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Review

The mechanism of action of glatiramer acetate in multiple sclerosis and beyond

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ABSTRACT

In multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE), the immune system reacts again self myelin constitutes in the central nervous system (CNS), initiating a detrimental inflammatory cascade that leads to demyelination as well as axonal and neuronal pathology. The amino acid copolymer glatiramer acetate (GA, Copaxone) is an approved first-line treatment for MS that has a unique mode of action. Accumulated evidence from EAE-induced animals and from MS patients indicates that GA affects various levels of the innate and the adaptive immune response, generating deviation from the pro-inflammatory to the anti-inflammatory pathway. This review aims to provide a comprehensive perspective on the diverse mechanism of action of GA in EAE/MS, in particular on the *in situ* immunomodulatory effect of GA and its ability to generate neuroprotective repair consequences in the CNS. In view of its immunomodulatory activity, the beneficial effect of GA in various models of other autoimmune related pathologies, such as immune rejection and inflammatory bowel disease (IBD) is noteworthy.

Contents

Abbreviations: MS, Multiple sclerosis; EAE, Experimental autoimmune encephalomyelitis; CNS, Central nervous system; BBB, Blood brain barrier; MBP, Myelin basic protein; MOG, Myelin oligodendrocyte glycoprotein; PLP, Myelin proteolipid protein; GA, Glatiramer acetate; APC, Antigen presenting cells; MHC, Major histocompatibility; TCR, T-cell receptor; APL, Altered peptide ligand; Th, T-helper; Tregs, T-regulatory cells; Foxp3, Forkhead box P3; IL, Interleukin; IFN, interferon; TGF, Transforming growth factor; NT, Neurotrophin; BDNF, Brain derived neurotrophic factor; IGF, Insulin-like growth factor; MRI, Magnetic resonance imaging; MTR, Magnetization transfer ratio; DTI, Diffusion tensor imaging; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy; OPCs, Oligodendrocyte progenitor cells; NPCs, Neuronal progenitor cells GVHD, Graft versus host disease; IBD, Inflammatory bowel disease; CD, Crohn's disease.

