-injection groups, two-way ANO-

VA was used, followed by post hoc Bonferroni's multiple comparisons test. Finally, the unpaired Student's t-test was used to make comparisons in two grouped experimental designs. Variables were tested for normality (Kolmogorov–Smirnov) and variance homogeneity (Bartlett's test) and transformed when necessary. The minimum level of statistical significance was set at $p < 0.05$. For clarity, the number of animals (N) and the statistical analyses used in each case are addressed in the corresponding figure legend. Statistical tests were performed using Graph Pad Prism 5.00 for Windows (Graph Pad Software, San Diego California USA).

3. Results

3.1. Long-term expression of IL-1β induces reversible neutrophil recruitment and demyelination

We previously demonstrated that the long-term expression of IL-1β in the striatum induces neuroinflammation characterized by PMN neutrophil recruitment and reversible demyelination (Ferrari et al., 2004). The peak of neuroinflammation, demyelination and rat IL-1β expression detected by qRT-PCR and ELISA occurs 21 days after the adenovector injection (Fig. 1A–E). According to these data, IL-1β mRNA decreases as the lesion recovers, and IL-1β protein synthesis follows mRNA expression (Fig. 1D–E). A reduced but active lesion was observed in the single-injection group 30 dpi, where inflammation was lower than in the single-injection group 21 dpi (Fig. 1B), but MHC-II-positive cells could still be observed within the lesion (Fig. 1F). The tissue had completely recovered in the single-injection 50 dpi group, where inflammation was reduced to a small scar (Fig. 1B), and no MHC-II signal was observed in the lesion (Fig. 1G). The animals did not demonstrate signs of ongoing disease (normal fur, normal activity and movement, normal food consumption) at any time point studied. No inflammatory response was observed in animals injected once with the Ad-βGal after 21 days (see Suppl. Fig. S2A).

3.2. Re-injection of AdIL-1β when the first lesion is unresolved has a diminished effect

3.2.1. Inflammatory and demyelinating response to the long-term re-exposure to IL-1β

To study the effect of a second IL-1β stimulus on a previously injured region, we injected the AdIL-1β in the striatum 30 days after the first stimulus and analyzed the tissue 21 days after the second stimulus (see timeline in Fig. 2A). In this case, the tissue had not fully recovered from the earlier injury at the time of the second injection. The inflammatory volume showed that the response to the second IL-1β event was diminished when the first stimulus had also been IL-1β (IV = 11.51 ± 2.09), compared to the group that had been injected first with the control vector Ad-β Gal and then with the AdIL-1β (IV = 47.67 ± 3.64) ($p < 0.001$ for βGal/IL-1β vs. IL-1β /IL-1β) (Fig. 2B). The inflammatory volume of the βGal/IL-1β group was similar to that observed for the animals from the single-injection 21 dpi group (IV = 44.81 ± 4.01; Fig. 1B). A similar inflammatory response was also observed when the animals were injected with the dilution buffer as the first stimulus and AdIL-1β as the second one (see Suppl. Fig. S2B). No inflammatory response was observed when the second stimulus was Ad-βGal (Fig. 2B). None of the animals presented signs of ongoing disease (normal fur, normal activity and movement, normal food consumption).

The inflammatory response of animals injected with IL-1β/IL-1β was characterized by an inflammatory infiltrate mostly composed of microglia/macrophages and scarce neutrophils located in the blood vessels. Very few inflammatory cells were observed in the parenchyma (see inset Fig. 2G). This response is completely different from that observed for βGal/IL-1β and single-injection 21 dpi groups. The inflammatory response in animals injected with β gal/IL-1β was characterized by dilated blood vessels with a large amount of marginated PMN neutrophils and scarce microglia/macrophages. Cuffed and recruited neutrophils to the brain parenchyma were widespread throughout the entire striatum. The lesion exhibited an extensive edematous area with neutrophils and microglia/macrophages (see inset Fig 2E and F). A pattern similar to that described for βGal/IL-1β animals could be observed in the single-injection 21 dpi group.

Similar results were observed when analyzing demyelination; again, the demyelinating volume was higher in animals injected with βgal/IL-1β. Indeed, a decreased demyelination volume was observed in IL-1β/IL-1β animals compared to βGal/IL-1β ($p < 0.001$ for βgal/IL-1β vs. IL-1β/IL-1β) (Fig. 2C). Taking into consideration the differences between these two groups, we next investigated whether remyelination was similar. For that, we sacrificed the animals 50 days after the second adenovector injection and looked at semi-thin sections. Representative pictures (Suppl. Fig. S2) showed that both βGal//IL-1β and IL-1β/IL-1β animals had remyelinated axon bundles.

In accordance with what we observed for inflammation, IL-1β mRNA was highly expressed in βGal/IL-1β animals, and almost no detectable IL-1β levels were observed in the IL-1β/IL-1β group by qRT-PCR ($p < 0.001$ for βgal/IL-1β vs. IL-1β/IL-1β) (Fig. 2D).

3.2.2. Axonal damage as response to a second chronic exposure to IL-1β when the first lesion is still active

We analyzed the presence of axonal pathology using the APP antibody as a marker of axonal injury. We identified axonal damage in the lesions demonstrated by the appearance of APP positive axons either inside or in the edges of the lesions of βGal//IL-1β and IL-1β/IL-1β animals. The morphology of APP-positive axons varied from small swellings to large spheroid end bulbs and varicose fibers (Fig. 3A–B). The number of APP-positive axons correlated with inflammation and demyelination. APP-positive degenerative axons were statistically lower in IL-1β/IL-1β compared to βGal/IL-1β (Fig. 3C) ($p < 0.01$). Hence, a higher inflammatory and demyelination volume correlates with an increased number of degenerative axons. No APP-positive axons or spheroids were found in the normal white matter of either ipsi or contralateral hemispheres, confirming that APP stained only damaged axons.

3.2.3. Glial response to a second exposure to IL-1β

In an attempt to comprehend the cellular components involved in the diminished response to the second IL-1β stimulus, we used the GFAP molecular marker to analyze astrocyte reactivity and MHC II expression for microglia/macrophage response.

Astroglia and microglia response followed the course of inflammation and demyelination.

Qualitative assessment indicated that more activated astroglia were seen in βGal/IL-1β animals compared to IL-1β/IL-1β (Fig. 4B and C). The astroglial response in the βGal/IL-1β group is similar to that observed at the single-injection 21 dpi group (Fig. 4A).

Microglial response also exhibited the same pattern described for astrocytes, inflammation and demyelination response. MHC-II-positive cells were statistically higher in βGal/IL-1β compared to IL-1β/IL-1β ($p < 0.001$ for βGal/IL-1β vs. IL-1β/IL-1β) (Fig. 4E–G), demonstrating a decreased microglial response to the second stimulus. There was no difference in MHC-II-positive cells between the IL-1β/IL-1β and IL-1β/βGal groups. Animals in the βGal/IL-1β had significantly more MHC II-positive cells than the ones in the βGal/βGal group ($p < 0.001$ for βGal/IL-1β vs. βGal/βGal) (Fig. 4G).

Fig. 2. A second injection of interleukin-1β (IL-1β) decreases neuroinflammation in the first experiment. (A) Experimental animals were re-injected in the striatum when the first inflammatory event was still unresolved (30 days) and sacrificed 21 days after the second stimulus was administered. (B) Rats in the IL-1β/IL-1β group had less inflammation volume than animals from the βGal/IL-1β group. Two-way ANOVA with Bonferroni's multiple comparisons post hoc test. (C) The same result was observed for demyelination volume. Two-way ANOVA of transformed values (squared root of proportions), with Bonferroni's multiple comparison post hoc test. (D) IL-1β mRNA levels: the IL-1β/IL-1β group had a diminished response to the second inflammatory stimulus. Two-way ANOVA with Bonferroni's multiple comparison post hoc test. Representative pictures of the inflammatory lesion in the single- injection 21 dpi. group (E), βGal/IL-1β (F), and IL-1β/IL-1β groups (G). Insets in each photograph are higher magnifications (100*x*) where the relative amount of polymorphonuclear neutrophils (PMN) (black arrowheads) and macrophages (white arrows) can be observed. Red Oil-O stained sections of representative animals in the single-injection 21 dpi. (H), βGal/IL-1β (I), and IL-1β/IL-1β groups (J). $^{***}p < 0.001$. $N = 6–8$/group for histology; $N = 6$/group for real-time PCR. Scale bars: (E–J) 1 mm.

