Redundant regulatory mechanisms in autoimmune diseases: The example of experimental autoimmune encephalomyelitis

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ABSTRACT

In multiple sclerosis (MS) and in its animal model, Experimental Autoimmune Encephalomyelitis (EAE), autoagressive and regulatory cells traffic into the Central Nervous System (CNS), and may alter the course of the disease. Consequently the role of immunocompetent cells is major determinants in EAE pathogenesis for instance, CD4$^+$ T helper 1 cells, have been identified as a key element in determining the course of the disease; however other cells, can also induce EAE, and have pathogenic and regulatory roles in EAE pathogenesis (induction and recovery). Experimental autoimmune encephalomyelitis models are also useful tools in understanding the generation and organization of the myelin-specific autoimmune repertoires and immunoregulatory loops involved in spontaneous recovery. The aim of the present work is to outline how the pathogenic and the regulatory elements prevail in EAE, and correlate them with other autoimmune disorders. These effects of pathogenic and regulatory cells, need to be considered for efficacious therapy. A necessary step for the design of antigen-specific immunotherapies in the treatment of chronic autoimmune disorders in humans is to learn how manipulate the immune system, to know the biology of its cell populations.

Keywords: EAE, Myelin Oligodendrocyte Glycoprotein (MOG), effector T cells, regulatory T cells, immunotherapy, autoimmune diseases

RESUMEN

Mecanismos regulatorios redundantes en enfermedades autoinmunes: El ejemplo de la encefalitis autoinmune experimental. En la Esclerosis Múltiple (MS) y en su modelo animal Encefalitis Autoinmune Experimental (EAE) células autoreactivas y reguladoras penetran el Sistema Nervioso Central (SNC), y determinan el curso de la enfermedad. Consecuentemente el balance entre células inmunocompetentes será el principal determinante en la patogénesis de la EAE. El subconjunto de células CD4$^+$, ha sido identificado como un elemento clave en determinar el curso de la EAE y MS, sin embargo otras células tienen funciones patogénicas y/o reguladoras determinantes en la patogénesis de la EAE (inducción y remisión). Los modelos animales de EAE son herramientas útiles para comprender la generación y la organización del repertorio autoinmune específico de la mielina y los lazos inmunoreguladores involucrados en los procesos de remisión espontánea. El propósito de este trabajo de revisión es resaltar que en la EAE y en otras enfermedades autoinmunes prevalecen simultáneamente elementos patogénicos y reguladores. Para instaurar una terapia efectiva es necesario tener en consideración el alcance que tienen los efectos de las células patogénicas y reguladoras. Aprender como manipular el sistema inmune, conociendo la biología de sus poblaciones celulares, es un paso imprescindible en el diseño de inmunoterapia específica de antígeno, para el tratamiento de los desórdenes autoinmunes.

Palabras Claves: EAE, MOG, células T efectoras, células T reguladoras, inmunoterapia, enfermedades autoinmunes

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory and demyelinating disease of the Central Nervous System (CNS) and is one of the better studied models of organ-specific autoimmune disease. EAE shares many clinical and histological features with the human disease Multiple Sclerosis (MS) [1-4]. CNS inflammation in both MS and EAE are characterized by disruption of the Blood Brain Ba-rrier (BBB) by activated autoreactive myelin-specific T cells, leading to tissue destruction and subsequent neurological dysfunction [5].

The CNS is an immune privileged site protected by the BBB, which isolates nervous tissues from immune competent cells. Unstimulated leukocytes do not readily adhere to the vascular endothelium of BBB but inflammatory signals may induce the expression of proteins on the endothelial cell surfaces that promote the adhesion and extravasation of activated immune cells from the circulation into brain tissue [6]. Thus during inflammatory disorders such as autoimmune diseases, immune competent cells can penetrate BBB and reach targets where they will continue or amplify the immune reaction.

The arrival of myelin-specific T cells in the CNS, implies recognition of single or a limited number of related self-determinants, which are normally presented by microglial cells [7], resulting in the expansion of T cell clones [8]. This activated response

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then expands against self-determinants of the same molecule, (or other molecules) within the nervous system [9]. These antigenic spreading phenomena correlate to progression of EAE and probably relapse in MS [10].

During the inflammatory process in EAE ectopically organized lymphoid structures in CNS are induced by lymphotoxins such as TNFβ. This lymphoid neo-organogenesis may in turn re-stimulate neuroantigen-specific T cells, driving their clonal expansion. Moreover, lymphoid neo-organogenesis provides the site in which the amplification of the autoimmune process and determinant spreading occurs, permitting access to more highly specialized lymphoid structures [11-14].

Another event is T cell apoptosis, which may be exceptionally high during acute EAE. Apoptosis not only affects autoreactive effector T-cell populations but also secondarily recruited lymphocytes, could be responsible for the spontaneous remissions observed in the course of these diseases. Interferon Gamma (IFNg), is a prototypical cytokine of T helper 1 (Th1) cells, and is involved in EAE regulation by its role in apoptosis [15-17].

EAE can be induced by challenge with encephalitogenic proteins, peptides or even T cells clones, representing monophasic or polyphasic clinical courses in which ascending paralysis is usually followed by spontaneous recovery.

The lack of spontaneous CNS-specific autoimmunity in normal individuals implies the presence of specific regulatory mechanisms maintaining immune homeostasis. To regulate the immune response and lower the potential for autoimmunity, the immune system has several mechanisms to control the outgrowth and differentiation of activated cells. Professional regulatory T cells evolved redundant mechanisms, including apoptosis-mediated clonal deletion, anergy, and secretion of soluble factors such as cytokines, which in turn diminish the autoreactivity and sustain spontaneous recovery.

EAE is good model for studying the inflammatory response generated and regulated by the immune system. A comparable diversity of clinical forms with MS, can also be observed in variants of EAE animal models, which represent the stages in the course of MS [18].