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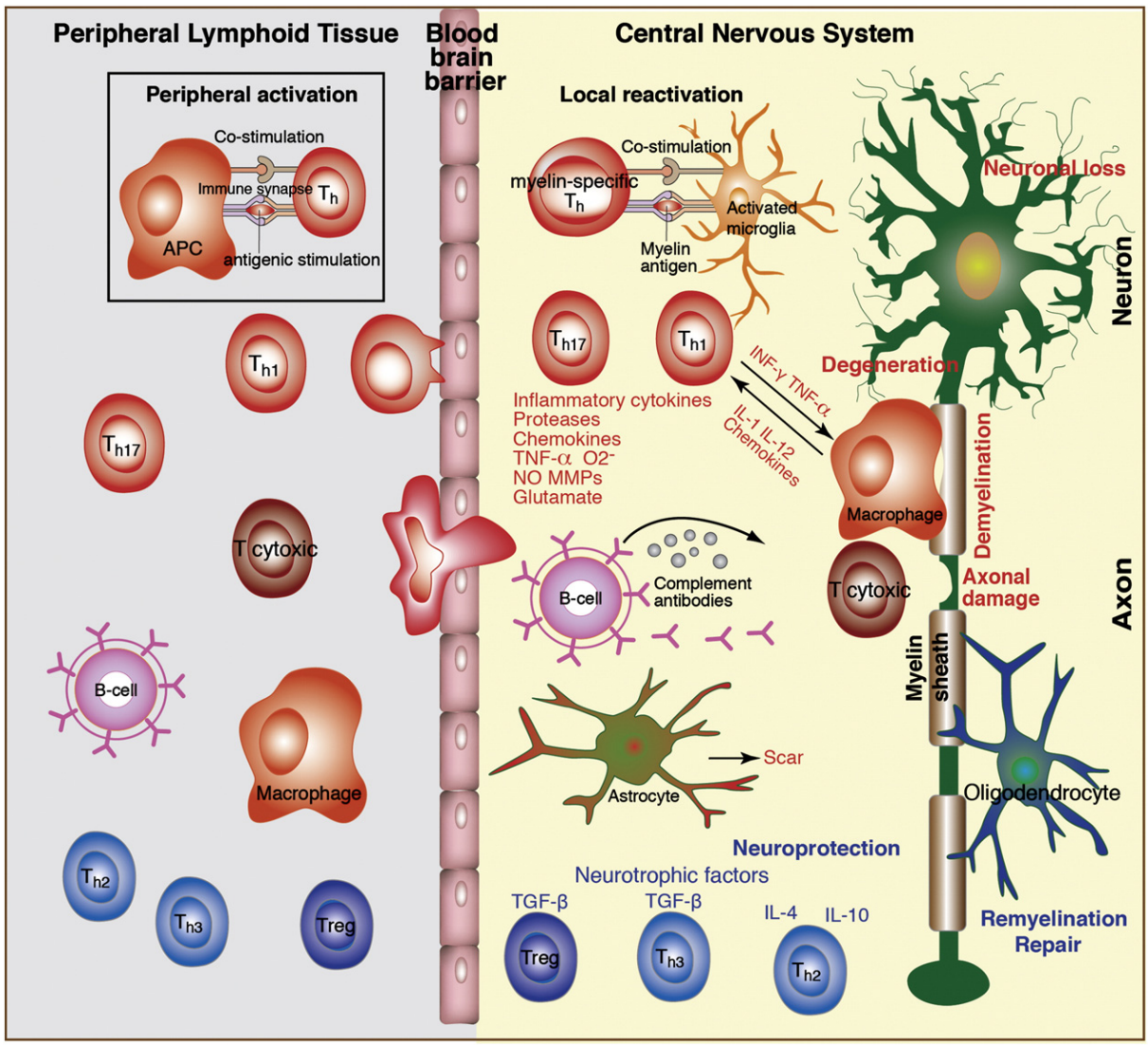
1. Introduction — the pathology of multiple sclerosis and the development of glatiramer acetate

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and a leading cause for disability in young adults, with female predominance [1]. The most typical clinical progression pattern is a phase of relapsing and remitting symptoms (relapsing remitting MS, RRMS) that frequently develops to a progressive disease course (secondary progressive MS, SPMS). A fraction of patients shows disease progression from the beginning (primary progressive MS, PPMS) which represents a somewhat different pathology [2]. Essential data on MS has been obtained by using

the animal model - experimental autoimmune encephalomyelitis (EAE), induced by immunization with myelin antigens such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or myelin proteolipid protein (PLP). EAE induction by the different encephalitogenic antigens or their peptides in susceptible animal strains leads to development of various disease forms (acute, relapsing remitting or progressive) that mimic the different patterns of MS. In spite of certain discrepancies, the EAE model has been proven as an essential tool for testing novel therapies as well as for the elucidation of their mechanism of action [3].

Traditionally MS has been considered an autoimmune disease, in which the immune system reacts against the body's own constituents, in this case against the myelin in the CNS, initiating a vicious inflammatory cascade [4,5]. Several myelin encephalitogenic epitopes were identified in MS and EAE, such as the peptides comprising the 84–102 amino acids of MBP, the 35–55 amino acids of MOG, and the 139–151 amino acids of PLP [6]. In addition, epitope spreading toward multiple encephalitogenic determinants occurs with disease progression [7]. Immune cells of both the adaptive and innate systems are involved in the inflammatory network that mediates the disease [8,9]. T-helper (Th)-1 and Th-17 cells, cytotoxic T-cells, B-cells and macrophages enter the CNS through the blood brain barrier (BBB) and the plexus choroideus, secreting pro-inflammatory cytokines, chemokines and other inflammatory substances. CNS resident cells, such as microglia and astrocytes, are stimulated upon tissue damage and further facilitate T-cells activation and scar formation. All these cell populations maintain the inflammatory milieu and mediate tissue injury, leading to multifocal demyelination, impairment of nerve fiber conductivity, as well as loss of axons, neurons and oligodendrocytes (illustrated in Fig. 1). Defective T-cell apoptosis also plays a role in the development of the disease and its chronic evolution [10]. Besides the detrimental role of the immune system in MS/EAE pathology, immune cells such as Th2/3 and T-regulatory cells mediate anti-inflammatory protective pathways that suppress the disease. During the recent years it has become clear that MS is a multifaceted heterogeneous disease with different patterns of tissue damage [2]. Thus, in addition to the detrimental inflammation, widespread axonal and neuronal pathology is a central component of MS [9,11]. There is evidence that axonal transection and neuronal damage, occur even at early disease stage, supporting neurodegenerative disease course [12]. Diffuse abnormalities in the grey matter and in normal-appearing brain tissue are currently recognized as central components of MS [13].

