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Using EAE to better understand principles of immune function and autoimmune pathology

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) in which myelin becomes the target of attack by autoreactive T cells. The immune components of the disease are recapitulated in mice using the experimental autoimmune encephalomyelitis (EAE) model. EAE is classically induced by the immunization of mice with encephalitogenic antigens derived from CNS proteins such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Immunization of susceptible mouse strains with these antigens will induce autoreactive inflammatory T cell infiltration of the CNS. More recently, the advent of clonal T cell receptor transgenic mice has led to the development of adoptive transfer protocols in which myelin-specific T cells may induce disease upon transfer into naïve recipient animals. When used in concert with gene knockout strains, these protocols are powerful tools by which to dissect the molecular pathways that promote inflammatory T cell responses in the central nervous system (CNS). Further, myelin-antigen-specific transgenic T cells may be cultured *in vitro* under a variety of conditions prior to adoptive transfer, allowing one to study the effects of soluble factors or pharmacologic compounds on T cell pathogenicity. In this review, we describe many of the existing models of EAE, and discuss the contributions that use of these models has made in understanding both T helper cell differentiation and the function of inhibitory T cell receptors. We focus on the the step-by-step elucidation of the network of signals required for T helper 17 (Th17) cell differentiation, as well as the molecular dissection of the Tim-3 negative regulatory signaling pathway in Th1 cells.

Keywords

Multiple sclerosis; experimental autoimmune encephalomyelitis; CD4⁺ T lymphocyte; myelin; interleukin-17; Sgk1; Tim-3; Bat3

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Declarations

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Multiple sclerosis is a chronic, debilitating neurologic disorder characterized pathologically by central nervous system (CNS) white matter inflammation, demyelination and, in cases of chronic disease, astroglial scarring. The formation of demyelinated lesions leads to axonal injury and neurological deficit [1]. While the precise mode of disease etiology remains elusive, it is widely believed that MS pathology is initiated by the infiltration of autoreactive T cells into the CNS and subsequent immune attack on CNS myelin. The immune hypothesis of MS pathology is supported by the near-uniform presence of T cells, B cells and macrophages in MS plaques [1]. Evidence from the clinic also supports a central role for the immune system in MS disease course: glatiramer acetate, natalizumab and interferon-beta, all of which modulate the immune response, are front-line therapies [2].

MS is a highly prevalent disease, with rates of incidence estimated at between 177 to 350 per 100,000 in the United States and Canada [3], and as a result has been the subject of intensive research. Experimental autoimmune encephalomyelitis (EAE) is an umbrella term referring to multiple different models of CNS autoimmunity in vertebrate animals. Besides rats and mice, these have included species as diverse as non-human primates [4] and guinea pigs [5]. However, the bulk of the literature has centered on the study of CNS autoimmunity in rats and mice, with the latter being the focus of this review. Use of these models has greatly expanded our insight into the mechanisms that are responsible for MS pathology. EAE models have also allowed us to gain broad insight into the function of delayed type immune responses in general, and inflammatory T cell responses to CNS myelin in particular. In this review, we will describe the various mouse models of MS and their characteristics. In addition, we will highlight some key advances in our understanding of autoimmune pathology that have resulted from studies using EAE.

Clinical history of MS

Aspects of the pathology of MS were described as early as the mid-1800's with Cruveilhier in particular noting the existence of MS lesions in the CNS [6]. However, the first comprehensive description of the disease was provided in the 1860's by Charcot, who emphasized the requirement of multiple distinct attacks in the proper characterization of MS [7, 8]. Indeed, MS must be distinguished clinically from acute disseminated encephalomyelitis (ADEM), an idiopathic condition of acute central nervous system inflammation. The critical unit of diagnosis is a neurologic event and its subsequent resolution [9]. The McDonald criteria for the diagnosis of MS define a neurologic event as being an attack that lasts at least 24 hours, with subsequent attacks having to occur at least 30 days after the preceding attack in order to be considered separate events [10].

With this definition in mind, it is possible to classify subtypes of MS pathology on the basis of disease course. Approximately 85% of patients initially present with a relapsing-remitting disease course (RR-MS), in which clearly defined, repeated, attacks are followed by remissions. There may or may not be cumulative neurological deficit upon remission, and this pattern may last for years. However, of these patients, about 50% will transition to a secondary progressive form in which disease-associated deficits accumulate without relapse. Approximately 15% of total patients present a primary-progressive course in which disease worsens progressively after the initial attack, without remission [11].

While developing a single model of EAE that encompasses the full spectrum of MS symptoms remains a challenge, individual models of EAE are able to recapitulate specific elements of MS pathology. Models of active immunization and passive transfer in the SJL/J, C57BL/6 and NOD strains will be the focus of this review.