Autoantigens as immunogens in EAE induction: The relevance of Myelin Oligodendrocyte Glycoprotein (MOG)

EAE can be induced by a diversity of CNS antigens. EAE has been induced in rodents and other species by sensitization with a number of myelin related proteins, including myelin basic protein (MBP) [19], proteolipid protein (PLP) [20, 21], myelin-associated glycoprotein (MAG) [22], myelin oligodendrocyte basic protein (MOBP) [23], and with peptides of these proteins, known as immunodominant epitopes. Recently, myelin oligodendrocyte glycoprotein (MOG) induced EAE has attracted increasing attention [24, 25].

MOG is an exposed antigen of myelin, is specifically expressed in the CNS on the outermost lamellae of the myelin sheath (Fig. 1), as well as the cell body and processes of oligodendrocytes [26]. MOG is an important target for autoimmune responses and is responsible of inflammatory demyelination in the CNS [27-29]. The encephalitogenic properties of MOG are associated with the generation of autoreactive MOG-specific T cells and the induction of antibody responses, which promote central nervous system demyelination. Antibodies against MOG cause demyelination in vitro and in animals with induced EAE [30-32], and have also been found in active lesions of patients with multiple sclerosis [33]. Moreover MOG appears as a regulator of the classical complement pathway, due to its capacity to bind C1q. Activation of the classical complement system is known to play an important role in autoimmune demyelination [34, 35].

Contrary to MBP or PLP specific T cell responses, occurring in both MS patients and controls, peripheral blood lymphocytes of MS patients exhibit a predominance of T cell responses to MOG, which is seldom observed in control donors [36], MOG appears as a prevalent antigenic molecule among myelin proteins. Autoantibodies to MOG have a remarkable predictive value of the course of MS. The initial detection of serum antibodies against MOG after a clinically isolated syndrome, predicts early conversion to MS. The absence of these antibodies indicate that the patients may remain disease-free for several years [37].

In susceptible animals, immunization with native or recombinant MOG elicits a severe EAE that mimics many of the clinical, pathological, and immunological features of MS, even if MOG derived peptides, or passive transfer of MOG-specific T cells, and autoantibodies against MOG are used in EAE induction [25, 31].However, different outcomes of immune response had been demonstrated after EAE-induction immunizing with the MOG$_{35-55}$ peptide in diverse mouse strains.

MOG$_{35-55}$ induces strong immune response in the context of H-2^b, leading to clinical EAE in B6 mice. H-2^s mice, as SJL do not develop disease in response to MOG$_{35-55}$, but instead mount a vigorous response to a different peptide, MOG$_{92-106}$, which is clini-

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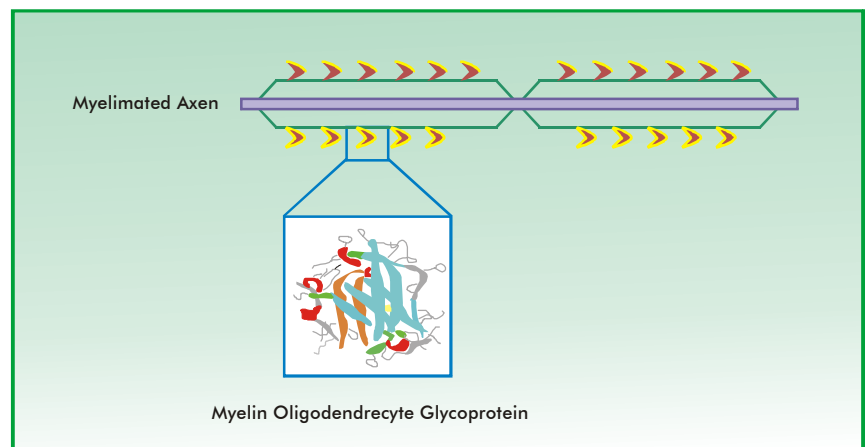
Figure 1. Myelin Oligodendrocyte Glycoprotein is the most exposed protein of myelin. Tridimentional model was reproduced with permission of PhD student L. Alonso.

cally manifested as relapsing-remitting EAE [24, 38-40]. We demonstrated the relevance of MOG as autoantigen during EAE induction is demonstrated by comparing immunized B6 mice with MOG$_{35-55}$ and spinal cord homogenate (SCH), depleted of CD25$^+$ T cells. As depicted in figure 2 and table 1 the onset of the disease is not different, contrary to the severity and clinical course. The recovery in SCH immunized mice is earlier than in the MOG immunized group (unpublished results).

Susceptibility changes among mouse strains even if they are Major Histocompatibility Complex (MHC) congeneic. For instance, SJL/J mice, a prototypical strain used to study EAE bearing the same haplotype of B10.S mice, while is resistant to both active and passive induction of EAE [41]. Another example is the congeneic partner NOD and NOD.B6*Idd3***,** (III mice). The NOD strain (that spontaneously develop autoimmune diabetes) is also susceptible to EAE. Conversely its H-2 congeneic, NOD.B6*Idd3* (III mice) is both resistant to diabetes and EAE [42].

This suggests, that genes outside the MHC, like anti-inflammatory or pro-inflammatory cytokines, might also modulate disease susceptibility. On the other hand, EAE-resistant mice produce predominantly anti-inflammatory cytokines upon immunization with MOG$_{35-55}$, both in the peripheral lymphoid tissue and in the nervous system, and such immune response may be implicated in enhancing self-tolerance and consequently inhibiting EAE [41, 42]. The mechanism involved in the different disease manifestations could include partial toleration events due to autoantigens expression outside the CNS, different migration pattern within the target organ, a differential competence of encephalitogenic T cells to infiltrate the CNS and inherent properties of the T cells themselves, such as the cytokine expression [18].

Adjuvants and immunoenhancers for EAE induction

For efficient EAE inductions with encephalitogenic peptides an inflammatory component which, antigenic presentation and disrupts the mechanisms of peripheral tolerance is required. On the other hand, it is necessary to override BBB for the autoreactive cells generated in the periphery to reach targets in the CNS.

In 1950 Incomplete Freund's adjuvants (IFA) was first used in the induction of EAE [43], later with the use of Complete Freund's adjuvant (CFA) fewer injections were required to reach the same goals**.** Killed *Mycobacterium tuberculosis* (MT H$_{37}$Ra) contained in CFA, is the source of CpG motifs and heat shock proteins (HSP), which are a danger signal for an adaptive response. It allows the generation of autorreactive T cells and changes the physiological context of inflammation. The 70-kDa HSP has been suggested as a potential autoantigen in MS [44]. However, these are not the only danger signals, in terms of severity and incidence of EAE induction, necessary to reach an efficient model of disease [44, 45]. Still it is necessary to change the accessibility of the CNS and permeabilising BBB for peripheral autoreactivity reaching targets in brain tissue.