Until about 20 years ago, only symptomatic treatments were available for MS patients. Indiscriminate immunosuppression with its



hazardous risks and severe side effects was also attempted in order to restrain the excessively active immune system [14]. The development of the first disease-modifying therapies (DMTs) and their approval in the 1990s has altered the natural history of the disease. These are the recombinant versions of interferon (IFN)-β [15,16] and the synthetic copolymer glatiramer acetate (GA). The later is the subject matter of this review.

In the late 1960s, Sela, Arnon, Teitelbaum and colleagues at the Weizmann Institute in Israel, conducting basic research on the immunological properties of synthetic polymers and copolymers, made a serendipitous drug discovery [17]. Hypothesizing that synthetic polypeptides with amino acids analogous to those of the autoantigen MBP will induce EAE, they designed several such copolymeric mixtures. However, none of the copolymers was encephalitogenic, instead, they were found to be protective against EAE. Copolymer 1 (Cop 1), now called glatiramer acetate (GA; Copaxone), randomly composed of L-alanine, L-lysine, L-glutamic acid and L-tyrosine, in a molar ratio of 4.2:3.4:1.4:1.0, proved to have the greatest activity in ameliorating EAE. Furthermore, the effect of GA was not restricted to a particular species, disease type, or encephalitogen used for EAE induction, and it ameliorated disease in guinea pigs, rabbits, various mouse strains, and in two kinds of monkeys [18]. Since this discovery, extensive preclinical research, pivotal controlled clinical trials, and long-term assessments, established the efficacy and the safety of GA as a disease-modifying therapy for MS [19–22]. GA at a daily subcutaneous dose of 20 mg has been found to alter the natural history of RRMS by reducing the relapse rate and affecting disability. These consequences were consistent with magnetic resonance imaging (MRI) findings from various clinical centers [23]. Currently, GA is one of the most widely prescribed first-line treatments for RRMS.

GA is the first and so far the only therapeutic agent ever to have a copolymer as its active ingredient. Due to the complexity and variability of its polypeptides mixture, a clear definition of its active component has not been established. Moreover, it has been claimed that the overall "pool" of various amino acid sequences facilitates multiple ways of action and is thus required for its therapeutic activity. In some cases diverse findings led to contrasting conclusions and controversies as to which is the "key" process mediated by this drug. Due to these unique characteristics, GA has been often referred to as a drug with an "unclear" or "elusive" mechanism of action. Nevertheless, over the last 4 decades, results from ours as well as from other laboratories, obtained in multiple *in vitro* and *in vivo* systems, clarified the immunological mechanism of action of GA (Table 1). Furthermore, our recent studies revealed neuroprotective repair consequences of GA treatment in the CNS. The purpose of this review is to provide a comprehensive overview and elucidate the current understanding on the diverse mechanism of action of GA in EAE/MS. In addition findings on the effect of GA in additional autoimmune related pathologies are presented.

2. Peripheral immunomodulatory mechanisms

The immunological mechanism by which GA induces its therapeutic effect was extensively investigated over the years in EAE-induced animals and in MS patients. These studies indicated that GA acts by immunomodulating various levels of the immune response, which differ in their degree of specificity.