Relapsing EAE (R-EAE) in the SJL/J strain

EAE may be induced in mice through either active immunization with protein or peptide, or by passive transfer of encephalitogenic T cells. In either case, the relevant immunogen is derived from self-CNS proteins such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG). Immunization of SJL/J mice with the immunodominant epitope of PLP (PLP₁₃₉₋₁₅₁), induces a relapsing-remitting disease course [12], while disease induced by the immunodominant MOG₃₅₋₅₅ peptide in C57BL/6J mice tends to be of a chronic nature [13] (Figure 1). Classic EAE is characterized by an ascending paralysis beginning at the tail [14], followed by hind limb paralysis and forelimb paralysis and is assessed semi-quantitatively using a 5-point scale [15–17]. In some cases, an “atypical” form of EAE is seen. This is particularly the case when models of adoptive myelin-reactive T cell transfer are used. Atypical EAE, which is also scored using a 5-point scale, is characterized less by muscular paralysis and more by ataxia and loss of coordination [18].

Although much early work was done in guinea pigs and rats, by the early- to mid-eighties, mice began to be extensively used as a model for EAE. Using both MBP-derived peptides and MBP-reactive T cell clones, Zamvil et al. were able to induce EAE in SJL/J and PL/J mice [19]. They later demonstrated that an acetylated N-terminal-derived peptide from MBP was sufficient to induce EAE in PL/J mice [20]. These initial studies seemed to indicate that MBP was the most relevant source of encephalitogenic antigen in the CNS. At around the same time, Miller and colleagues were able to induce a relapsing EAE disease course in SJL/J mice by immunizing them with syngeneic mouse spinal cord homogenate. Notably, *in vivo* clearance of CD4⁺ T cells through use of L3T4 monoclonal antibody resulted in a dramatic reduction in disease severity [21], as could depletion of TcR Vβ2-expressing T cells thought to be largely specific to PLP [22]. These findings pointed to a role for CD4⁺ T cells in disease.

Initial studies suggested that MBP is the only encephalitogenic protein that could induce EAE, and that disease induced by spinal cord homogenate and other myelin antigens, including PLP, was due to contamination with MBP. However, subsequent studies demonstrated that SJL/J mice could be tolerized to spinal cord homogenate via pre-treatment with purified PLP, but not MBP, protein [23]. Furthermore, induction of EAE could be induced by synthetic myelin PLP₁₃₉₋₁₅₁, which had no homology to MBP, further strengthening the concept that multiple myelin proteins may have the ability to induce EAE. PLP₁₃₉₋₁₅₁ was identified as an immunodominant epitope for the induction of EAE in the SJL strain [24, 25], and the first clinical signs of disease could be noted 12–18 days after a single immunization. More than half of immunized mice showed signs of relapse after recovery from the initial attack (Figure 1A). Notably, R-EAE could also be induced through the passive transfer of PLP₁₃₉₋₁₅₁-reactive T cell lines and clones to naïve hosts. Passive transfer resulted in a mixture of disease courses, with some animals developing monophasic disease and others showing a pattern of remission and relapse [15]. Additional studies found evidence of epitope spreading in the R-EAE model. Mice immunized with PLP₁₃₉₋₁₅₁ developed splenic lymphocyte responses to PLP₁₇₈₋₁₉₁, and these PLP₁₇₈₋₁₉₁-reactive T cells could induce EAE upon adoptive transfer. Further, SJL/J recipients of T cells reactive to encephalitogenic peptide MBP₈₄₋₁₀₄ displayed delayed type responses when challenged with PLP₁₃₉₋₁₅₁ [24], and PLP₁₇₈₋₁₉₁-driven EAE can result in activation of PLP₁₃₉₋₁₅₁-reactive T cells in the CNS of diseased animals [26]. These findings are particularly intriguing given evidence of autoantigenic heterogeneity in MS [27].

Classic models of adoptive transfer involve the immunization of donor mice with PLP-derived peptides, isolation of peripheral lymphoid cells after 7 to 10 days of culture, *in vitro*

restimulation and subsequent transfer to naïve recipients. While providing proof of the central role of CD4⁺ T cells to pathogenesis, these models suffer from several limitations. While peripheral lymph node cells are restimulated with specific antigen (such as PLP₁₃₉₋₁₅₁) prior to transfer, the population is essentially polyfunctional. This makes it difficult to isolate the contribution of T cells directed against a specific antigenic epitope. Further, the encephalitogenic capacity of transferred T cells necessarily reflects the *in vivo* condition in donor animals. The decreased ability of knockout T cells to transfer disease could, for example, reflect defective antigen presentation in the donor animals rather than result from lesions in T cell function. This, combined with the pleiotropic nature of immune gene expression, makes it difficult to use these adoptive transfer models to isolate the contributions of individual genes to encephalitogenic T cell function.