The induction of EAE in mice or rats requires that encephalitogenic antigens are mixed together with
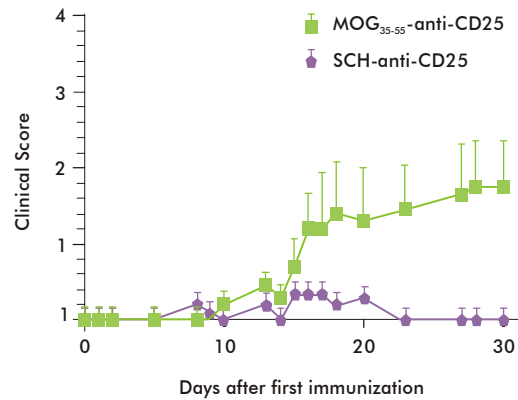

Figure 2. The SCH-anti-CD25 EAE induced group (circles) have a median clinical score of 0.55 ± 0.1, a maximal clinical score of 1 and the total recovery were at day 22th. Contrary, the MOG$_{35-55}$-anti-CD25 induced EAE (square) have a median clinical score of 2.15 ± 1.2, a maximal clinical score of 4 with the course of the disease being progressive, with not recovery.

MT H$_{37}$Ra, but it is also indispensable to introduce Pertussis Toxin (PT).

Coinjection of PT with neuroantigens in CFA enriched with *Mycobacterium tuberculosis,* enhanced the incidence and severity of the disease. The mechanism by which PT facilitates the induction of EAE has been attributed to opening up the BBB enhancing its vascular permeability and promoting the migration of pathogenic T cells to the CNS. This interpretation has recently come under scrutiny, and the generation of autoimmune Th1 cells has been suggested as the primary mode of action [46].

Indeed, PT has pleiotropic effects on the immune system, such as T cells mitogenesis, augmentation of cytokine and antibody production, and the promotion of delayed type hypersensitivity responses [47, 48]. PT also induces T cell differentiation and clonal expansion in EAE, via the activation of Antigen Presenting Cells (APC) in lymphoid tissues and the CNS, providing both stronger co-stimulatory signals and growth factors for autoreactive T cells [49]. It has been suggested that the mechanisms of PT binding on the surface of APC, might be either cross-linking cell surface molecules on T cells, or directly stimulating T cells together with the co-stimulatory molecules expressed on APC [50].

By mapping of EAE-modifying loci in mice, *eae9* has been identified as a PT-controlled locus, which

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Table 1. Active immunizations using syngeneic spinal cord homogenate (SCH) and myelin oligodendrocite glycoprotein synthetic peptide (MOG$_{35-55}$) with depletion of regulatory cells (CD4$^+$CD25$^+$) produce different forms of EAE in terms of clinical course, severity and recovery. The SCH-αCD25 EAE induced group have total recovery at day 22th. Contrary the MOG-αCD25 induced EAE have a progressive course of disease and do not recover.

Groups	Incidence (%)	Onset Day (Mean ± SD)	Clinical Score	
			Mean ± SD	Maxim
Control	0	0	0	0
MOG$_{35-55}$-αCD25	100	14 ± 1.8	2.15 ± 1.2	4
SCH-αCD25	100	13 ± 4.8	0.55 ± 0.1	1

overrides genetic checkpoints in the pathogenesis of EAE. Surprisingly *eae9* is located in a region encoding lymphoid tissue-homing-chemokine receptor CXCR5 and Interleukin 18 (IL-18) (Interferon gamma inducing factor) [51].

CXCR5 is expressed at the CNS in astrocytes, microglial cells, oligodendrocytes, neurons, endothelial cells [52], and in invariant NK (iNK) T cells, which are involved in the transition from innate to adaptive immunity at the site of inflammation and in secondary lymphoid tissues [53-55]. The ligand of CXCR5, the chemokine CXCL13, is involved in the regulation of the compartmentalization of T and B cells and might be involved in lymphoid neogenesis of CNS in MS and EAE onset [52, 56]. Recent reports state that gene expression of CXCL13 is markedly and persistently upregulated in the CNS of mice with relapsing-remitting and chronic-relapsing EAE [57].

IL-18, is produced by monocytes/macrophages, dendritic cells, B cells and other APC cells as well as astrocytes and microglia. IL-18 also promotes NK cell and Th1 cell activity and may bridge innate and adaptive immune responses. Anti IL-18 antibodies may even prevent EAE, IL-18-deficient (IL-18-/-) mice are defective in mounting autoreactive Th1 and autoantibody responses, and are resistant to MOG_{35-55} peptide-induced autoimmune encephalomyelitis [58, 59].

Thus, the role of PT in EAE induction is wider than initially thought and exemplifies how after microbial infections the interactions between innate and adaptive immune systems in response to self-antigens are favorable. It convincingly explains the observed relationship between MS relapses and infectious diseases [60].

Pathogenic roles of CD4⁺ T cells

It was once thought that autoreactive CD4 T cells have a major role in autoimmune disease [61, 62], however the frequency of such self-autoreactive T cells are similar in normal individuals to those afflicted with autoimmunity [63]. Also in transgenic mice, with artificial high frequency of self-reactive T cells, the development of spontaneous autoimmune diseases is uncommon [64, 65]. These findings suggest that the mere presence of CD4 autoreactive T cells is not sufficient for the development of autoimmune pathologies [66].

Direct evidence for the role of CD4+ T cells in EAE induction has come from adoptive transfer studies in which myelin specific CD4 T cell lines and clones were shown to induce chronic relapsing encephalomyelitis and paralysis after transfer [45, 61, 62].

Most investigations in the past of EAE models focused on CD4 T helper 1 (Th1) cells and the resulting cascade of cytokines and chemokines involved in pathogenesis. Certainly, it has been demonstrated that CD4⁺ myelin specific T cells induced EAE predominantly via production of Th1 cytokines [67], but not always, because CD4 T helper 2 (Th2) myelin specific T cells, could trigger EAE.