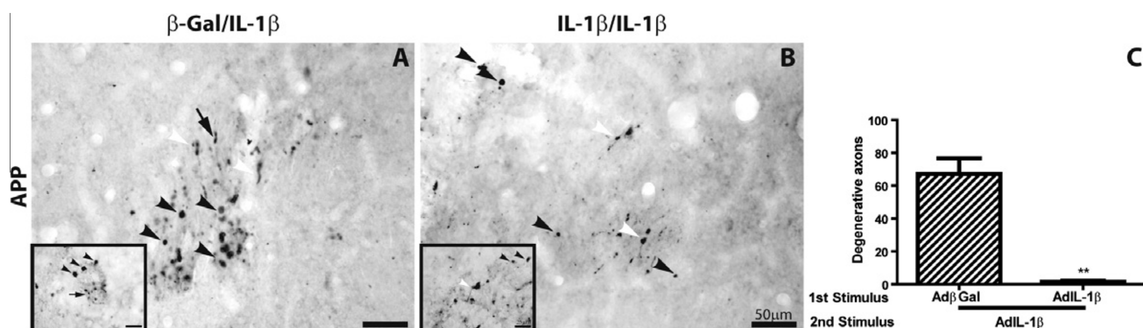

Fig. 3. Axonal damage in the first experiment. Axonal injury was identified as APP-positive axons in both βGal/IL-1β (A), and IL-1β/IL-1β groups. The morphology of APP-positive axons varied from small swellings (white arrowheads) to large spheroids end bulbs (black arrowheads) and varicose fibers (black arrows). (C) APP-positive degenerative axons were statistically lower in IL-1β/IL-1β compared to βGal/IL-1β. Unpaired t test with Welch's correction, $^{**}p < 0.01$. $N = 4–5$/group. Scale bars: (A and B) 50 μm; insets 25 μm.

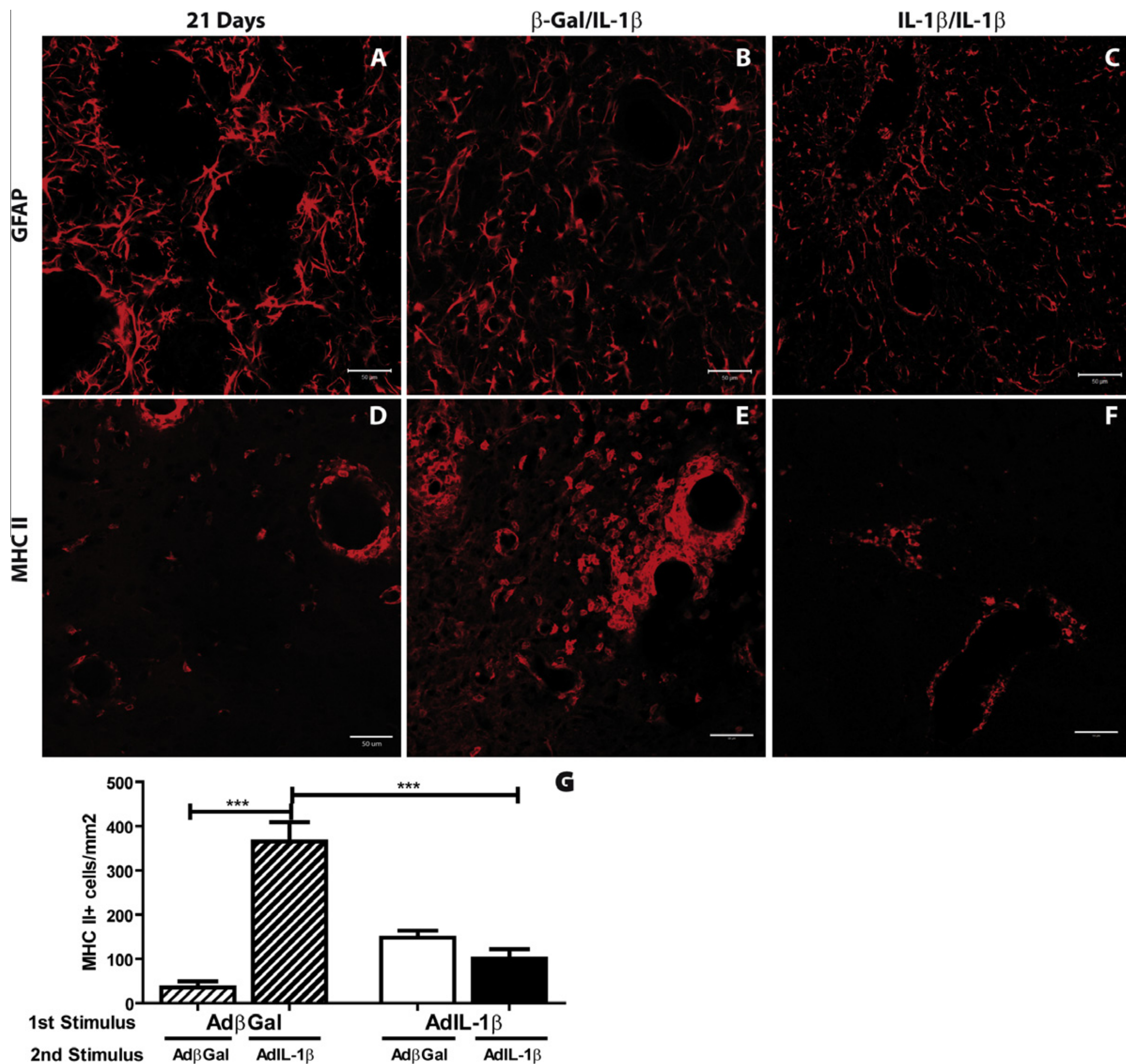

Fig. 4. Glial response to a second IL-1β stimulus. (A–C) Glial fibrillary acidic protein (GFAP) immunofluorescence shows that rats injected with AdIL-1β 30 days after the first lesion (C) have less astrocytic activation than the single-injection 21 dpi group (A) and βGal/IL-1β (B) groups. (D–F) Immunofluorescence against the macrophage/microglia marker MHC II (major histocompatibility complex class II). Representative micrographs show less MHC II-positive cells in the IL-1β/IL-1β group. (G) Quantification of MHC II-positive cells. Two-way ANOVA of transformed values (squared root of proportions), with Bonferroni's multiple comparisons post hoc test, $^{***}p < 0.001$. N = 5–8/group. Scale bars: (A–F) 50 μm.

3.3. Re-injection of AdIL-1β when the first inflammatory response is resolved has no differential effect

3.3.1. Inflammatory and demyelinating response to the second chronic IL-1β stimulus

Taking into consideration the results from the first set of experiments, we wondered if the diminished response to the second AdIL-1β was related to the fact that it was administered when the first lesion was only partially recovered. Therefore, as a second approach to relapsing events in the injured brain, we injected the second stimulus once the induced damage by the first stimulus in the striatum was completely restored, that is, 50 days after the first injection. The tissue was analyzed 21 days after the second stimulus, as in the first experimental paradigm (see timeline Fig. 5A). No statistical differences in the volume of inflammation were observed between βGal/IL-1β compared to IL-1β/IL-1β animals (p = 0.47 for interaction, $p > 0.05$ for βGal/IL-1β vs. IL-1β/IL-1β) (Fig. 5B). None of the animals presented signs of ongoing disease (normal fur, normal activity and movement, normal food consumption). Moreover, the inflammatory response was similar in both studied groups (Fig. 5E and F), characterized by the presence of dilated vessels filled with marginated neutrophils and scarce macrophages (insets Fig. 5E and F). Cuffed and recruited PMN neutrophils were widespread throughout the striatal parenchyma. In addition, a large edematous area could also be observed in animals of both groups. Qualitatively, the inflammatory response is similar to that observed in the single-injection 21 dpi group (Fig. 5D), indicating that the tissue reaction is different when the stimulus is still active or if the first inflammatory event is fully restored. Taken together, these results suggest that when a second chronic IL-1β stimulus is applied 50 days after the first one, the dynamics and features of the tissue response are similar to those after the first injection. However, re-stimulation when the first lesion has recovered only partially (30 days) induces a diminished and qualitatively different response.