The development of T cell receptor (TcR) transgenic mouse models in over the past 20 years has greatly facilitated the study of antigen-specific T cell responses. The TcR α - and β -chains specific for a given epitope are expressed in the mouse germline. As they express a functional TcR, the T cells from the resulting transgenic animals escape Rag1- or Rag2-mediated recombination of their TcR loci and express transgenic TcRs at a very high frequency. T cells from TcR transgenic mice can be >95% specific for a defined epitope that may be either ectopic or self-derived [28]. MBP Ac(1-11) specific TcR transgenic mice were the first to be generated, and were shown to develop spontaneous paralytic disease on the PL/J and B10.PL backgrounds [29]. More recently, we have exploited TcR transgenic technology to generate 2 TcR transgenic mouse lines on the SJL/J background (5B6 and 4E3) that are specific for PLP₁₃₉₋₁₅₁. Both strains develop spontaneous EAE at a high frequency, and we were unable to maintain the 4E3 strain on the SJL/J background past the 5th generation because of spontaneous EAE in the progeny. 5B6 animals display infiltration of antigen-specific CD4⁺ T cells into both the meninges and CNS parenchyma [30]. More recently, Wekerle et al. have generated a MOG₉₂₋₁₀₆-specific TcR transgenic line (TCR¹⁶⁴⁰) that develops spontaneous R-EAE on the SJL/J background [31].

However, studying specific genes and pathways on the SJL/J or PL/J backgrounds turned out to be quite challenging, because of the emergence of the C57BL/6J (B6) strain as the preferred background for the generation of gene knockout animals. To study the effect of gene deletions on the SJL/J or PL/J strains would thus require extensive and time-consuming backcrosses. This led to the search for an appropriate EAE model on the C57BL/6J background.

EAE models of active MOG peptide immunization on the C57BL/6J background

The relative ease of transgenesis and genetic ablation in the C57BL/6J (B6) strain has made it the *de facto* strain of choice for studying immune and autoimmune responses [32]. Thus, there was great interest in developing models of EAE in H-2^b-restricted animals, as this would facilitate the study of how individual genes and molecular pathways affect disease progression. In an important initial study, Ben-Nun and colleagues [33] studied the ability of multiple MOG-derived peptides to induce EAE in B6 mice. While several peptides could induce T cell responses upon *ex vivo* restimulation, only MOG₃₅₋₅₅ was able to induce CNS autoimmunity upon immunization. While this study reported that pertussis toxin (PT) could enhance disease onset and severity but was not absolutely required, concomitant administration of PT is now commonly used in conjunction with this model. Importantly, it was reported that MOG₃₅₋₅₅ induced a chronic form of the disease that did not remit [33] (Figure 1B). While this observation has been independently corroborated [13], it is also possible that the dose of MOG₃₅₋₅₅ peptide can influence the course of disease. Berard et al. [17] have reported that while a relatively high dose of peptide and adjuvant (300 μ g

MOG₃₅₋₅₅, 4 mg•ml⁻¹ of *M. tuberculosis* extract in adjuvant, 300 ng PT) induces chronic non-remitting EAE in B6 mice, lower doses of peptide/adjuvant (50 µg MOG₃₅₋₅₅, 0.5–1 mg•ml⁻¹ *M. tb.*, 200 ng PT) can promote a relapsing-remitting disease course (Figure 1B). These findings suggest that careful titration of peptide and dosage under specific environmental conditions may be required in order to standardize the type of disease seen upon MOG₃₅₋₅₅ immunization.

In the years following the development of the MOG₃₅₋₅₅ EAE model, a synergy emerged between use of this system and the new abundance of gene knockout strains. This allowed for a number of studies on the role of individual immune factors in regulating autoimmune responses against CNS myelin, including cytokines [34–36], T cell surface receptors [37, 38], signaling molecules [39] and transcription factors [40]. Several studies used knockout strains to elucidate the contribution of entire cell types to MOG₃₅₋₅₅-induced EAE such as B cells [41], CD11c⁺ monocytes [42], CD8⁺ T cells [43] and γδ T cells [44].

MOG₃₅₋₅₅-driven EAE has also permitted analysis of the role that CD8⁺ T cells play in CNS autoimmunity. Accumulating evidence suggests that CD8⁺ T cells have a role in MS pathology. CD8⁺ T cells outnumber CD4⁺ T cells in MS lesions by as much as 10 to 1 [45], and CNS antigen-reactive T cells can be readily detected in the peripheral blood of MS patients [46]. Taken together, these data suggest that CD8⁺ T cells proliferate in response to myelin antigens, and can traffic to sites of inflammation in the context of CNS autoimmunity. Ford and Evavold [47] isolated peripheral immune cells from MOG₃₅₋₅₅-immunized mice, restimulated them with peptide, and then adoptively transferred sorted CD8⁺ T cells into recipient animals. They found that MOG₃₅₋₅₅-specific CD8⁺ T cells were as capable of inducing EAE in naïve recipients as their CD4⁺ counterparts. As CD8⁺ T cells respond to 10–14mer peptides presented by MHC class I molecules [48], the authors reasoned that there was a core CD8⁺-reactive epitope contained within amino acids 35–55 of MOG. Indeed, they were able to identify MOG₃₇₋₄₆ as a minimal peptide that can induce specific CD8⁺ T cell responses in B6 mice.