Lafaille and colleagues [68] and Pedotti and collaborators [69] have independently shown that transfer of *in vitro* generated Th2 cells from MBP-specific TCR transgenic mice to Recombinant Activation

Gene 1 (RAG-1) knockout and to **ab** T cell-deficient mice, was able to induce EAE, but only with a longer preclinical phase as compared with the transfer of Th1 cells. In normal or γδ T cell-deficient mice, they found resistance to EAE induced by Th2 cells [68]. Moreover, the coadmininstration of MBP-specific Th2 and Th1 cells did not abrogate disease induction in any recipient animals [68, 69]. This indicates that disease induction by activated Th1 cells cannot be prevented by previously activated Th2 cells. This has serious implications because it was thought that immuno-modulators, which polarize the response to Th2, could resolve the autoreactivity mediated by Th1 cells. At this point the protective pattern of autoimmunity, mediated by Th2 should be carefully evaluated and correctly classified, because the pattern of Th2 cytokines has evolved and does not only depend of Il-4 or IL10 as was once believed.

Regulatory role of CD4⁺ T cells

Induced EAE by an active challenge is usually followed by spontaneous recovery. The improvement process probably depends on cellular interactions between encephalitogenic T cells and regulatory cells. The course of passive EAE was unremitting in T-Cell-deficient mice, but when these animals were reconstituted with spleen cells from syngeneic wild-type mice, the course of clinical disease mirrored that of wild-type mice, thus restoring the regulatory activity to normal [70].

B6 TCRβ-chain knockout mice that were adoptively transferred with an MOG_{35-55} encephalitogenic T cell line, failed to recover from acute phase of passive EAE and the disease progressed more rapidly, resulting in death for most. In contrast, wild-type B6 mice normally recovered from acute disease, followed by one or more relapses [71]. This would suggest that specialized regulatory T cells are involved in the counter balance of adaptive immune response.

Regulatory CD4⁺CD25⁺ T cells populations, do not contain previously activated CD4⁺ T cells and inhibit T cells proliferation in a TCR-dependent manner, perhaps through direct T-T cell interactions [72, 73]. Several mechanisms of action for CD4⁺CD25⁺ regulatory T cells have been postulated, fundamentally those mediated by Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) [74] and Interleukin 10 (IL-10) [75]. Another distinctive characteristic of CD4⁺CD25⁺ T cells is its an exclusive transcription factor foxp3 [76]. The transfer of CD4⁺CD25⁺ regulatory T cells has been reported to suppress EAE mediated by naïve MOG-specific T cells, in recombination-activating gene–1-deficient TCR-transgenic mice [77, 78]. This indicates that regulatory T cells may block both the initiation of autoimmune responses and inhibit the function of established autoreactive effector cells.

In experiments of EAE induction, treatment with anti-CD25 antibody following immunization resulted in a significant enhancement of disease severity and mortality (unpublished results) [75]. Conversely, transfer of CD4⁺CD25⁺ regulatory T cells from naive mice decreased the severity of active EAE. IL-10-deficient mice were unable to suppress active EAE,

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suggesting that natural regulatory function are related to mechanism that involves Il-10 [79]. Supplementation of regulatory T cells significantly reduced the severity of the clinical disease both for active and adoptive EAE induction, perhaps by promoting a disease-protective immune response and preventing CNS inflammation by increased expression of Interstitial Cell Attachment Molecule 1 (ICAM-1) and P-selectin [80]. In support of this, the treatment with recombinant IFN-β1b has a short-term up regulating effect on soluble ICAM-1[81].

In the spinal cord of regulatory T cells recipients, CNS inflammation as the degree of lymphocyte infiltration was substantially reduced [80]. Although transferred regulatory T cell populations were not detected within either the brain nor spinal cord during the peak of EAE. It is possible to find more donor cells in draining lymph nodes of regulatory T cell vs. non-regulatory T cells recipients, suggesting a differential trafficking with regulatory populations, this is supported by elevated ICAM-1 levels [80]. In brain endothelial cells, ICAM-1-mediated signaling is a crucial regulatory step in the process of lymphocyte migration through the BBB, and as such it represents an additional phase in the multistep paradigm of leukocyte recruitment [81, 82].

CTLA-4 is a key co-stimulatory molecule for activating $CD25^+CD4^+$ regulatory T cells to exert suppression and control of self-reactive T cells. In vivo blockade of CTLA-4 suffices to break natural self-tolerance and elicit pathological autoimmunity [83].In experimental allergic encephalomyelitis, CTLA-4 blockade during the onset of clinical symptoms mar-kedly exacerbated the disease, increasing mortality. These enhancements of disease severity were associa-ted with high production of the encephalitogenic cytokines Tumor Necrosis Factor alpha (TNF-α), Interferon Gamma (IFN-γ) and Interleukin 2 (IL-2), suggesting that the regulatory role of CTLA-4 is in over attenuating inflammatory cytokine production [84].

Other types of antigen-specific $CD4^+$ T cells exist without a defined phenotype, implicated in the regulation of the effector function of autoimmune T cells. For instance, high Transforming Growth Factor Beta (TGF-β) producing T cells [85], high IL-10, high IL-4, high IFN-α [86] or high IL-10, low IL-4 producing regulatory T cells class 1 (Tr1) [9]. The Tr1 regulatory cell, not only are the ultimate effector population in the regulation of autoimmunity, but they also induce naive T cells to provide long-term inhibition of autoreactivity [9, 10].

Pathogenic role of CD8+ T cells in EAE

Contrary to what was once believed, not only are there roles for CD4 Th1 and Th2 cells in EAE induction, CD8 +T cells can also induce this disease. In fact, brain lesions in EAE and Multiple sclerosis patients include inflammatory infiltrates of both $CD4^+$ and $CD8^+$ T cells.