A similar response was observed when demyelination was analyzed (Fig. 5C). In this case, βGal/IL-1β animals were statistically different from IL-1β/IL-1β injected animals ($p < 0.05$ for βGal/IL-1β vs.

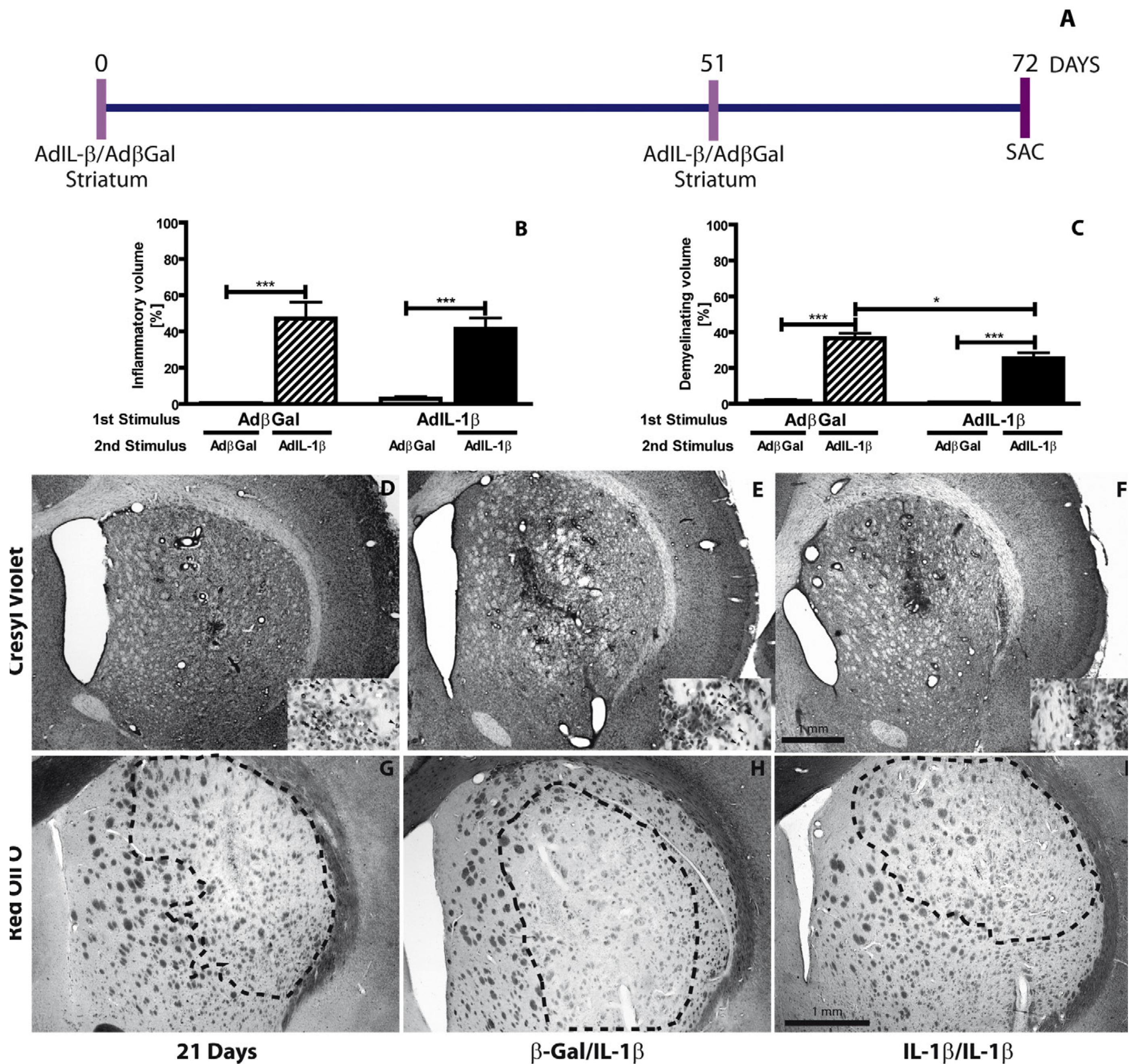

Fig. 5. A second IL-1β stimulus, once the first lesion is resolved, has an effect that is similar to the first lesion. (A) In this experiment, the second pro-inflammatory stimulus was administered 50 days after the first one, once the primary lesion had completely resolved. Animals were sacrificed 21 days after the second intrastriatal injection. (B and C) Animals in the βGal/IL-1β and IL-1β/IL-1β groups presented no difference in the inflammatory volume (Two-way ANOVA with Bonferroni's multiple comparisons post hoc test) (B), and little difference in demyelinating volume (Two-way ANOVA of transformed values [squared root of proportions], with Bonferroni's multiple comparison post hoc test) (C). Representative pictures of the inflammatory lesion in the single-injection 21 dpi group (D), βGal/IL-1β (E), and IL-1β/IL-1β groups (F). Insets in each photograph are at higher magnification (100×), where the relative amount of polymorphonuclear neutrophils (PMN) (black arrowheads) and macrophages (white arrows) can be observed. Red Oil-O stained sections for representative animals in the single-injection 21 dpi group (G), βGal/IL-1β (H), and IL-1β/IL-1β groups (I). $^{***}p < 0.001$; $^{*}p < 0.05$. $N = 5$/group. Scale bars: (E–J) 1 mm.

IL-1β/IL-1β). However, the difference between these two groups was lower compared to animals injected 30 days after the first stimulus (see Fig. 2).

3.3.2. *Axonal damage as a response to the second IL-1β stimulus applied once the primary lesion is resolved*

The presence of degenerative axons was evaluated in the secondary lesions from the 50–21 dpi experiment. As was described previously, the morphology varied from varicose fibers to spheroids (Fig. 6A and B). The number of APP-positive axons was lower in IL-1β/IL-1β compared to βGal/IL1β Fig. 6C $p < 0.05$). The number of injured axons correlated with the trend shown for demyelination, where the βGal/IL-1β group exhibited higher demyelination volume. In particular, higher demyelination volume involved increased axonal degeneration.

3.3.3. *Glial response to the re-exposure to IL-1β once the tissue damage is resolved*

The glial response followed the course of inflammation and demyelination. GFAP immunoreactivity was similar in IL-1β/IL-1β

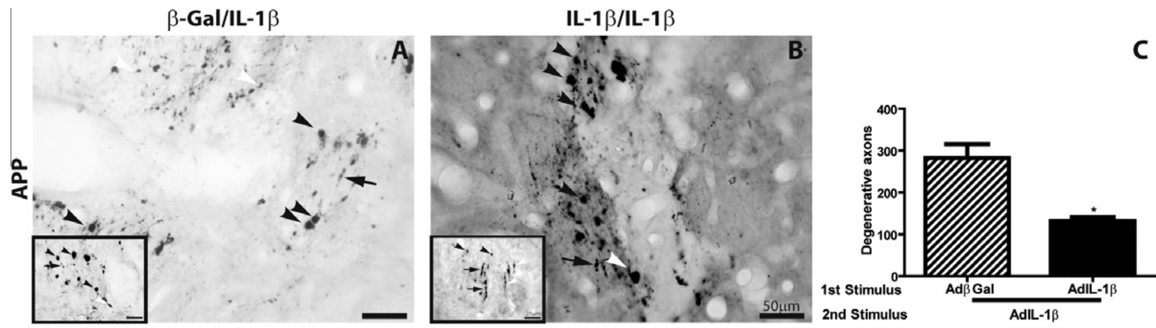

Fig. 6. Axonal damage in the second experimental design. Axonal injury was identified as APP positive axons in both βGal/IL-1β (A), and IL-1β/IL-1β groups. The morphology of APP positive axons varied from small swellings (white arrowheads) to large spheroids end bulbs (black arrowheads) and varicose fibers (black arrows). (C) APP-positive degenerative axons were statistically lower in IL-1β/IL-1β compared to βGal/IL-1β. Unpaired t test, $^{*}p < 0.01$. $N = 4$/group. Scale bars: (A and B) 50 μm; insets 25 μm.