Transgenic T cell models of MOG-driven EAE

2D2 transgenic mice

Much as had been the case for EAE research in the SJL/J strain, there was an emerging need to generate TcR transgenic models of MOG-driven EAE that could allow one to study the contribution of specific genes to T cell driven CNS autoimmunity. While T cell or bone marrow transfers into, and subsequent peptide immunization of, immunodeficient strains can yield important insights [16], such an approach is cumbersome. Our group has developed a class II-restricted TcR transgenic model to study MOG₃₅₋₅₅-driven EAE. An epitope-reactive T cell clone (2D2) was isolated from MOG₃₅₋₅₅-immunized B6 mice and its α- and β-chains were knocked in to the B6 germline by transgenesis [49]. The resulting 2D2 T cells are Vα3.2⁺ and Vβ11⁺, and react specifically to MOG₃₅₋₅₅. Peptide immunization of 2D2 mice resulted in more severe EAE than in nontransgenic littermate controls, and surprisingly, a low percentage (~4%) of 2D2 transgenic animals develop spontaneous EAE,

Intriguingly, a high percentage of 2D2 animals (>30%) develop spontaneous optic neuritis (ON), an inflammatory disease of the optic nerve that frequently results from inflammation and demyelination of the nerve [49]. Isolated ON is frequently an early symptom of MS [50]. Immunization of 2D2 mice with a suboptimal dose of MOG₃₅₋₅₅ resulted in a high frequency of optic neuritis yet no signs of classical EAE.

Wekerle and colleagues had previously described a B cell heavy chain knock-in mouse strain (IgH^{MOG}; also known as TH) that contains a high frequency of anti-MOG antibody-

secreting B cells in the peripheral repertoire. Upon active immunization with recombinant MOG, IgH^{MOG} animals develop EAE at a higher frequency than do nontransgenic littermates [51]; however, the mice never develop spontaneous EAE, emphasizing the need for myelin-specific T cell expansion in the periphery in order for disease to progress. The logical next step was to exploit the availability of the 2D2 and IgH^{MOG} strains to determine the effect of MOG specificity of both B cells and CD4⁺ T cells in the same animal. While IgH^{MOG} animals do not develop spontaneous EAE [51] and 2D2 mice only develop EAE at a low frequency [49], a high frequency of IgH^{MOG}x2D2 mice (over 60%) display spontaneous EAE. Histologically, the disease in these animals is characterized by the presence of inflammatory lesions in the spinal cord and optic nerve, and also by the relative sparing of the brain from inflammation. This histopathological pattern is reminiscent in some, but not all, respects to human Devic's disease/neuromyelitis optica (NMO) [52, 53], a type of CNS inflammation that is distinct from classical MS and that is prevalent in Asian populations [54]. Taken together, these findings indicate that the 2D2 model may provide a flexible platform by which to study multiple phenotypes of clinical MS [49].

Additionally, the generation of the 2D2 strain created the possibility that clonally homogenous MOG-specific T cells could be stimulated *in vitro* under a variety of conditions prior to adoptive transfer. In earlier adoptive transfer models of EAE, T cells were isolated from primed peptide-immunized mice, restimulated *ex vivo* with specific antigen, and transferred to hosts [15]. These models, while useful, carry several distinct disadvantages. Notably, the clonal heterogeneity of these T cell populations made it impossible to ensure that the transferred cells were entirely specific to the antigen in question. Further, *in vivo* crosstalk between different cell types during priming made it difficult to isolate the effects of various treatments and gene deletions on T cell pathogenicity. Finally, peptide immunization gives rise to multiple T helper cell lineages *in vivo*, making it challenging to isolate the contribution of specific T cell subsets to disease. Jäger et al [55] developed a model in which 2D2 T cells are stimulated *in vitro*, prior to transfer, in the absence of antigen-presenting cells (APCs). Naïve (CD62L^{hi}) 2D2 CD4⁺ cells are first purified using fluorescent label-assisted cell sorting (FACS) techniques. They are then stimulated *in vitro* using antibodies to CD3 and the costimulatory ligand CD28. As 2D2 T cells are clonally specific for MOG₃₅₋₅₅, one can be certain that the activated cells are directed against the target antigen. Cells are then transferred to naïve host animals. The study's focus was on the capacity of different T helper subsets to induce EAE. To this end, *in vitro* stimulated 2D2 T cells were cultured with various combinations of cytokines and blocking antibodies to generate Th1, Th2, Th17 and Th9 subsets. Intriguingly, we were able to show that Th1, Th17 and Th9 cells each induce EAE with distinct pathologic signatures [55]. Further, this transfer model provides an ideal system by which to study the effects of pharmacological compounds on T cell-driven CNS autoimmunity. As the 2D2 T cells are activated in the absence of APCs, the effect(s) of the compound(s) in question will be only directed towards MOG₃₅₋₅₅-reactive T cells.