Immunochemistry of T cells interactions with its cognate antigens and novel genetic studies about susceptibility to disease have shown evidence that involves both CD4 and CD8 in the pathogenesis of the autoimmune diseases. The gene products of Hu-

man Leukocyte Antigen (HLA) class II and I, are elevated on inflamed oligodendroglial cells. Oligoclonal $CD4^+$ and $CD8^+$ T cell populations within MS plaques, and $CD8^+$ T cell clones specific for myelin antigens, have been isolated from MS patients [45, 87, 88].

MBP-specific CD8 T cells isolated from wild-type mice are able to mediate severe CNS autoimmunity that exhibits similarities to MS not seen in myelin-specific CD4 T cell–mediated EAE. Intravenous injection of cytotoxic $CD8^+$ T cell clones injures the brain inducing ataxia, spasticity and hind limb paralysis. Neuropathology also revealed CD8 perivascular cuffs in the vascular walls of the brain [89]. Adoptive transfer of CD8-enriched MOG-specific T cells, induce a much more severe and permanent disease, with brain lesions being more progressive and destructive than disease actively induced by immunization with $pMOG_{35-55}$, demonstrating the encephalitogenic potential of $CD8^+$ MOG-specific T cells [45, 90]. These data are evidence that support an essential role for CD8 T cells in autoimmune demyelination.

However, it is crucial to recognize that there are differences between CD4-induced EAE and CD8-induced EAE. These differences appear in attempts to modulate disease with agents intended to abrogate the cytokines TNFα and IFNγ.

When EAE is induced with $CD4^+$ T cells, diseases might be blocked with anti-TNFα antibody or agents

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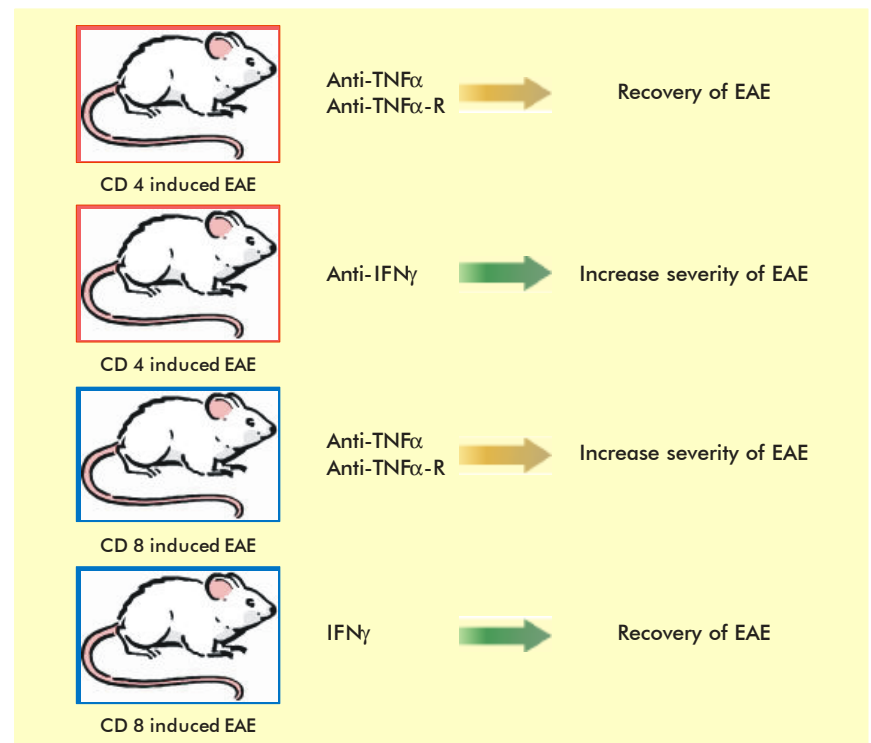
Figure 3. There are differences between the outcome of CD4+ T cells induced EAE and CD8+ T cells induced EAE related to the therapeutic approach. In CD4+ induced EAE, diseases might be blocked with TNFα antibody or agents that blocked TNFα receptor (TNFR); on the contrary, the use of anti-IFNγ impaired the course of disease. In contrast, in CD 8 induced EAE, the disease is stopped by administration of recombinant IFNγ and the intervention with TNFR system has no effect.

that blocked TNFα receptor (TNFR). In this case, the use of anti IFNγ impaired the progression of the disease. In contrast, in a CD8-induced model of EAE, the disease is arrested by administration of recombinant IFNγ and intervention on TNFR system has no effect [91] figure 3.

Multiple Sclerosis has several clinical forms, some of them share a resemblance to CD8 T cells induced-EAE and others are similar to CD4 T cell induced EAE [92, 93]. For the design of novel therapeutic tools, the identification of the molecular and cellular events involved in the pathogenesis will determine in the success of clinical trials. Thus, the selection of EAE models based in its different pathogenic mechanisms to asses immune-system interventions require the exhaustive understanding of molecular and cellular events driving the course of the disease.

Regulatory roles of CD8+ T cells

Early studies of EAE in CD8 deficient mice suggested that CD8 T cells with undefined antigen specificity might function as suppressors or regulatory T cells in CNS autoimmune disease [60, 94].

The ability of CD8+ T cells to regulate CD4+ T cell responses have been mostly attributed, to the CD8+ T cells' production of cytokines [95] but other studies have identified specific cognate interactions between regulatory CD8+ T cells and activated CD4+ T cells. During antigen driven CD4+ T cell responses *in vivo*, CD8+ T cells specifically regulate CD4+ T cells in a T cell antigen receptor (TCR) Vβ-specific manner [96-98].

After antigen activation CD4+ T cells express membrane $Q_{a-1}/TCR_{V\beta}$ motifs that are recognized by the αβTCR expressed by precursor regulatory CD8+ T cells. Q_{a-1} is a mouse homolog of human HLA-E, and is only expressed at low levels on resting T cells but is increased after antigen activation. Qa-1 ligand is composed of Qa-1-β₂-microglobulin heterodimers that contain peptides derived from TCR Vβ. Q_{a-1} restricted CD8+ T cells may eliminate a subpopulation of activated autoreactive CD4+ cells through TCR-dependent recognition of self peptide-Q_{a-1}.complexes [99]. These CD8+ T cells are induced to differentiate and down-regulate CD4+ T cells expressing the particular $Q_{a-1}/TCR_{V\beta}$ motifs [100]. CD8 effector activity is supported by cell activation and population expansion [101]. Recently it has been demonstrated that TCR Vβ-derived peptides associated with Q_{a-1} on activated autoreactive CD4+ cells can activate CD8-dependent suppression and inhibit autoimmunity [99].