Fig. 7. Glial response to a second chronic IL-1β event, when the first lesion has been resolved. (A–C) Representative photographs of astroglial activation (as evidenced by glial fibrillary acidic protein [GFAP]). There is no difference between the single-injection 21 dpi group (A), βGal/IL-1β (B), and IL-1β/IL-1β groups (C). (D–F) Similar result seen for the presence of macrophages/microglia stage 4 cells; representative micrographs show no difference between the three groups. (G) Quantification of MHC II (major histocompatibility complex class II)-positive cells showed no difference between the βGal/IL-1β and IL-1β/IL-1β groups (G). Two-way ANOVA of transformed values (squared root of proportions), with Bonferroni's multiple comparisons post hoc test. $^{**}p < 0.01$; $^{***}p < 0.001$. $N = 5$–6/group. Scale bars: (E–J) 50 μm.

compared to βGal/IL-1β-injected animals in the single-injection 21 dpi group (Fig. 7A–C). The microglial response exhibited a similar pattern: MHC-II positive cells increased in βGal/IL-1β and in IL-1β/IL-1β animals compared to βGal/βGal and IL-1β/βGal respectively ($p < 0.05$ for βGal/IL-1β vs. IL-1β/IL-1β and for βGal/βGal vs. IL-1β/βGal) (Fig. 7E–G). Moreover, there was no difference between

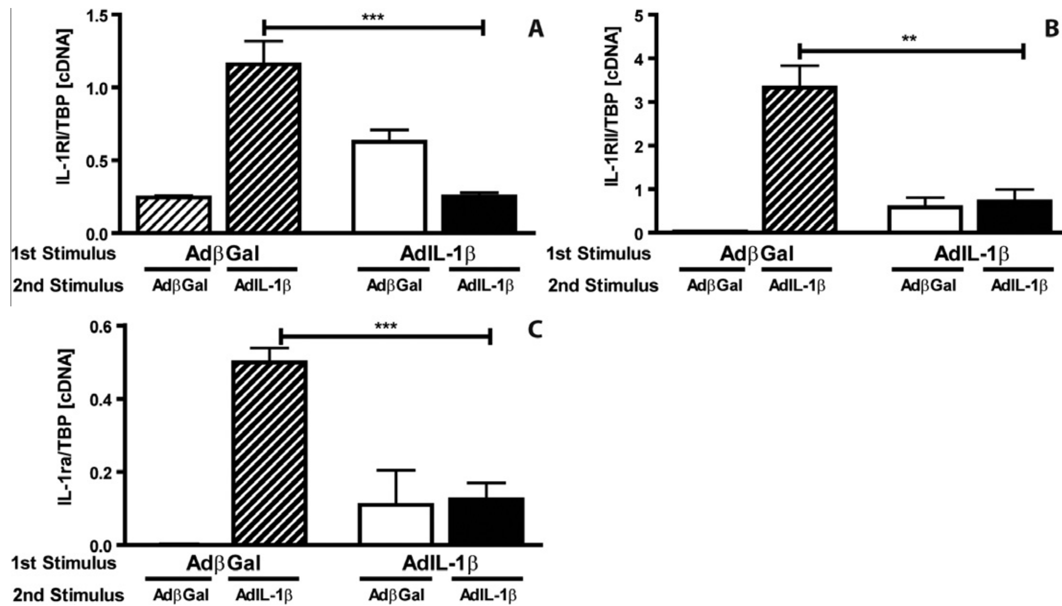

Fig. 8. Variation in the mRNA levels of interleukin-1 (IL-1) receptors and IL-1 receptor antagonist (IL-1ra) mRNA levels were measured for the first experimental design: where the second pro-inflammatory stimulus is administered with the first lesion yet unresolved. Values are expressed as relative cDNA over housekeeping (TATA binding protein [TBP]) expression. (A) IL-1 receptor I (IL-1RI), two-way ANOVA of Ln transformed values. (B) IL-1 receptor II (IL-1RII), two-way ANOVA of Ln transformed values. (C) IL-1ra, two-way ANOVA. $^{**}p < 0.01$; $^{***}p < 0.001$. $N = 5–6$/group.

the groups that received the AdIL-1β as a second stimulus and the single-injection 21 dpi group (Fig. 7D–F). It appeared that the MHC-II expression response was independent of the presence of the first stimulus at this time point.

3.4. IL-1 receptor response after the second IL-1β stimulus when the tissue damage is unresolved

IL-1β exerts its effects via binding to the 80 kDa IL-1 receptor I (IL-1RI). A second IL-1 receptor, the type II receptor (IL-1RII), is also able to bind IL-1β but acts as a decoy receptor. Another component of the IL-1 family is the IL-1 receptor antagonist (IL-1ra), which is a highly selective competitive receptor antagonist that binds the functional IL-1RI but does not elicit a response. All of these members of the IL-1 family are endogenously expressed in the CNS and can be modified under several pathological conditions (Rothwell and Luheshi, 2000).

We hypothesized that the diminished effect observed after the second injection of AdIL-1β could be due to differential behavior in the expression of the IL-1 receptors and/or IL-1ra. To answer this, we studied the mRNA expression of these molecules by qRT-PCR at different time points and in different experimental groups.

Initially, we investigated whether differential expression of the receptors at the time of the second injection (30 or 50 days) could explain the reduced response to the second AdIL-1β when injected after 30 days. The single-injection 30 and 50 dpi groups demonstrated that there was no difference in the expression of IL-1RI ($p = 0.81$), IL-1RII ($p = 0.66$) or IL-1ra ($p = 0.87$) (see Suppl. Fig. S4).

Given that there were no differences in the expression of IL-1 receptors or IL-1ra between the two time points chosen for the second injection, we wondered if the differential expression of these molecules was evident only in the presence of the second stimulus. We only analyzed the first experiment, where unexpected diminished response was seen in the IL-1β/IL-1β group. All samples were taken 21 days after the second adenovector administration. The experimental group that received the control adenovirus on both occasions (βGal/βGal) demonstrated that there was no effect of the adenoviral vector *per se* because the expression of both receptors and IL-1ra exhibited a similar response to the second injection to that found in single-injection 50 dpi animals once the lesion was completely recovered (Fig. 8 and Suppl. Fig. S4).

Animals that were injected with the inflammatory stimulus only on the second occasion (βGal/IL-1β) showed a significant up-regulation of IL-1RI, indicating that in response to the AdIL-1β, there is a high number of functional receptors available to transduce the IL-1 signal (Fig. 8A). However, there was also an increase in the expression of the molecules that regulate the IL-1β-induced inflammatory activity (IL-1RII and IL-1ra) (Fig. 8B and C). This pattern of expression may be due to the fact that we are looking at the peak of the inflammatory response, and therefore, both the reinforcing and the undermining elements are present. Interestingly, the response of both receptors and IL-1ra is completely different when the first stimulus is the AdIL-1β. In this case, the IL-1β/IL-1β animals showed a statistically decreased response compared to the βGal/IL-1β group ($p < 0.001$ for IL-1RI between βGal/IL-1β; $p < 0.01$ for IL-1RII between βGal/IL-1β; $p < 0.001$ for IL-1ra between βGal/IL-1β), demonstrating that the presence of the first inflammatory stimulus reduced the response to the second AdIL-1β (Fig. 8A–C).