1C6 transgenic mice

It has been known for some time that the NOD strain mice, which develop diabetes spontaneously, are susceptible to EAE upon active immunization with MOG₃₅₋₅₅. Further, these animals display a relapsing-remitting disease course that is followed by a chronic non-remitting stage [56], very similar to what is observed in human MS (Figure 1C). As a significant number of MS patients transition from RR-MS to a secondary progressive phase of disease [57], the EAE course seen in NOD mice renders it a highly attractive model with which to recapitulate human pathology. We therefore set out to generate a CD4⁺ TcR transgenic model for MOG₃₅₋₅₅-driven EAE on the NOD background, in much the same manner that we had previously generated the 2D2 transgene on the B6 background.

Surprisingly, the resulting mice (named 1C6) possessed both CD4⁺ and CD8⁺ MOG₃₅₋₅₅-reactive T cells. The 1C6 model was thus the first to produce MOG-reactive transgenic CD8⁺ T cells, and provided a unique opportunity to study the role of CD8⁺ T cell-mediated EAE [58]. We transferred purified 1C6 CD4⁺ T cells, CD8⁺ T cells, or both, to lymphocyte-deficient NOD. Scid and immunized them with MOG₃₅₋₅₅. All three groups developed EAE, though mice receiving 1C6 CD8⁺ T cells alone developed disease at a lower incidence and of lessened severity [58].

1C6 mice were then crossed to the IgH^{MOG} strain to generate, for the first time, a strain in which the three major lymphocyte subtypes bore antigenic specificity for the same CNS antigen. In contrast to 1C6 animals, IgH^{MOG} × 1C6 mice developed EAE at a high frequency (>60%); further, in contrast to IgH^{MOG} × 2D2 mice [53], IgH^{MOG} × 1C6 mice displayed classic EAE rather than Devic's-like pathology, with the majority of inflammatory lesions seen in the spinal cord [58]. Thus, the 1C6 strain allows one, for the first time, to study the interplay between MOG-specific CD4 T cells, CD8 T cells and B cells in the same mouse strain.

Key insights derived from use of EAE models

Role of the Th17 subset in EAE

Over 20 years ago, Mosmann and Coffman first described two subtypes of T helper cells that could be discriminated on the basis of cytokine production. T helper 1 (Th1) cells were shown to produce interferon (IFN) γ [59], while Th2 cells were described as producing interleukin (IL)-3, IL-4, IL-5 and IL-13 [59–61]. The Th1/Th2 paradigm soon came to dominate our understanding of effector CD4⁺ T cell responses. Th1 cells were shown to clear intracellular pathogens, while Th2 cells were shown to clear extracellular pathogens and parasitic infections [62]. In the context of autoimmunity, Th1 responses were thought to promote pathology while Th2 responses were thought to inhibit it [63].

A series of initial studies showed that myelin-specific Th1, but not Th2, cells were capable of inducing EAE upon adoptive transfer. These findings, coupled with the expression of the benchmark Th1 cytokine IFN γ in CNS lesions in EAE, helped construct the paradigm of EAE being a Th1-mediated disease. However, subsequent studies showing that mice deficient in IFN γ develop highly severe EAE proved an inconvenient truth in light of the Th1/Th2 paradigm [64–66]. These findings were in contrast to the resistance to EAE of *IL-12p40*^{−/−} mice, which lack one subunit of the key Th1 differentiation factor IL-12 [67].

This Gordian knot began to be severed by the discovery of the cytokine IL-23, a heterodimer consisting of the p40 subunit of IL-12 with a novel p19 subunit [68]. The resistance of *IL-12p40*^{−/−} mice to EAE could thus be reinterpreted to demonstrate a critical role for IL-23, and not IL-12, in EAE. Indeed, *IL-12p35*^{−/−} [67, 69, 70] and *IL-12R β 2*^{−/−} [71] mice, which solely lack IL-12 mediated signaling, were susceptible to MOG₃₅₋₅₅ driven EAE, while *IL-23p19*^{−/−} mice were resistant [70]. Thus, findings from work on the EAE model now hinted at the existence of a distinct T helper subset, potentially induced by IL-23, that could promote autoimmunity (summarized in Figure 2A).

Further EAE studies identified a critical role for the inflammatory cytokine IL-17, and IL-17-producing T cells, in IL-23-mediated autoimmune pathogenesis. CNS-infiltrating T cells produced IL-17, yet corresponding T cells from *IL-23p19*^{−/−} mice did not. Further, *in vivo* blockade of IL-17 [72, 73], but not IFN γ [73], reduced the severity of EAE. It is important to note that Th1 responses are capable of driving CNS autoimmunity; transfer of Th1 differentiated 2D2 T cells to naïve hosts induce robust EAE [55].