Distinct functional subsets of CD8-T cells exist with divergent roles in CNS autoimmunity, contribute to disease in different ways, as both pathogenic and regulatory cells.

Pathogenic roles of B cells in EAE

The importance of the B cell function in autoimmunity of the CNS had been neglected in spite of early experiments showing that rats depleted of B cells, were rendered resistant to EAE [102]. However, B cells and antibodies (Ab) are essential players in the pathogenesis of EAE and MS. Inappropriate activation of B cells by cross-reactive, or self-mimicking

pathogens could explain the reversal of tolerance. It is thus conceivable that natural Ab are the source of pathological Ab, generated by affinity maturation through somatic mutation and immune globulin (Ig) class switch [103].

The identification of MOG as a major target for autoantibody-mediated demyelination in EAE, revived interest in the role of antibody in the pathogenesis of MS. Intravenous injection of a MOG-specific monoclonal antibody in rats with EAE, induces extensive demyelination, enhances the inflammatory response and dramatically increases disease severity. Conversely, in the absence of MOG-specific monoclonal antibody, the pathology of these disease is purely inflammatory, confirming the demyelinating potential of MOG-specific antibody [104].

Unless the BBB is compromised, circulating anti-MOG antibodies are unable to enter the CNS and initiate demyelination. Mice vaccinated with MOG encoding DNA constructs, despite high titres of anti-MOG antibody in the circulation, do not develop any spontaneous neurologic deficit, nor subclinical pathological changes in the CNS [105].Therefore, in the pathogenesis of EAE the role of B cells and antibodies is secondary to induction of an encephalitogenic T-cell responses to MOG.

Regulatory role of B cells

Studies with B cell deficient mice showed that B cells are necessary for recovery from EAE [105, 106]. Induction of EAE in B10.PL mice rendered deficient of B cells by disruption of the μ heavy chain transmembrane exon (B10.PLμMT), showed that these mice have a similar incidence of EAE induction compared to controls. However B10.PLμMT had greater variation in the speed of disease onset and severity. They also failed to completely recover as compared to B10.PL in which spontaneous recovery was the norm [105]. On the other hand, B cell deficient mice immunized with a MBP peptide have a long chronic disease course, while wild-type mice show EAE paralysis followed by full recover [106]. It suggests that B cells are not required for the activation of encephalitogenic T cells and subsequent induction of EAE, but may play a pivotal role in the immune regulation of the disease.

It is common to observe in healthy humans and mice autoantibodies to self-antigens [107-110], and its presence across strains and species [111-113]. Even in cord blood of newborns auto-antibodies had been detected, suggesting that their synthesis might be independent of stimulation by foreign antigens [114]. Autoantibodies, both of the IgG and IgM isotypes, are detectable in the sera of C57BL/6 mice, but it is known that these strains do not spontaneously develop autoimmune disease. Conversely the C57BL/6 strain is susceptible to induction by immunization to EAE [115].

It has been suggested that autoantibodies may be associated with mechanisms that might prevent autoimmune disease. Transfer of autologous B cells expressing encephalitogenic determinants induced specific unresponsiveness and protected mice from induction of EAE, even when the transfer was after the disease onset. These protected animals were unre-

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sponsive to encephalitogenic determinants as measured by a delayed type hypersensitivity (DTH) [116, 117].

Pathogenic roles of natural killer T cells

Natural Killer (NK) T Cells recognize lipid antigens on the major histocompatibility complex (MHC) class I-like molecule CD1 and immediately secrete large amounts of IFNγ, characteristic of a type 1 response and simultaneously IL-4 type 2 responses. All of them can influence the fate of the immune response, because they induce activation of both innate and adaptive responses and the activation of Natural Killer (NK) cells, dendritic cells, T cells and B lymphocytes [118, 119].

Activation of Vα14 NKT cells in the context of CD1 alters the cytokine profile of T cells reactive to myelin antigens and their ability to induce EAE. Thus, the stimulation of CD1 with α-Galactosilceramide, which is a potent activator of NK T cells both *in vitro* and *in vivo*, can either enhance or prevent disease. Depending on the nature of NKT cell response in different murine strains, IFNγ secretion will be involved in the exacerbation, whereas IL-4 will play an important role in protection. The balance between IFNγ and IL-4 secretion in response to activation via CD1 determines whether the activation of Vα14 NK T cells will enhance or diminish the disease [53].

Innate pathways of immunity are crucial to the up regulation of co-stimulatory molecules on APC as well as in providing an initial cytokine milieu necessary for the development of acquired immunity. NK T cells that rapidly secrete cytokines can thus influence the outcome of immune response not only in infectious but also in autoimmune diseases [53, 120].

In mice, the acquisition of NKT-cell competence to secrete IL-4 and IFN-γ *in vivo* depends on co-stimulation: stimulation through CD80/CD86 is required for IL-4 and IFN-γ secretion, and stimulation through CD40 is required for IFN-γ secretion [121]. Consequently, blockade of CD86 polarized NKT cells toward a TH2-like phenotype (with concomitant suppression of EAE), and activation of APCs by treatment with CD40 biased them towards a Th1-like phenotype and exacerbated EAE [54].

Regulatory role of NK-T cells

The observation that B6 mice depleted of NK T cells, followed by immunization with MOG$_{35-55}$, achieved EAE with increased severity as compared with non depleted B6 mice [122] indicating a regulatory role for NK-T cells.

However it is unlikely that NK T cells are the only regulatory populations. Mounting evidence in mice where NK T cells are found at normal or increased levels (recombination-activating gene–1 knockout mice or IL-7Receptor knockout mice) has shown that passive EAE is more severe in these strains that in wild-type mice. This indicates that NKT cells are involved in a regulatory activity either alone or in concert with NK cells. In vivo activation of NK cells, as assessed by production of IFNγ, is dependent on the presence of an intact NKT cell population. The role of NK or NKT cells in the regulatory process is strengthened by the findings

that EAE in the absence of IFNγ is more severe [16, 123-127]. It has also been demonstrated that, activated CD4$^+$ T Cells in the spleen and central nervous system of IFNγ-KO mice during EAE markedly increased in vivo proliferation and significantly decreased ex vivo apoptosis [16].