The initial prerequisite step is the binding of GA to major histocompatibility (MHC) class II molecules. *In vitro* studies on murine and human antigen presenting cells (APCs) indicated that GA undergoes a rapid and efficient ("promiscuous") binding to various MHC class II molecules, and even displaces other peptides from the MHC binding groove [24]. This competition for binding to the histocompatibility molecules can prevent the presentation of other antigens and hinder their T-cell activation. Several groups have demonstrated that GA induces generalized alterations of various types of APCs, such as dendritic cells and monocytes, so that they preferentially stimulate protective anti-inflammatory responses. Hence, dendritic cells from GA-treated MS patients produced less TNF-α, less IL-12, and more IL-10, compared to those of untreated patients [25]. GA induced a broad inhibitory effect on monocyte reactivity [26], and promoted the development of anti-inflammatory type II monocytes characterized by increased secretion of IL-10 and TGF-β, as well as by decreased production of IL-12 and TNF [27]. Furthermore, GA-induced type II monocytes were able to transfer protection from EAE [27]. This modulation on the level of the innate immune system is the least specific step in the immunological processes affected by GA, and can be beneficial for the inhibition of response to several myelin antigens. In addition, in the case of the MS immunodominant encephalitogenic epitope of MBP (comprising amino acids 82–100), GA acts in a strictly antigen-specific manner. Using MBP 82–100 specific T-cell clones from MS patients and from EAE-induced mice, it was shown that GA inhibits their activation by T-cell receptor (TCR) antagonism, acting as an altered peptide ligand (APL) [28].

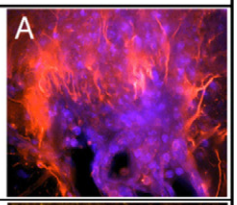
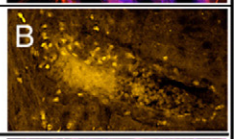
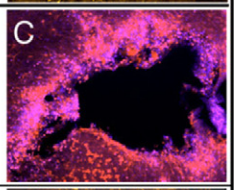
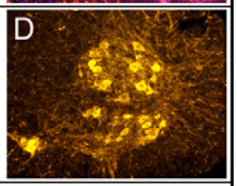
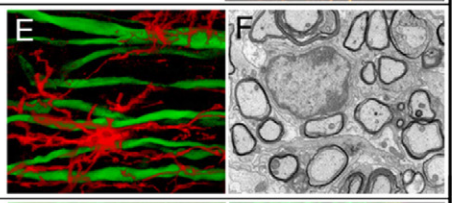
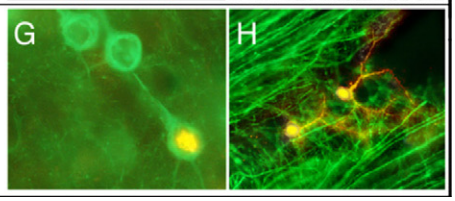
Most studies attribute the primary mechanism for GA activity to its ability to skew T-cell response from the pro-inflammatory to the anti-inflammatory pathway. It has been long known that GA-treated animals develop specific T cells in their peripheral immune system [18]. Furthermore, T-cell lines and hybridomas could be isolated from spleens of mice rendered unresponsive to EAE by GA [29]. Both cell types acted as modulatory suppressor cells, as they inhibited the response of MBP-specific effector cells *in vitro*, and adoptively transferred protection against EAE *in vivo*. T-cell lines/clones induced by GA progressively polarized toward the T-helper (Th) 2/3 subtype, secreting high amounts of anti-inflammatory cytokines such as interleukin (IL) -4, -5, -10, and transforming growth factor (TGF)-β, until they completely lost the ability to secrete Th1 pro-inflammatory cytokines such as INF-γ [30]. In several cases, the secretion of Th2/3 cytokines by the GA-induced T-cell lines was obtained in response to either GA or MBP. Other myelin antigens such as PLP and MOG could not activate the GA-specific T-cells, yet EAE induced by PLP and MOG could be suppressed by GA as well as by GA-induced T-cells. These results are indicative of "bystander suppression mechanisms" induced by GA [31] which are especially important in view of the epitope spreading occurring in MS/EAE [6,7]. A shift from a pro-inflammatory Th1-biased cytokine profile toward anti-inflammatory Th2-biased profile was observed also in GA-treated MS patients [32,33], indicating that such GA-specific cells are involved in the therapeutic effect of this drug in MS.