4. Discussion

In this study, we demonstrated that the response to a second pro-inflammatory stimulus is altered depending on whether the primary lesion is still active or completely resolved. If the primary lesion is active when the second stimulus is applied, the secondary inflammatory response to the chronic expression of IL-1β is decreased. This diminished response to the re-exposure of AdIL-1β is seen compared to both the single-injection 21 dpi group, which had no second stimulus, and to the group that received the control adenovector in the first injection. Demyelination followed a similar pattern to that described for inflammation: there was less demyelination 21 days after the second stimulus only when both the first and second stimuli were IL-1β. The demyelinating lesion was more profound when the first stimulus was the Ad-βGal and the second

stimulus was the AdIL-1β. However, the diminished effect as a consequence of the re-exposure to IL-1β was not seen if the second injection was applied once the first lesion was restored.

We have generated an alternative, focally induced demyelination model that allows us to study the effect of a single molecule of the innate immune system. This model could be a useful tool for the dissection of mechanisms that either enhance or counteract remyelination in the absence of T cells. Several animal models of demyelination have helped in understanding the pathophysiology of MS (Denic et al., 2011). As reviewed in Blakemore and Franklin (Blakemore and Franklin, 2008), animal models can be divided in two groups: those which attempt to replicate the disease as accurately as possible, such as virus-induced encephalomyelitis and EAE, and others that provide a more reductionist approach that allows studying specific aspects of the disease (e.g., ethidium bromide, lysolecithin, cuprizone). In this model, the repeated IL-1β stimulus demonstrated that the state of the primary lesion determines the course of the second one. This model would allow assigning functional relevance to individual pathological features of repeated demyelinating events. Even though the proposed model does not replicate the complex RRMS pathology, it may contribute to the study of specific inflammatory molecular (IL-1β) and cellular (PMN neutrophils) mediators and their downstream components without the complexity of the environment described in MS.

4.1. A second burst of IL-1β decreased the inflammatory response if it occurred when the first lesion was still active

The neuroinflammatory response of a second administration of the AdIL-1β decreased when the first stimulus was also AdIL-1β. This effect could not be observed when the first stimulus was the Ad-βGal; indeed, βGal/IL-1β animals had significantly increased injury compared to animals that received IL-1β as first stimulus.

The refractoriness to re-stimulation with the same cytokine has been studied previously and is known as tachyphylaxis (Colditz, 1985; Cybulsky et al., 1988), a phenomenon of local desensitization of any tissue to a specific inflammatory agent (Cybulsky et al., 1988). Tachyphylaxis could be viewed as a mechanism involved in the down-regulation of an inflammatory event as a response to chronically high levels of a cytokine (Cannon and Dinarello, 1985). In particular, this phenomenon was described for the most important inflammatory cytokines IL-1β and TNF-α (Colditz, 1985; Cybulsky et al., 1988; Pober et al., 1986; Ulich et al., 1990), and some of these authors have described a decreased neutrophil infiltration as a result of this desensibilization. Our results showed a reduction in infiltrated neutrophils: the inflammatory infiltrate after the second burst of IL-1β was characterized by macrophages and scarce neutrophils, a totally different response to that observed when a unique dose of IL-1β was injected. However, the possibility of the involvement of other mediators in this phenomenon cannot be ruled out. Although a single cytokine is induced experimentally, we cannot dismiss the possible up-regulation of other mediators. In fact, we have previously shown that the long-term expression of IL-1β did not induce TNF-α (Ferrari et al., 2004), but we did not study the up-regulation of other cytokines.

The decreased response to the second IL-1β stimulus may be due to differential activity of the IL-1 receptors and/or its receptor antagonist, IL-1ra. The behavior of the receptors and other members of the IL-1 family could be synergistic with the diminished response to the second stimulus, as IL-1β induces its effect through IL-1RI, whereas IL-1RII and IL-1ra counteract the action of IL-1. We found that IL-1RI, IL-RII and IL-1ra showed reduced expression in response to the second injection of the AdIL-1β, 30 days after the first one.

IL-1 family members are endogenously expressed in the CNS. Their expression can be modified under several pathological conditions (Rothwell and Luheshi, 2000). Differential regulation of IL-1 receptors as a response to central or peripheral pro-inflammatory stimuli has been described by several authors (Docagne et al., 2005; Ericsson et al., 1995; Stern et al., 2000; Turrin et al., 2001; Wang et al., 1997). Therefore, IL-1 family members could act synergistically with the decreased inflammatory response after a second IL-1β stimulus. The expression of functional IL-1R in the CNS is mostly found on endothelial cells and in neurons of very few specific structures of the brain (Ching et al., 2007; Ericsson et al., 1995). In particular, IL-1RII is known to be expressed in neutrophils (Warabi, 2007). The actions of IL-1β in the brain are primarily mediated by endothelial cells, and endothelial IL-1RI is reported to mediate the recruitment of neutrophils across the BBB into the brain parenchyma (Ching et al., 2005; Li et al., 2011). Thus, if a re-exposure to IL-1β overexpression induced a down-regulation of IL-1RI transcription and if endothelial IL-1RI was required to recruit leucocytes, then it would not be surprising if the second burst of IL-1β in the brain did not induce the expected inflammatory response. Further analysis regarding the pattern of IL-1RI expression (endothelial cells, microglial cells, astrocytes or neurons) could help in understanding the mechanisms behind the mild effect of the IL-1β stimulus.

4.2. A second burst of IL-1β when the first lesion was still active decreased demyelination

Although remyelination occurs in MS plaques, the failure of this process is a primary feature of MS pathology (Patrikios et al., 2006; Prineas et al., 1993). Our results demonstrated a decreased demyelinating response after the second IL-1β stimulus. These results may be attributed to several causes:

4.2.1. Previous inflammatory events may have a positive effect on myelin

The presence of IL-1β during the first event may have induced the expression of pro-myelinating molecules (Mason et al., 2001; Watzlawik et al., 2010), which, in turn, partially modified the demyelinating effect of the second IL-1β stimulus. In accordance with this hypothesis, priming with IL-1 prior to EAE induction has been seen to reduce the symptoms of the disease (Huitinga et al., 2000).

4.2.2. The presence of activated microglia from the previous lesion impaired demyelination

Activated microglia may be required to facilitate remyelination, as they are involved in the clearance of the myelin debris and because they can stimulate the production of growth factors that improve the remyelinating environment (Kotter et al., 2001, 2005; Li et al., 2005).

4.2.3. Reduced presence of MHC-II activated macrophages/microglia

MHC-II activated microglia induce cytokine secretion, which enhances demyelination (Hiremath et al., 2008). Our results also demonstrated that MHC-II expression decreased in IL-1β/IL-1β animals, where demyelination is milder compared to controls.

4.2.4. Decreased and qualitatively different infiltrate induced a milder demyelinated lesion

Neutrophil recruitment is known to be important in the demyelinating process (Carlson et al., 2008; Ferrari et al., 2004; Naegele et al., 2011). Primed neutrophils have been described in MS patients (Naegele et al., 2011). Moreover, CXC chemokine receptor type 2 (CXCR2)-positive neutrophils are known to contribute to the development of lesions in the EAE and cuprizone animal

models (Carlson et al., 2008; Liu et al., 2010). In addition, the presence of neutrophils and IL-1β expression were demonstrated in the onset of the pathology in EAE (Soulika et al., 2009). Therefore, neutrophil recruitment is a sign of very early spinal cord inflammation. Whether its quick disappearance is restricted to EAE or also occurs in acute MS lesions remains to be studied (Soulika et al., 2009).