In the protection against microbes NK cells collaborate with adaptive immunity and enhance Th1 activity through producing IFNγ, but a possible role of NK cells in immunoregulation has been suggested because the impairment of this type of cell results in exacerbation of neurological disorders [128, 129].The administration of the immunomodulatory drug quinoline-3-carboxamide, that enhances NK cell activity, suppressed the clinical and histological signs of chronic relapsing EAE [130]. During EAE, the proportion of NK cells in the peripheral blood increased, but the absolute number of NK cells in the spleen at this period decreased to one fifth of normal animals. These findings suggest that NK cells of the spleen are recruited toward the CNS via the blood stream [131]. Other evidence supports NK cells regulating EAE in an independent pathway of NK-T cells. Knockout mice for the gene β2-microglobulin (β2-m) and recombinant activation gene- 2 (RAG–2) can be more susceptible to EAE, particularly when NK cells are deleted [114]. One way speculate that depression of NK cell activity may lead to an enhancement or induction of autoimmune disease, in those subjects with prior defect in the regulatory system [122, 132, 133].

Pathogenic role of γδ T cells

Gamma delta T cells (γδ T Cells) are a distinct lymphocyte population that can exhibit reactivity with overexpressed heat shock proteins at inflammatory sites.

Mounting evidence shows that γδ T Cells contribute to the development of EAE by accelerating the inflammatory process in the CNS. γδ T Cells have also been shown in CNS lesions of SJL mice, adoptively sensitized to develop EAE [134] and in active MS plaques [135, 136].The depletion of γδ T Cells reduced clinical and pathological signs of disease, associated with reduced expression of IL-1 beta, IL-6, TNF-alpha, lymphotoxins and IFNγ [137]. The expression of activation markers on γδ T Cells and a cytokine profile biased towards a Th1 pattern [134], confirms a contributory role for these cells in the pathogenesis of EAE.

Regulatory role of γδ T cells

In support of a preventive role of γδ T Cells in the recurrence of EAE, EAE mice administered the T-Cell receptor (TCR) gamma delta specific monoclonal antibody, to deplete γδ T Cells *in vivo*, inducing aggravation and disease recurrence [134], suggesting a preventive role in relapse of EAE.

On the other hand, after treatment with mycobacterial antigens previous to immunization with MBP, a moderate increase of γδ T cells with suppression of the immune response and a reduction in EAE severity results. Immune suppression may be due to the production of TGF beta by γδ T lymphocytes [138] and presumes a role of γδ T cells in maintenance of self-tolerance.

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Pathogenic role of dendritic cells

Dendritic Cells (DC) are capable of breaking the state of «self-ignorance» and inducing aggressive auto-reactive T cells initiating adaptive immunity, and frequently leading to autoimmunity. However, in the development of autoimmune diseases, different types of DC exhibit distinct properties for inducing Th1/Th2 cell responses [139].

DC have been identified in the inflamed CNS [140]. The transfer of DC presenting a self-peptide MOG$_{35-55}$ into naive mice induced EAE. Interestingly in the lymph nodes and spleens of these mice there were MOG$_{35-55}$-specific T cells of the Th1 phenotype [141]. This indicates that DC presenting a self-antigen can induce the organ-specific autoimmune disorder EAE.

Regulatory role of dendritic cells

There seems to be a relevant role for DC in the initiation of regulatory events of tissue specific immune response in the CNS. DCs isolated from mice with EAE exhibit a phenotype similar to immature bone marrow-derived DC, characterized by intermediate surface MHC class II and low expression of the co-stimulatory molecule CD80. They are unable to prime naive T cells, inhibit T cell proliferation stimulated by mature bone marrow-derived DC, and have a Th2 cytokine profile mediated by TGFβ and IL-10. Thus it is possible that DCs may contribute to preserving immune privilege within the inflamed CNS [140].

Other evidence in favour of the immunosuppressive role of DC is the result of transferring LPS-stimulated DCs to mice with EAE. These cells, which have a mature phenotype with upregulated CD40, CD80, and CD86, significantly suppressed the severity of clinical signs and inflammation in the CNS, compared to immature DC-injected mice and PBS-injected mice. Lymphocytes from LPS-stimulated DC-injected mice, produced lower level of IL-12, IFN-gamma, but a higher level of IL-10, as compared to immature DC-injected and non-DC-injected mice [142].

Overproduction of Nitric Oxide (NO) and IFNγ by DC induced decreasing autoreactive T cell by increasing apoptosis in such cells. While spontaneous remission of EAE has been associated with prominent apoptosis mediated by IFNγ [16, 143].

Contrary to what was once believed, DCs are able to promote Th2 differentiation and have the potential for suppression of inflammatory demyelination.

Summarizing

Regulation of the immune system and specifically of autoimmune responses may occur at different physiological levels. One of them is the ability to evoke regulatory cells, which normally affect the passage from basic physiologic autoimmunity, toward inflammatory or pathologic autoinmune response. Cells with regulatory capacity may have multiple phenotypes. Regardless of the more relevant of them, due to their high capacity to arbitrate regulatory events, are CD4$^+$CD25$^+$ T cells, it is also possible to induce other regulatory cells, which will efficiently control pathological autoreactivity. In this sense, interventions in the immune system with IFNα has proved to be useful in generating a regulated or immunosuppressive environment mediated by Tr1's cytokines

[144]. It is also possible to induce antigen-specific tolerance prospectively as a result of prolonged delivery of subcutaneous infusion of low doses of peptides, which are able to transform mature T cells into CD4$^+$CD25$^+$ regulatory T cells [145]. Altered Peptides Ligands (APL), such Copaxone [146, 147] and other peptides, even those derived from autoantigens [148] could re-direct the immune response and promote selective stimulation of regulatory cells [149].