The effect of GA on the T-cell subset is not restricted to the Th2/3 versus Th1 pathways. Several studies demonstrated the effect of GA on Th-17 and on T-regulatory (Tregs) cells, which are pivotal effectors of disease exacerbation and suppression, respectively [34]. Thus, it was shown that *in vitro* exposure of peripheral $CD4^+$ T-cells, from healthy humans or from GA-immunized mice, to GA, resulted in elevated level of Tregs, through activation of the transcription factor forkhead box P3 (Foxp3). Furthermore, GA treatment led to increased Foxp3 expression in $CD4^+$ T-cells of MS patients, whose Foxp3 level was low at baseline [35]. Pretreatment of mice with GA, before EAE induction, resulted in increased Foxp3 expression on Tregs during the mild disease which was developed subsequently. These Tregs were more effective in EAE prevention than Tregs isolated from untreated mice [36]. GA treatment to EAE-induced mice resulted in elevation of Tregs and reduction of Th-17 cells, as demonstrated by the detection of their specific transcription factors, Foxp3 and RORγt, respectively, on both the mRNA and the protein levels [37,38]. In addition to its effect on the $CD4^+$ T-cell subset, GA affects also $CD8^+$ T-cells. The regulatory function of these cells which was impaired in MS untreated patients, was drastically improved after several month of GA treatment, to the levels observed in healthy individuals [39,40].

B-cells are involved in both the pathogenesis and modulation of MS and EAE, by secreting antibodies and cytokines as well as by their efficient antigen presentation [41]. GA-treated patients

Table 1

Immunomodulatory and neuroprotective effects of glatiramer acetate in MS and EAE

Peripheral immunomodulation	Competition for MHC	Promiscuous binding to various MHC class II molecules, displacement of myelin antigens from the MHC binding groove [24].
	Alteration of the innate immune response	Inhibitory effect on monocytes reactivity, deviation of dendritic cells and monocytes to produce less TNF-α and IL-12, more IL-10 and TGF-β, and to stimulate Th2 anti-inflammatory responses [25–27].
	T-cell receptor antagonism	Inhibition of the activation of T-cells specific to the 82-100 epitope of MBP [28].
	T-cell deviation	Induction of specific Th2/3-cells that secrete high amounts of IL-4, IL-5, IL-10, and TGF-β [29–34]. Elevation of the prevalence and function of T-regulatory cells, activation of the transcription factor Foxp3 [35,36]. Reduction of Th-17 cells and their transcription factors RORγt [37,38]. Improvement of the regulatory function of $CD8^+$ T-cells [39,40].
	Modification of B-cells	Induction of antibodies with beneficial rather than neutralizing activity [42]. Bias toward production of anti-inflammatory cytokines such as IL-10 [43]. Down-regulating of chemokine receptors [44].
Immunomodulation in the CNS	Secretion of anti-inflammatory cytokine	GA-specific Th2/3 cells cross the BBB and secrete in situ anti-inflammatory cytokines. Bystander expression of IL-10 and TGF-β by resident astrocyte and microglia. Reduction in the overall expression of IFN-γ [46,47,49,50]. 
	Th-17 and T-regulatory cells	Decrease in the amount of Th-17 cells. Increase in T-regulatory cells [38]. 
Neuroprotection	Elevation of neurotrophic factors	GA-specific T-cells express BDNF in the brain [49]. Restoration of the impaired expressions of BDNF, NT-3, NT4, IGF-1, and IGF-2 [53–55,59]. 
	Reduced CNS injury	Prevention of demyelination [60–62]. Preservation of retinal ganglion cells [63]. Inhibition of motor neuron loss [62]. Preservation of brain tissue integrity by the MRI parameters MTR and DTI [65]. Reduced formation of "black holes" [69]. Increase in NAA:Cr ratio [70]. 
	Remyelination	Augmented remyelination [62]. Increased proliferation, maturation and survival of oligodendrocyte progenitor cells and their accumulation in the lesions [55,61]. 
	Neurogenesis	Elevated proliferation, migration and differentiation of neuronal progenitor cells and their recruitment into injury sites [64]. 