4.3. A second burst of IL-1β decreased the number of injured axons

Axonal damage is a hallmark of both MS and experimental animal models (Haines et al., 2011; Irvine and Blakemore, 2006; Kornek et al., 2000; Moreno et al., 2011; Trapp et al., 1998). We evaluated the presence of axonal damage in the proposed model, demonstrating that the number of degenerative axons decreased in the IL-1β/IL-1β group compared to the βGal/IL-1β group when the primary lesion was still active. The axonal damage correlated with the course of inflammation, MHC-II expression and demyelination. The relationship between the presence of IL-1β and the accumulation of APP was also demonstrated in an animal model of diffuse axonal injury (Yang et al., 2011). The correlation between injured axons, inflammation and demyelination was previously shown by several authors (Bitsch et al., 2000; Haines et al., 2011; Kuhlmann et al., 2002; Mancardi et al., 2001; Raine and Cross, 1989). Even if acute axonal injury can be seen in areas with no demyelination, a positive correlation with the number of macrophages and CD8-positive lymphocytes was demonstrated. However, there was no correlation between axonal damage and tumor necrosis factor alpha (TNF-α) or inducible nitric oxide synthase expression (Bitsch et al., 2000; Haines et al., 2011). In addition, in EAE, a temporal and spatial coordination between neutrophil-enriched inflammatory infiltrate, IL-1β expression, and the onset of axonal injury was observed (Soulika et al., 2009). Therefore, axonal damage could be an early sign associated with both inflammation and demyelination (Mancardi et al., 2001), as demonstrated in this study.

In summary, IL-1β expression within the lesions could facilitate remyelination or at least diminish the demyelination process in relapsing events. The inhibition of IL-1β activity in a MS lesion should be careful analyzed, especially considering that anti-IL-1 treatments were not demonstrated to be beneficial either in MS patients or in the EAE animal model (Warabi, 2007). Irrespective of the mechanism involved, our model of AdIL-1β re-administration during an active lesion provides an experimental set-up to identify key molecules related to the diminished toxic effects of a second IL-1β mediated injury to the brain parenchyma.

4.4. A second burst of IL-1β did not affect inflammatory or demyelinating responses when the first lesion was fully restored

The presence of a previous but completely restored lesion did not influence the response to a second IL-1β stimulus. Our results demonstrated that if the relapsing event was far enough to allow the lesion to be completely repaired, the second response mimicked the first one. Growth and anti-inflammatory factors induced during the first inflammatory event may be required for the diminished secondary response, as noted previously.

Accordingly, a model of repeated and focal demyelination within the CNS, in which the lesion is allowed to repair itself, is not characterized by impaired remyelination or oligodendrocyte progenitor cell depletion (Penderis et al., 2003). Interestingly, in MS lesions, Bramow et al. (2010) suggested that the first episode of demyelination and remyelination leaves the damaged area vulnerable to a new episode of demyelination when comparing primary and secondary progressive disease (Bramow et al., 2010). These contradictory results could be explained by the fact that we generated a reductionist model based on the repeated long-term expression of IL-1β, which lacks the complexity of progressive human lesions as described by Bramow et al., 2010.

Axonal damage seemed slightly decreased as a response to a second stimulus when the first lesion is resolved. Fewer injured axons were observed in the IL-1β/IL-1β group.

Interestingly, these results correlated with demyelination, where a slight DV increase was observed in βGal/IL-1β group when compared with the IL-1β/IL-1β group. As previously noted, it is possible that both inflammation and the degree of demyelination influenced the number of damaged axons in our model. However, these data contradict other data obtained from MS patients, which demonstrated that axonal damage correlates with inflammation but not with active demyelination (Bitsch et al., 2000; Haines et al., 2011). In addition in RRMS and SPMS, axonal damage is higher in early stages of the disease compared to older lesions. However, in PPMS, no differences were found over time (Kuhlmann et al., 2002). These data suggest that axonal damage could also be influenced by the state of the previous lesion.

Thus, the fact that a previous inflammatory event took place does not necessarily determine the response to a second one; it is possible that the level of activity of the first lesion defines the reaction to a second one.

5. Conclusions

Repeated IL-1β induced inflammatory episodes diminished the secondary response depending on whether the primary lesion was active. We demonstrated that if the primary lesion was active, the reinjection of the AdIL-1β produced a diminished secondary lesion, but if the primary lesion was resolved, then the secondary response was similar to the first one.

The limited knowledge about interaction between the immune and the nervous system is still the main impediment in developing therapeutic tools. Indeed, even if MS is described as a T-cell-mediated autoimmune disease, the early infiltration of neutrophils has been related to the onset of the pathology in both MS and experimental animal models. Clinical therapies demonstrate that anti-inflammatory and immunosuppressive treatments may sometimes provide temporary relief of the symptoms, but no available treatment has shown to slow down the progression of the pathology or stop the disease. Animal models of neuroinflammation should allow studying specific inflammatory mechanisms involved in relapsing/remitting episodes. The analysis of repeated episodes in the CNS is crucial to achieve a better comprehension of relapsing and remitting events. Moreover, studying the impact of specific components of the inflammatory response on degenerative processes should help develop new therapeutic tools in demyelinating diseases.

6. Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2012.07.007.

References

Aggelakis, K., Zacharaki, F., Dardiotis, E., Xiromerisiou, G., Tsimourtou, V., Ralli, S., Gkaraveli, M., Bourpoulas, D., Rodopoulou, P., Papadimitriou, A., Hadjigeorgiou, G., 2010. Interleukin-1B and interleukin-1 receptor antagonist gene polymorphisms in Greek multiple sclerosis (MS) patients with bout-onset MS. Neurol. Sci. 31, 253–257.

Andersson, P.B., Perry, V.H., Gordon, S., 1992. Intracerebral injection of proinflammatory cytokines or leukocyte chemotaxins induces minimal myelomonocytic cell recruitment to the parenchyma of the central nervous system. J. Exp. Med. 176, 255–259.

Anthony, D.C., Ferguson, B., Matyzak, M.K., Miller, K.M., Esiri, M.M., Perry, V.H., 1997. Differential matrix metalloproteinase expression in cases of multiple sclerosis and stroke. Neuropathol. Appl. Neurobiol. 23, 406–415.

Argaw, A.T., Zhang, Y., Snyder, B.J., Zhao, M.L., Kopp, N., Lee, S.C., Raine, C.S., Brosnan, C.F., John, G.R., 2006. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. J. Immunol. 177, 5574–5584.

Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T., Bruck, W., 2000. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain 123 (Pt 6), 1174–1183.

Blakemore, W.F., Franklin, R.J., 2008. Remyelination in experimental models of toxin-induced demyelination. Curr. Top. Microbiol. Immunol. 318, 193–212.

Blond, D., Campbell, S.J., Butchart, A.G., Perry, V.H., Anthony, D.C., 2002. Differential induction of interleukin-1beta and tumour necrosis factor-alpha may account for specific patterns of leukocyte recruitment in the brain. Brain Res. 958, 89–99.

Borzani, I., Tola, M.R., Caniatti, L., Collins, A., De Santis, G., Luiselli, D., Mamolini, E., Scapoli, C., 2010. The interleukin-1 cluster gene region is associated with multiple sclerosis in an Italian Caucasian population. Eur. J. Neurol. 17, 930–938.

Bramow, S., Frischer, J.M., Lassmann, H., Koch-Henriksen, N., Lucchinetti, C.F., Sorensen, P.S., Laursen, H., 2010. Demyelination versus remyelination in progressive multiple sclerosis. Brain 133, 2983–2998.

Brosnan, C.F., Cannella, B., Battistini, L., Raine, C.S., 1995. Cytokine localization in multiple sclerosis lesions: correlation with adhesion molecule expression and reactive nitrogen species. Neurology 45, S16–21.

Cannon, J.G., Dinarello, C.A., 1985. Increased plasma interleukin-1 activity in women after ovulation. Science 227, 1247–1249.

Carlson, T., Kroenke, M., Rao, P., Lane, T.E., Segal, B., 2008. The Th17-ELR+CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. J. Exp. Med. 205, 811–823.

Codarri, L., Fontana, A., Becher, B., 2010. Cytokine networks in multiple sclerosis: lost in translation. Curr. Opin. Neurol. 23, 205–211.

Colditz, I.G., 1985. Kinetics of tachyphylaxis to mediators of acute inflammation. Immunology 55, 149–156.

Comi, G., Filippi, M., 2005. Clinical trials in multiple sclerosis: methodological issues. Curr. Opin. Neurol. 18, 245–252.