The immunization by attenuated autoreactive T cells (T cell vaccination) can induce T-cell-dependent inhibition of autoimmune responses, mediated by the specific recognition of activated CD4$^+$ T cells by suppressive CD8$^+$ cells [99]. These findings support the hypothesis of Jerne of the Immune Network, or the Homunculus Immunologic Theory of Cohen and reinforce the fact of specificity of regulation between T-B cells populations.

Another innovative approach has been treatment with anti-CD3 monoclonal; it has been shown to elicit regulatory cells which in turn counterbalanced the autoreactivity phenomena in diabetes mellitus [150, 151], and psoriatic arthritis [152]. It has also been shown in EAE that non-mitogenic anti-CD3 directly induces a state of immune unresponsiveness in activated pathogenic autoreactive effector cells and increases the absolute number of CD4$^+$CD25$^+$ regulatory T cells [153].

To induce a regulatory response with the aim of counterbalancing pathological autorreactivity, it is important to consider the stage of the disease, so that during early stages in MS or induction of EAE the activation status of APC and B cells as APC are the key elements for intervention. Conversely, during the advanced course of the disease, the targets of therapy are CD4$^+$, CD8$^+$ T cells and B cells as antibody producing cells.

These different approaches used independently, may generate cells with regulatory capacity, but their physiological relevance may be diminished *in vivo*.

We believe that all these therapeutic approaches could be more successful if they would be combined, as strategies, on a sequential schedule that allows the

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Therapeutic Approach	Effect
Immunosuppressive Therapy	Depletion of autoreactive clones
Immunosuppressive with autologous peptides	Expands clones of desire specificity
IFNα Treatment	Polarization toward a regulated context mediated by anti-inflammatory cytokines
T cell vaccination	Induce T cell-dependent inhibition of autoimmunity
Anti-CD3 Treatment	Induce T cell-dependent inhibition of autoimmunity

Box 1. Combined Therapy: Depletion of autoreactive clones with immunosuppressor therapy, followed by the intervention with peptides, which preferentially expand some clones of desirable specificity, together with IFNα, which will warrant polarizations toward a regulated system. Immunization with attenuated-autoreactive T cells or Anti-CD3 treatment induces an immune network of regulatory cells to control autoreactivity.

reprogramming of immune system. An example of Combined Therapy is depicted in box 1.

Combined Therapy should be more safe, specific and durable in terms of elimination of pathological autoreactivity. Combined Therapy, besides its redundancy, may improve by its robustness.

Conclusions

The successful therapy of MS and other chronic and inflammatory autoimmune diseases will be the selective supression or functional interference with disease causing cells. Regrettably there is not an universal and precise protocol for testing immunomodulators as therapeutics.

The identification of downstream cellular targets and molecular mechanisms of T cell action, both as effector and regulatory functions, during first stage or during an advanced course of disease, further enhance the development of treatments that inhibit immunopathology.

Although EAE has proven to be a particularly useful animal model to understand mechanisms of both immune-mediated CNS pathology and progressive clinical course, it is necessary that a correct interpretation and comprehension of each physiopathological event involved in induction, reversion and amelioration of EAE. It is also indispensable to use this information in the design of Combined Therapy. The divergent role for immunocompetent cells, together with their functionally distinct subsets, contribute to CNS autoimmunity in different ways, both in pathogenic and regulatory cells (Fig 4).
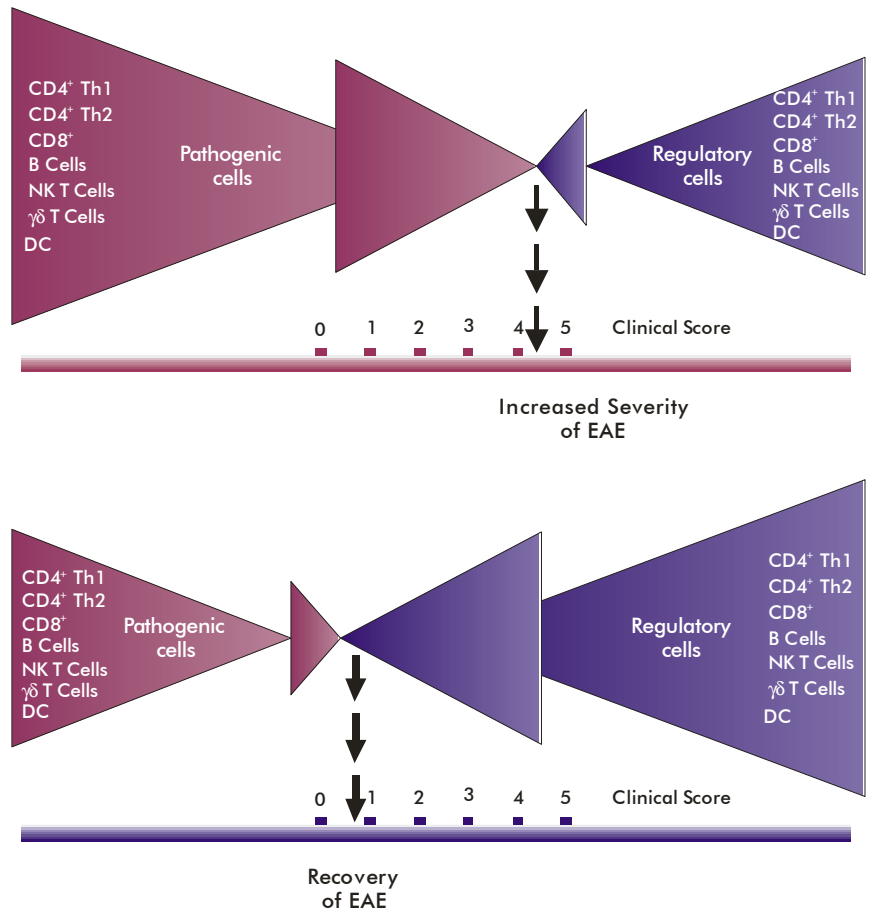
Acknowledges

Figure 4. During EAE induction autologous pathogenic cells effect a cascade of events responsible for brain damage. Identical phenotypic cells are involved in recovery process. The balance between effectors pathogenic and regulatory cells would be based more on function dependent than phenotype.

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