Inserts demonstrate *in situ* consequences of GA in the CNS: A. GA-specific T-cells (blue) expressing IL-10 (red); B. infiltration of Foxp3 expressing T-cells (yellow); C. GA-specific T-cells (blue) expressing BDNF (red); D. intact formation of motor neurons; E. oligodendrocyte progenitor cells (red) extending processes between transected fibers (green); F. remyelination zone of newly myelinated axons surrounding an oligodendrocyte; G. BrdU expressing neuronal progenitors (yellow) born during GA/BrdU injection in a lesion

developed GA-specific antibodies that did not interfere with GA activity in terms of MHC binding or T-cell stimulation, and eventually declined 6 month after treatment initiation [42]. The high proportion of IgG1 versus IgG2 antibody isotype as well as the switch to IgG4 observed in the treated patients reflected the Th1 to Th2 shift induced by GA. Moreover relapse-free patients developed higher GA-antibody titers, suggesting a beneficial rather than neutralizing activity of anti-GA antibodies. Recently, it has been demonstrated that the effect of GA on B cells contributes to its therapeutic activity, leading to their biases toward the production of anti-inflammatory cytokines such as IL-10 [43]. These B cells ameliorated EAE by down-regulation of chemokine receptors associated with trafficking of inflammatory cells into the CNS [44].

The above cumulative findings from many laboratories established the broad immunomodulatory effect of GA on various subsets of the immune system.

3. Immunomodulation in the CNS

The significant outcome of a therapy is obviously its effect in the diseased organ – in the case of MS – the ability to induce effective modulation of the pathological processes in the CNS. The initial immunological activity of GA apparently occurs in the periphery (at the injection sites and in the corresponding draining lymph nodes). An indication for dendritic uptake of GA and its delivery to the CNS has been demonstrated [45]. However, since GA is rapidly degraded in the periphery, it is unlikely that its sufficient amounts can reach to the CNS to compete effectively with myelin antigens or initiate specific immune response. Most views thus currently accept that the therapeutic effect of GA is mediated by the GA-induced immune cells that penetrate the CNS. The presence in the CNS of GA-specific T-cells, induced in the periphery either by parenteral or by oral treatment, was demonstrated by their actual isolation from the brains of actively sensitized mice, as well as by their localization in the brain following passive transfer to the periphery [46,47]. Thus, specific *ex-vivo* reactivity to GA, manifested by cell proliferation and by Th2 cytokine secretion, was found in whole lymphocyte population isolated from brains of EAE induced mice treated by GA. Moreover, highly reactive GA-specific T-cell lines, that secreted *in vitro* IL-4, IL-5, IL-10 and TGF-β in response to GA, and cross-reacted with MBP at the level of Th2 cytokine secretion, were obtained from brains and spinal cords of GA-treated mice. The ability of the GA-induced cells to cross the blood–brain barrier (BBB) and accumulate in the CNS was confirmed by the injection of labeled GA-specific T-cells into the periphery and their subsequent detection in the brain [46,47]. Preferential recruitment of GA-induced T-cells into inflamed organs, namely the brain in the case of EAE and the intestine in the case of inflammatory bowel disease, was also demonstrated (Fig. 2). There is currently a consensus that the brain is not an immune privileged site and that activated T cells, regardless of their specificity, penetrate the CNS, especially in the course of MS/EAE when the BBB is dispirited [48]. While cross-reactivity of the GA-specific T-cells with MBP [29,30] is not essential for the entrance into the CNS, it may enable their *in situ* re-activation.

