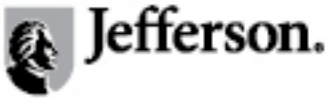

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Mechanisms of immunological tolerance in central nervous system inflammatory demyelination

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Abstract

Multiple sclerosis is a complex autoimmune disease of the central nervous system that results in a disruption of the balance between pro-inflammatory and anti-inflammatory signals in the immune system. Given that central nervous system inflammation can be suppressed by various immunological tolerance mechanisms, immune tolerance has become a focus of research in the attempt to induce long-lasting immune suppression of pathogenic T cells. Mechanisms underlying this tolerance induction include induction of regulatory T cell populations, anergy and the induction of tolerogenic antigen-presenting cells. The intravenous administration of encephalitogenic peptides has been shown to suppress experimental autoimmune encephalomyelitis and induce tolerance by promoting the generation of regulatory T cells and inducing apoptosis of pathogenic T cells. Safe and effective methods of inducing long-lasting immune tolerance are essential for the treatment of multiple sclerosis. By exploring tolerogenic mechanisms, new strategies can be devised to strengthen the regulatory, anti-inflammatory cell populations thereby weakening the pathogenic, pro-inflammatory cell populations.

Keywords

interleukin-10; interleukin-27; immune tolerance; multiple sclerosis; regulatory T cells

Introduction

Multiple sclerosis (MS) is a chronic, autoimmune disease of the central nervous system (CNS). It is primarily characterized by inflammatory damage to the myelin sheath and axonal degeneration, leading to neurological disability.[1] Normal immune function in the CNS is characterized by a combination of pro- and anti-inflammatory signals. These signals become dysregulated by increased pro-inflammatory stimulus in MS, leading to local tissue damage and the formation of lesions. The underlying immune process is also thought to be heavily reliant on $CD4^+$ T cells.[2] MS pathology has been extensively studied using the animal model of experimental autoimmune encephalomyelitis (EAE). In this model, EAE can be actively induced with an injection of a myelin protein and complete Freund's adjuvant, resulting in the production of pathogenic, myelin-specific T cells. These cells,

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which have developed in the peripheral lymphoid organs, infiltrate the CNS. In the CNS, infiltrating autoreactive T helper (Th $CD4^+$) cells encounter their cognate antigens presented by antigen-presenting cells (APC).[3–11] The result is reactivation of $CD4^+$ T cells, which, in turn, activate APC by cell–cell contact and pro-inflammatory cytokines. These pro-inflammatory signals recruit immune cells, such as $CD8^+$ T cells, macrophages/dendritic cells (DC), mast cells and activated microglia, which cause local tissue damage.[7–10]

It has been known for decades that inflammation in the CNS can be suppressed by various immunological tolerance mechanisms. Our laboratory, along with others, has established methods to elucidate these mechanisms, which include induction of regulatory T populations, immune deviation and induction of APC. These APC produce immunoregulatory cytokines, such as interleukin (IL)-10, transforming growth factor-β (TGF-β) and IL-27.[9, 10] Immune tolerance can be induced in EAE by the administration of encephalotogenic antigens in a variety of tolerogenic forms and by various routes.[12–14] In the present article, we review the key mechanisms underlying immune tolerance in MS and EAE, and their impact on future therapeutic intervention in these diseases.

Pathogenic Th1 and Th17 cells

A significant amount of MS research has been focused on $CD4^+$ (Th) cells based on the hypothesis that they play a central role in CNS autoimmunity. In EAE animals, the immune responses that develop after immunization are largely governed by interferon-γ $(IFN\text{-}\gamma)^+$ Th1 cells, which are the most abundant $CD4^+$ T cells observed in the CNS of animals after immunization with myelin peptides.[3, 15] Additionally, Th1, but not Th2 myelin-specific cells, were able to induce EAE when adoptively transferred into recipient mice.[16, 17] Furthermore, in MS relapse, elevated levels of Th1 cytokines have been observed in MS patients when compared with healthy controls, whereas Th2 cytokines are present during remission in MS patients.