Cybulsky, M.I., McComb, D.J., Movat, H.Z., 1988. Neutrophil leukocyte emigration induced by endotoxin. Mediator roles of interleukin 1 and tumor necrosis factor alpha 1. J. Immunol. 140, 3144–3149.

Ching, S., He, L., Lai, W., Quan, N., 2005. IL-1 type I receptor plays a key role in mediating the recruitment of leukocytes into the central nervous system. Brain Behav. Immun. 19, 127–137.

Ching, S., Zhang, H., Belevych, N., He, L., Lai, W., Pu, X.A., Jaeger, L.B., Chen, Q., Quan, N., 2007. Endothelial-specific knockdown of interleukin-1 (IL-1) type 1 receptor differentially alters CNS responses to IL-1 depending on its route of administration. J. Neurosci. 27, 10476–10486.

Denic, A., Johnson, A.J., Bieber, A.J., Warrington, A.E., Rodriguez, M., Pirko, I., 2011. The relevance of animal models in multiple sclerosis research. Pathophysiology 18, 21–29.

Depino, A.M., Earl, C., Kaczmarczyk, E., Ferrari, C., Besedovsky, H., del Rey, A., Pitossi, F.J., Oertel, W.H., 2003. Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. Eur. J. Neurosci. 18, 2731–2742.

Dinarello, C.A., 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27, 519–550.

Dincic, E., Zivkovic, M., Stankovic, A., Obradovic, D., Alavantic, D., Kostic, V., Raicevic, R., 2006. Association of polymorphisms in CTLA-4, IL-1ra and IL-1beta genes with multiple sclerosis in Serbian population. J. Neuroimmunol. 177, 146–150.

Docagne, F., Campbell, S.J., Bristow, A.F., Poole, S., Vigues, S., Guaza, C., Perry, V.H., Anthony, D.C., 2005. Differential regulation of type I and type II interleukin-1 receptors in focal brain inflammation. Eur. J. Neurosci. 21, 1205–1214.

Dujmovic, I., Mangano, K., Pekmezovic, T., Quattrocchi, C., Mesaros, S., Stojsavljevic, N., Nicoletti, F., Drulovic, J., 2009. The analysis of IL-1 beta and its naturally occurring inhibitors in multiple sclerosis: The elevation of IL-1 receptor antagonist and IL-1 receptor type II after steroid therapy. J. Neuroimmunol. 207, 101–106.

Edwards, L.J., Sharrack, B., Ismail, A., Tumani, H., Constantinescu, C.S., 2011. Central inflammation versus peripheral regulation in multiple sclerosis. J. Neurol. 258, 1518–1527.

Ericsson, A., Liu, C., Hart, R.P., Sawchenko, P.E., 1995. Type 1 interleukin-1 receptor in the rat brain: distribution, regulation, and relationship to sites of IL-1-induced cellular activation. J. Comp. Neurol. 361, 681–698.

Ferrari, C.C., Depino, A.M., Prada, F., Muraro, N., Campbell, S., Podhajcer, O., Perry, V.H., Anthony, D.C., Pitossi, F.J., 2004. Reversible demyelination, blood-brain barrier breakdown, and pronounced neutrophil recruitment induced by chronic IL-1 expression in the brain. Am. J. Pathol. 165, 1827–1837.

Furlan, R., Bergami, A., Brambilla, E., Butti, E., De Simoni, M.G., Campagnoli, M., Marconi, P., Comi, G., Martino, G., 2007. HSV-1-mediated IL-1 receptor antagonist gene therapy ameliorates MOG(35–55)-induced experimental autoimmune encephalomyelitis in C57BL/6 mice. Gene Ther. 14, 93–98.

Hagemeier, K., Bruck, W., Kuhlmann, T., 2012. Multiple sclerosis - remyelination failure as a cause of disease progression. Histol. Histopathol. 27, 277–287.

Haines, J.D., Inglese, M., Casaccia, P., 2011. Axonal damage in multiple sclerosis. Mt Sinai J. Med. 78, 231–243.

Hartung, H.P., Kieseier, B.C., Hemmer, B., 2005. Purely systemically active anti-inflammatory treatments are adequate to control multiple sclerosis. J. Neurol. 252 (Suppl 5), v30–v37.

Hauser, S.L., Doolittle, T.H., Lincoln, R., Brown, R.H., Dinarello, C.A., 1990. Cytokine accumulations in CSF of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. Neurology 40, 1735–1739.

Hiremath, M.M., Chen, V.S., Suzuki, K., Ting, J.P., Matsushima, G.K., 2008. MHC class II exacerbates demyelination in vivo independently of T cells. J. Neuroimmunol. 203, 23–32.

Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., Lassmann, H., Wekerle, H., 2005. The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. Ernst Schering Res Found Workshop, 23–38.

Holman, D.W., Klein, R.S., Ransohoff, R.M., 2011. The blood-brain barrier, chemokines and multiple sclerosis. Biochim. Biophys. Acta 1812, 220–230.

Howe, C.L., Mayoral, S., Rodriguez, M., 2006. Activated microglia stimulate transcriptional changes in primary oligodendrocytes via IL-1beta. Neurobiol. Dis. 23, 731–739.

Huitinga, I., van der Cammen, M., Salm, L., Erkut, Z., van Dam, A., Tilders, F., Swaab, D., 2000. IL-1beta immunoreactive neurons in the human hypothalamus: reduced numbers in multiple sclerosis. J. Neuroimmunol. 107, 8–20.

Irvine, K.A., Blakemore, W.F., 2006. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. J. Neuroimmunol. 175, 69–76.

Kanwar, J.R., 2005. Anti-inflammatory immunotherapy for multiple sclerosis/experimental autoimmune encephalomyelitis (EAE) disease. Curr. Med. Chem. 12, 2947–2962.

Kieseier, B.C., Hemmer, B., Hartung, H.P., 2005. Multiple sclerosis–novel insights and new therapeutic strategies. Curr. Opin. Neurol. 18, 211–220.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., Lassmann, H., 2000. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am. J. Pathol. 157, 267–276.

Kotter, M.R., Setzu, A., Sim, F.J., Van Rooijen, N., Franklin, R.J., 2001. Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. Glia 35, 204–212.

Kotter, M.R., Zhao, C., van Rooijen, N., Franklin, R.J., 2005. Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression. Neurobiol. Dis. 18, 166–175.

Kuhlmann, T., Lingfeld, G., Bitsch, A., Schuchardt, J., Bruck, W., 2002. Acute axonal damage in multiple sclerosis is most extensive in early disease stages and decreases over time. Brain 125, 2202–2212.

Li, Q., Powell, N., Zhang, H., Belevych, N., Ching, S., Chen, Q., Sheridan, J., Whitacre, C., Quan, N., 2011. Endothelial IL-1R1 is a critical mediator of EAE pathogenesis. Brain Behav. Immun. 25, 160–167.

Li, W.W., Setzu, A., Zhao, C., Franklin, R.J., 2005. Minocycline-mediated inhibition of microglia activation impairs oligodendrocyte progenitor cell responses and remyelination in a non-immune model of demyelination. J. Neuroimmunol. 158, 58–66.

Lindquist, S., Hassinger, S., Lindquist, J.A., Sailer, M., 2011. The balance of pro-inflammatory and trophic factors in multiple sclerosis patients: effects of acute relapse and immunomodulatory treatment. Mult. Scler. 17, 851–866.

Liu, L., Belkadi, A., Darnall, L., Hu, T., Drescher, C., Cotleur, A.C., Padovani-Claudio, D., He, T., Choi, K., Lane, T.E., Miller, R.H., Ransohoff, R.M., 2010. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. Nat. Neurosci. 13, 319–326.

Loma, I., Heyman, R., 2011. Multiple sclerosis: pathogenesis and treatment. Curr. Neuropharmacol. 9, 409–416.

Lucchinetti, C.F., Bruck, W., Lassmann, H., 2004. Evidence for pathogenic heterogeneity in multiple sclerosis. Ann. Neurol. 56, 308.