Accumulating evidence therefore suggested that much as IL-12 drives Th1 cell generation, IL-23 must be the differentiating factor for IL-17-producing T cells (Th17 cells) that could promote CNS autoimmunity. However, naïve T cells do not express IL-23 receptor (IL-23R) [74], and are poorly responsive to IL-23 [68]. In addressing this issue, our group found that IL-17-producing CD4⁺ T cells (Th17 cells) differentiated in the presence of two cytokines, transforming growth factor (TGF) β and IL-6. We generated a TGF β -transgenic strain, in which TGF β was selectively expressed under the control of the IL-2 promoter, and crossed it to the 2D2 strain. TGF β -Tg x 2D2 mice developed more severe EAE than 2D2 mice, and *ex vivo*-isolated TGF β -Tg x 2D2 T cells produced massive amounts of IL-17 [75].

These findings, which were published alongside others [76, 77], brought together the concept that IL-23 is not the differentiating factor for Th17 cells. Rather, a combination of cytokines (TGF β + IL-6) mediates the initial differentiation of Th17 cells. Further studies showed that Th17 differentiation is a multi-step process. Upon the induction of Th17 differentiation by TGF β and IL-6, the response is amplified by IL-21 [78, 79] and the phenotype is stabilized by IL-23 [80]. Th17 cells have been implicated in the pathogenesis of human autoimmune diseases such as psoriasis [81], arthritis [82], type I diabetes [83], and notably, MS [84] (the role of Th17 responses in human autoimmune disease is reviewed extensively by Maddur et al. [85]). Anti-Th17 pathway biologics are now being tested in the clinic. Ustekinumab, a human monoclonal antibody that targets IL-12p40 (thus blocking both IL-12- and IL-23-dependent signals), is a well-tolerated therapy for psoriasis that is also being tested for Crohn's disease [86]. Secukinumab is a human anti-IL17A monoclonal antibody that recently showed promising effects in a phase II clinical trial for psoriasis [87], and phase II clinical trials are in progress to determine whether it can modulate RRMS [88]. Excitingly, data suggest that it can reduce the frequency of gadolinium-enhancing lesions seen on MRI scans of RRMS brains (AIN457 MS Study group, presented at ECTRIMS 2012). Thus, the discovery of the Th17 paradigm of autoimmune inflammation, which was largely driven by work using the EAE model, has led to the development of promising therapeutics for human autoimmune disease.

Our lab has continued to use MOG₃₅₋₅₅-driven EAE to dissect the signals and players that contribute to Th17-driven autoimmune responses. We established a crucial role for IL-6 in promoting Th17 responses at the expense of regulatory T cell (T_{reg}) expansion. IL-6 synergizes with TGF β to drive IL-17 production [75], and *IL-6*^{-/-} mice develop mild EAE upon immunization, relative to WT controls. However, *in vivo* depletion of T_{reg} restored both disease severity and *in vivo* Th17 generation, indicating that IL-6 may control the balance between TGF β -mediated Th17/T_{reg} differentiation [79] (Figure 2B). To understand the molecular basis for the development of Th17 cells, we undertook a temporal transcriptional analysis of developing Th17 cells at 18 different time points and generated a regulatory network that governs the development of Th17 cells. In the process, we identified major nodes and subnodes that govern Th17 generation [89].

We identified serum glucocorticoid kinase 1 (Sgk1), a major node downstream of IL-23, as being essential for the optimal expression of IL-23 receptor on Th17 cells [89, 90]. Mice that lack Sgk1 specifically in CD4⁺ T cells develop EAE of lessened severity; importantly, so do mice lacking Sgk1 only in IL-17-producing T cells [90]. Sgk1 is a salt-sensing kinase that regulates sodium retention in cells, and is itself upregulated by the presence of salt [91]. Intriguingly, a high-salt diet can make WT mice susceptible to EAE of increased severity and earlier onset [90, 92]; by contrast, high salt food has negligible effect on the disease severity of mice lacking Sgk1 in CD4⁺ T cells [90]. Furthermore, exposure of human Th17 cells to increased NaCl in culture increases their ability to produce IL-17 [92]. In this series of studies, the EAE model has been used to draw a link between genetic susceptibility, environment, immune function and disease pathology.

Negative signaling receptors in EAE

While T cells proliferate rapidly upon recognizing their cognate antigen in the context of a normal immune response, it is also critical that such responses are rapidly shut down once a pathogen is cleared. T cells can be induced to express a plethora of negative regulatory receptors that act in an autocrine fashion to terminate their responses. This can take place either by initiating a program of cell death or by inducing the production of immunomodulatory cytokines. Dysregulation of negative receptor signaling by T cells is a key mechanism underpinning autoimmune pathologies [93].

The surface molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4) is one of the best studied of T cell negative receptors. It is a homolog of the costimulatory molecule CD28, but with a higher affinity for the CD28 ligands B7.1 and B7.2. CTLA-4 is rapidly upregulated on activated T cells, and downregulates T cell activation by out-competing CD28 for access to its ligands [94]. Its role in modulating autoimmunity has been well studied using the EAE model. *In vivo* blockade of CTLA-4 with a monoclonal antibody worsened the severity of both PLP- [95, 96] and MBP-mediated [97] EAE. Further, anti-CTLA-4 antibody could abrogate the relative resistance of Balb/C strain mice to EAE induction [98].