In the CNS of EAE-induced mice, GA-specific T-cells manifested intense expression of the two potent regulatory anti-inflammatory cytokines IL-10 and TGF-β, but no trace of the detrimental inflammatory cytokine IFN-γ [49]. Of special interest is the finding that IL-10 and TGF-β were expressed not only by the GA-specific T-cells but also by CNS resident cells in their vicinity, such as astrocytes and microglia. In contrast, the overall expression of IFN-γ in the brain tissue was drastically reduced. In addition, GA treatment resulted in drastic reduction in the occurrence of the pro-inflammatory Th-17 cells, with parallel elevation of Tregs in the CNS of mice with either chronic or relapsing–remitting EAE [38]. Analysis of GA-reactive T-cells from the cerebrospinal fluid (CSF) of GA-treated MS patients revealed pronounced anti-inflammatory profile [50]. These cumulative results indicate that GA induces a bystander immunomodulatory effect in the CNS and generates *in situ* pro-inflammatory to anti-inflammatory cytokine shift, thus restraining the immuno-pathological disease progression. However, the effect of GA is not restricted to anti-inflammation, as described in the following.

4. Neuroprotection and repair processes

An essential challenge for MS therapy is to target not only the inflammatory aspect of the disease but also its neuroaxonal pathology, aiming toward neuroprotective outcomes. By broad definition, neuroprotection is an effect that results in salvage, recovery, or regeneration of the nervous system, its cells, structure and function. During the recent years accumulated findings indicated that GA treatment generates neuroprotective consequences in the CNS.

4.1. Elevation of neurotrophic factors

The initial indication for neuroprotective activity was the ability of GA-induced cells to secrete not only anti-inflammatory cytokines, but also the potent brain derived neurotrophic factor BDNF. This was demonstrated for murine GA-specific T-cells originating from the periphery or the CNS, as well as for human T-cell lines [51–54]. Furthermore, GA-specific T-cells demonstrated extensive BDNF expression in the brain of EAE-induced mice [49]. In addition to the GA specific T-cells that penetrated the CNS, most of the BDNF positive cells were neurons and astrocytes that showed higher BDNF expression

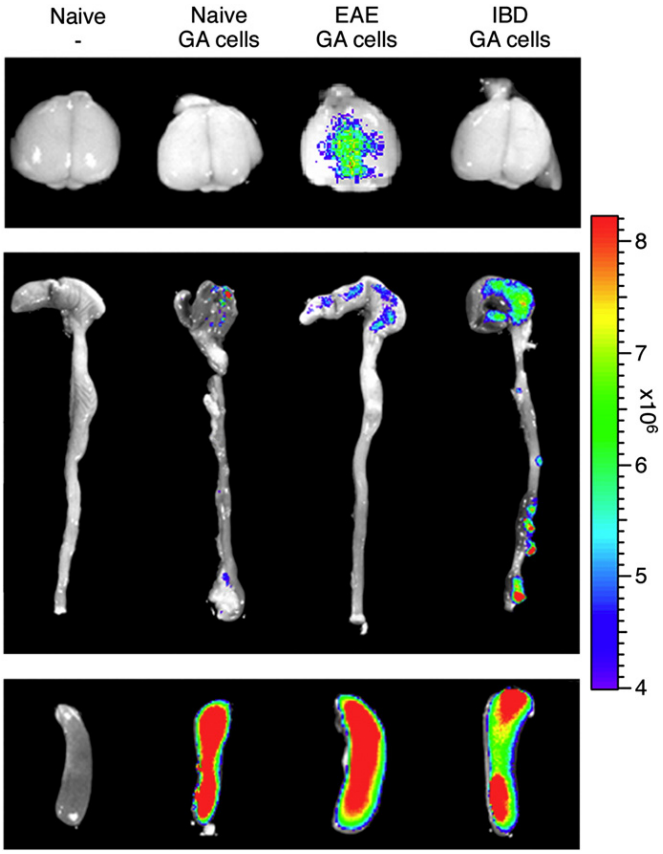


Fig. 2. Preferential recruitment of GA-induced T-cells into the inflamed organ, detection by *in vivo* imaging system (IVIS). TDIR-labeled GA specific cells were adoptively transferred to mice inflicted with EAE (MOG-induced model), or with inflammatory bowel disease (dextran-induced model), or to naïve mice. IVIS imaging depicts brains,

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