Ludigs, K., Parfenov, V., Du Pasquier, R.A., Guarda, G., 2012. Type I IFN-mediated regulation of IL-1 production in inflammatory disorders. Cell. Mol. Life Sci.

Mancardi, G., Hart, B., Roccatagliata, L., Brok, H., Giunti, D., Bontrop, R., Massacesi, L., Capello, E., Uccelli, A., 2001. Demyelination and axonal damage in a non-human primate model of multiple sclerosis. J. Neurol. Sci. 184, 41–49.

Mason, J.L., Suzuki, K., Chaplin, D.D., Matsushima, G.K., 2001. Interleukin-1beta promotes repair of the CNS. J. Neurosci. 21, 7046–7052.
Matsuki, T., Nakae, S., Sudo, K., Horai, R., Iwakura, Y., 2006. Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis. Int. Immunol. 18, 399–407.
Merson, T.D., Binder, M.D., Kilpatrick, T.J., 2010. Role of cytokines as mediators and regulators of microglial activity in inflammatory demyelination of the CNS. Neuromol. Med. 12, 99–132.
Moreno, B., Jukes, J.P., Vergara-Irigaray, N., Errea, O., Villoslada, P., Perry, V.H., Newman, T.A., 2011. Systemic inflammation induces axon injury during brain inflammation. Ann. Neurol. 70, 932–942.
Naegele, M., Tillack, K., Reinhardt, S., Schippling, S., Martin, R., Sospedra, M., 2011. Neutrophils in multiple sclerosis are characterized by a primed phenotype. J. Neuroimmunol.
Patrikios, P., Stadelmann, C., Kutzelnigg, A., Rauschka, H., Schmidbauer, M., Laursen, H., Sorensen, P.S., Bruck, W., Lucchinetti, C., Lassmann, H., 2006. Remyelination is extensive in a subset of multiple sclerosis patients. Brain 129, 3165–3172.
Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates. Academic Press, Orlando, FL.
Penderis, J., Shields, S.A., Franklin, R.J., 2003. Impaired remyelination and depletion of oligodendrocyte progenitors does not occur following repeated episodes of focal demyelination in the rat central nervous system. Brain 126, 1382–1391.
Perry, V.H., Cunningham, C., Holmes, C., 2007. Systemic infections and inflammation affect chronic neurodegeneration. Nat. Rev. Immunol. 7, 161–167.
Pober, J.S., Bevilacqua, M.P., Mendrick, D.L., Lapierre, L.A., Fiers, W., Gimbrone Jr., M.A., 1986. Two distinct monokines, interleukin 1 and tumor necrosis factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. J. Immunol. 136, 1680–1687.
Popescu, B.F., Lucchinetti, C.F., 2012. Pathology of demyelinating diseases. Annu. Rev. Pathol. 7, 185–217.
Prineas, J.W., Barnard, R.O., Revesz, T., Kwon, E.E., Sharer, L., Cho, E.S., 1993. Multiple sclerosis. Pathology of recurrent lesions. Brain 116 (Pt 3), 681–693.
Raine, C.S., Cross, A.H., 1989. Axonal dystrophy as a consequence of long-term demyelination. Lab. Invest. 60, 714–725.
Rothwell, N.J., Luheshi, G.N., 2000. Interleukin 1 in the brain: biology, pathology and therapeutic target. Trends Neurosci. 23, 618–625.
Ruggiero, V., 2011. Involvement of IL-1R/TLR signalling in experimental autoimmune encephalomyelitis and multiple sclerosis. Curr. Mol. Med..
Sarial, S., Shokrgozar, M.A., Amirzargar, A., Shokri, F., Radfar, J., Zohrevand, P., Arjang, Z., Sahraian, M.A., Lotfi, J., 2008. IL-1, IL-1R and TNFalpha gene polymorphisms in Iranian patients with multiple sclerosis. Iran. J. Allergy Asthma Immunol. 7, 37–40.
Saura, J., Pares, M., Bove, J., Pezzi, S., Alberch, J., Marin, C., Tolosa, E., Marti, M.J., 2003. Intranigral infusion of interleukin-1beta activates astrocytes and protects from subsequent 6-hydroxydopamine neurotoxicity. J. Neurochem. 85, 651–661.
Schiffenbauer, J., Streit, W.J., Butfiloski, E., LaBow, M., Edwards 3rd, C., Moldawer, L.L., 2000. The induction of EAE is only partially dependent on TNF receptor signaling but requires the IL-1 type I receptor. Clin. Immunol. 95, 117–123.
Shaftel, S.S., Carlson, T.J., Olschowka, J.A., Kyrkanides, S., Matousek, S.B., O'Banion, M.K., 2007. Chronic interleukin-1beta expression in mouse brain leads to leukocyte infiltration and neutrophil-independent blood brain barrier permeability without overt neurodegeneration. J. Neurosci. 27, 9301–9309.
Simi, A., Tsakiri, N., Wang, P., Rothwell, N.J., 2007. Interleukin-1 and inflammatory neurodegeneration. Biochem. Soc. Trans. 35, 1122–1126.
Soulika, A.M., Lee, E., McCauley, E., Miers, L., Bannerman, P., Pleasure, D., 2009. Initiation and progression of axonopathy in experimental autoimmune encephalomyelitis. J. Neurosci. 29, 14965–14979.
Stern, E.L., Quan, N., Proescholdt, M.G., Herkenham, M., 2000. Spatiotemporal induction patterns of cytokine and related immune signal molecule mRNAs in response to intrastriatal injection of lipopolysaccharide. J. Neuroimmunol. 109, 245–260.
Sutton, C., Brereton, C., Keogh, B., Mills, K.H., Lavelle, E.C., 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J. Exp. Med. 203, 1685–1691.
Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mork, S., Bo, L., 1998. Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. 338, 278–285.
Tsukada, N., Miyagi, K., Matsuda, M., Yanagisawa, N., Yone, K., 1991. Tumor necrosis factor and interleukin-1 in the CSF and sera of patients with multiple sclerosis. J. Neurol. Sci. 104, 230–234.
Turrin, N.P., Gayle, D., Ilyin, S.E., Flynn, M.C., Langhans, W., Schwartz, G.J., Plata-Salaman, C.R., 2001. Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. Brain Res. Bull. 54, 443–453.
Ulich, T.R., Guo, K.Z., Irwin, B., Remick, D.G., Davatelis, G.N., 1990. Endotoxin-induced cytokine gene expression in vivo. II. Regulation of tumor necrosis factor and interleukin-1 alpha/beta expression and suppression. Am. J. Pathol. 137, 1173–1185.
Wang, X., Barone, F.C., Aiyar, N.V., Feuerstein, G.Z., 1997. Interleukin-1 receptor and receptor antagonist gene expression after focal stroke in rats. Stroke 28, 155–161 (discussion 161–152).
Warabi, Y., 2007. Role of IL-1 and potential therapies in multiple sclerosis. Drug Discovery Today: Ther. Strategies 4, 19–24.
Watzlawik, J., Warrington, A.E., Rodriguez, M., 2010. Importance of oligodendrocyte protection, BBB breakdown and inflammation for remyelination. Expert Rev. Neurother. 10, 441–457.
Yang, X.F., Wang, H., Wen, L., 2011. From myelin debris to inflammatory responses: a vicious circle in diffuse axonal injury. Med. Hypotheses 77, 60–62.
Ysrraelit, M.C., Gaitan, M.I., Lopez, A.S., Correale, J., 2008. Impaired hypothalamic-pituitary-adrenal axis activity in patients with multiple sclerosis. Neurology 71, 1948–1954.
Zeis, T., Graumann, U., Reynolds, R., Schaeren-Wiemers, N., 2008. Normal-appearing white matter in multiple sclerosis is in a subtle balance between inflammation and neuroprotection. Brain 131, 288–303.
Zivadinov, R., Pirko, I., 2012. Advances in understanding gray matter pathology in multiple sclerosis: are we ready to redefine disease pathogenesis? BMC Neurol. 12, 9.