Programmed death-1 (PD-1) is another extensively studied inhibitory T cell receptor. Ligation of PD-1 results in inhibition of phosphoinositide 3' kinase (PI3K)-dependent inflammatory and proliferative signals [99]. It was therefore logical to hypothesize that loss of PD-1 signaling *in vivo* could enhance the development of EAE. Indeed, *in vivo* blockade of PD-1 [100] or its ligands [101] using a monoclonal antibody resulted in the worsening of EAE severity. Further, PD-1-deficient mice develop more severe EAE than controls upon immunization with MOG₃₅₋₅₅ [102]. Interestingly, *PD-1*^{-/-} T cells displayed enhanced production of inflammatory cytokines, but not increased proliferation, upon restimulation with MOG₃₅₋₅₅. These findings highlight the utility of the EAE model in uncovering the mechanistic bases for immune function.

Our group has had a long-standing interest in the function of T cell immunoglobulin and mucin domain-containing-3 (Tim-3), a negative regulatory receptor specifically expressed on IFN γ -producing T cells. Tim-3 is highly expressed on CD4⁺ T cells isolated from the CNS of PLP₁₃₉₋₁₅₁-immunized animals. Further, *in vivo* blockade of Tim-3 signaling worsened the severity on ongoing EAE, with macrophage and neutrophil infiltration of the parenchyma accompanied by significant demyelination of spinal cord neurons [103]. Future work demonstrated that *Tim-3*^{-/-} T cells were refractory to *in vivo* peripheral tolerance [104, 105]. Galectin-9 was identified as the ligand for Tim-3, and we found that knocking it down *in vivo* resulted in the exacerbation of MOG₃₅₋₅₅-driven EAE [106]. Taken together, these data suggest that absence or *in vivo* blockade of Tim-3 signaling can worsen the severity of EAE in multiple models of disease. Importantly, Tim-3 expression is downregulated on T cells isolated from the peripheral blood and cerebrospinal fluid of MS patients, suggesting that loss of Tim-3 mediated negative regulatory signaling correlates with human autoimmune disease [107]. Curiously, Tim-3 expression is higher in MS patients undergoing immunomodulatory therapy, as compared to patients who are not [108], suggesting that Tim-3 function and regulation are plastic and potentially targetable therapeutically.

While the significance of Tim-3 signaling in disease is clear (Figure 3), the signaling pathways downstream are less well understood. We have recently identified the adaptor molecule HLA-B-associated transcript 3 (Bat3) as a repressor of Tim-3 signaling. Bat3 physically binds to Tim-3, and overexpression of Bat3 protects Th1 cells from Tim-3 mediated cell death. Importantly, mice that lack Bat3 in the immune compartment develop less severe EAE upon active immunization of MOG₃₅₋₅₅ than wildtype controls [16]. As

Bat3 is not highly expressed in Th17 cells, and as the role of IL-17-driven responses in promoting EAE is well described [55, 75], we sought to develop a means by which to test the consequences of Bat3 expression in myelin-antigen reactive Th1 cells alone. To do so, we exploited retrovirally mediated gene transfer techniques. 2D2 cells were stimulated *in vitro* under conditions that promoted Th1 differentiation. While in culture, they were transduced by non-replicating retroviral particles that either encoded Bat3 cDNA or a Bat3-targeted shRNA construct. Transduced cells were then transferred to *Rag1*^{-/-} animals and monitored for signs of disease. Bat3-overexpressing 2D2 Th1 cells induced EAE of greater severity than controls, while Bat3 knockdown (Bat3KD) 2D2 T cells were completely ineffective in their ability to transfer EAE [16]. Intriguingly, approximately half of all Bat3KD 2D2 recipients developed peripheral lymphoid populations that were CD4⁺Tim-3^{hi}PD-1⁻IFN γ ^{dim}. This phenotype is highly reminiscent of that of dysfunctional exhausted T cells recently identified from the peripheral blood of HIV-1-infected individuals [109]. Indeed, the role of Tim-3 in mediating the dysfunction of exhausted T cells in HIV-1 [109], HCV [110] and cancers [111, 112] has been the subject of considerable interest in the last few years. Thus, by modulating the expression in a single molecule in myelin-antigen-reactive T cells, we may be able to shift an immune response from a self-destructive inflammatory one to one that is self-limiting in nature [16]. By manipulating the expression of inhibitory receptors on, and the cytokine balance of, inflammatory T cells, we hope to achieve our long term goal of inducing resolution of autoimmune diseases such as MS (Figure 3).

Concluding remarks

Dr. Abul Abbas, to whom this volume is dedicated, has worked for the last three decades in the field of autoimmunity and tolerance. He has made seminal contributions to our understanding of cytokine balance (Th1/Th2 paradigm), costimulatory molecules (CD28/CTLA-4) and regulatory T cell function (Foxp3⁺). He has also helped generate multiple transgenic models of autoimmunity using model antigens. Abul has an incredible talent for synthesizing complex information in a succinct and simple manner, and with his lectures he has engaged audiences all over the world. Blessed with a gregarious personality and infectious laugh, it is not a rarity to spot him at a conference, in the center of a large group of his many friends and colleagues. Finally, an editorial note that this dedicated issue to Abul Abbas is part of the Journal of Autoimmunity's focus each year on recognition of outstanding autoimmunologists and particularly contributors to the International Congress of Autoimmunity. Abul has graciously taught an introductory immunology course to a group of international students for many years at this congress. Our previous honorees have included Ian Mackay, Noel Rose, Harry Moutsopoulos, Pierre Youinou, and Chella David [114–116]. Above all, Abul has been a friend, supporter, source of counsel and mentor to many of us in the immunology community. We are immensely grateful to have him.

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Highlights

- Experimental autoimmune encephalomyelitis (EAE) is a well established mouse model of MS.
- Different models of EAE recapitulate different aspects of MS pathology
- EAE may be induced via active peptide immunization or via transfer of CNS-reactive T cells.
- EAE has yielded critical insights into our understanding of T cell differentiation.
- EAE has also increased our knowledge of the function of negative regulatory T cell receptors.

Figure 1. Variations in EAE disease course upon active immunization with encephalitogenic peptides

A. The immunization of SJL/J mice with PLP₁₃₉₋₁₅₁ can result in an initial paralytic attack, followed by multiple remissions and relapses [24]. B. Immunization of C57BL6/J mice with a high dose of MOG₃₅₋₅₅ causes a chronic disease course in which an initial attack does not resolve (solid line) [17, 33]. By contrast, immunization with a low dose of MOG₃₅₋₅₅ can induce either a multiphasic relapsing-remitting disease course (dotted line) or a single attack followed by a single remission (not shown) [17]. C. Immunization of NOD mice with MOG₃₅₋₅₅ results in a series of mild attacks/relapses followed by remissions. The disease ultimately transitions into a secondary chronic phase that is reminiscent of the secondary progressive phase of MS [56].

Figure 2. Use of MOG₃₅₋₅₅-driven EAE in gene knockout strains to identify a role for Th17 responses in CNS autoimmunity

A. IL-23-driven, and not IL-12-driven, responses are essential for EAE pathology. *IFN γ ^{-/-}* mice develop highly severe disease (top panel), despite the role of IFN γ as the key Th1 cytokine [64–66]. Further, *IL-12p35^{-/-}* [67, 69, 70] and *IL-12R β 1^{-/-}* [71] mice, which specifically lack signaling through the Th1-differentiating cytokine IL-12, are susceptible to EAE (second panel), while *IL-12p40^{-/-}* mice, which lack IL-12 and IL-23 expression, are resistant [67] (third panel). Importantly, *IL-23p19^{-/-}* mice, which lack IL-23 exclusively, are also resistant to EAE [70] (fourth panel). These studies collectively demonstrate a role for the Th17-promoting cytokine IL-23 in EAE pathogenesis. **B.** Role of TGF β and IL-6 in promoting EAE. TGF β Tg-CD4 Cre mice, which ectopically express TGF β in CD4⁺ T cells, develop highly severe EAE in comparison to control animals [75] (top panel). Further, while *IL-6^{-/-}* mice are relatively resistant to EAE (second panel), disease severity increases, and Th17 responses are restored, when CD25⁺ T_{regs} are depleted *in vivo* (bottom panel) [79]. These findings indicate that TGF β and IL-6 co-operate to promote Th17 responses, and that IL-6 does so in part by suppressing the activity of T_{regs}.

Figure 3. Role of Tim-3 in normal and pathologic immune responses

Upon encountering its cognate antigen in the context of an IL-12-rich milieu, a T cell will differentiate into a IFN γ ⁺ Th1 (CD4⁺) or Tc1 (CD8⁺) cell. A. In the case of an acute infection in which the antigen is ultimately cleared, Th1/Tc1 cells will secrete large quantities of the proinflammatory cytokines IFN γ , TNF α and IL-2, and upon terminal differentiation, will express high levels of Tim-3 [103]. Upon interaction with the Tim-3 ligand galectin-9 [106], these Tim-3⁺ T cells will undergo cell death and will be deleted from the repertoire. B. In instances of chronic viral infections [109, 110] and malignancies [111, 112], Tim-3 expression may become decoupled from the production of inflammatory cytokines. In the presence of persistent antigen, exhausted T cells progressively lose the capacity to produce IFN γ , TNF α and IL-2, and begin to express Tim-3. These exhausted T cells may persist in the periphery but ultimately undergo cell death [113]. C. In the case of autoimmunity, autoreactive Th1 cells produce great quantities of inflammatory cytokines and mediate tissue destruction. In the case of MS, these cells express display defective Tim-3 signaling [107, 108]. One possible mechanism for T cell dysfunction may be increased expression of Bat3, which represses Tim-3 signaling. Strategies that modulate the expression and function of Bat3 may result in augmentation of Tim-3 responses and subsequent amelioration of autoimmunity [16].