EAE has been considered a typical Th1-mediated disease, but recent data show that Th17 cells play an important role in the pathogenesis of EAE.[18, 19] Studies by Harrington *et al.* and Wang *et al.* first described this lineage of Th cells that express the cytokine IL-17A and whose development is driven by IL-23.[7,20] In CNS autoimmunity, immunization with myelin antigens induces the development of Th17 cells, and these myelin-specific cells traffic to the CNS, where they secrete IL-17A. IL-17A attracts various immune cells and, in particular, myeloid cells into the CNS, thus starting and propagating the inflammatory cascade.[10, 21–25]

There is evidence from MS patients supporting the pathogenic role of Th17 cells in disease development. These findings come from Tzartos *et al.*, who found a relative increase in Th17 cells in the lesions of active MS patients when compared with healthy controls.[26] In addition, Durelli *et al.* found a sevenfold increase in the fraction of Th17 cells in untreated patients with active MS compared with those with inactive MS and healthy controls.[27] In contrast, the Th1 cell population was not always found to increase. They also found that treatment with interferon-β led to apoptosis in the Th17, but not the Th1 cell population, a finding that has been confirmed by other research groups.[28] Suppression of these highly

pathogenic T cell subsets is crucial for long-lasting immune tolerance and attenuation of disease. Recent studies have shown that the newer MS agents, dimethyl fumarate and fingolimod, also decrease the population of Th17 cells.[29, 30] However, the immune mechanisms used by these drugs to maintain suppression of pathogenic Th cell subsets have not yet been elucidated.

Regulatory T cells

Immune tolerance has increasingly become a focus in MS research. In EAE, the immune system can become tolerized to myelin-specific antigens and anti-inflammatory mediators that inhibit pro-inflammatory signals, reducing inflammatory stress. A key component in this system is regulatory T cells.

Regulatory $CD4^+CD25^+$ T cells (Tregs) are part of the $CD4^+CD25^+$ effector T cell population. They are distinguished from these cells by the expression of the forkhead/winged helix family transcription factor forkhead box P3 (FoxP3), FR4 and constitutive expression of CTLA-4 (CD152).[31–37] In mice, the lymphocyte activation gene-3 (LAG-3) is also constitutively expressed on the surface of Treg cells.[38] In addition to the thymic-derived or “natural” (nTregs), Tregs can also be induced (iTregs) in the periphery. iTregs can be $FoxP3^+$, but, under a variety of conditions, they develop in the periphery from conventional $CD4^+$ T cells after antigen stimulation.[39] T helper 3 (Th3) cells are a population of iTreg cells that produce larger amounts of TGF-β and occur primarily after exposure to antigen through the oral route.[40] T regulatory 1 (Tr1) cells are a subset of iTregs, and are similar to nTregs in that they are both anergic *in vitro* and express CTLA-4. Induction of these cells can occur through stimulation by immature DC, in the presence of IL-10.[41]

Tregs are able to exert their suppressive effects on immune responses by limiting activation, proliferation and survival of various immune cells. These functions are exerted through direct cell–cell contact and cytokine production, and the depletion of Tregs in mice leads to autoimmunity.[42, 43] This autoimmunity can be prevented by the administration of $CD4^+CD25^+$ T cells to newborn mice.[43] In humans, a mutation in the FoxP3 gene causes immune dysregulation, polyendocrinopathy and enteropathy, and X-linked syndrome, which results in the early onset of one or more autoimmune diseases.[44, 45]

$FoxP3^+$ Tregs in MS/EAE

A role for Tregs in the modulation of neuroinflammatory responses and maintenance of self-tolerance is supported by both animal and human studies. In EAE, enhanced disease severity and mortality were observed when Tregs were depleted after treatment with anti-CD25 antibodies.[46] Adoptive transfer of Tregs into mice immunized with myelin oligodendrocyte glycoprotein (MOG) 35–55 conferred significant protection from EAE induction.[47] In addition, the authors observed increased frequencies of $MOG_{35–55}$-specific Th2 cells and decreased CNS infiltration.[48] In a recent report, Joller *et al.* identified a subpopulation of Tregs that express a co-inhibitory molecule, TIGIT, which on ligation induced expression of the effector molecule fibrinogen-like protein 2, and induced Treg-cell mediated suppression of Th1 and Th17 cells.[49] The aforementioned studies show that Tregs inhibit priming and

expansion of pathogenic Th1 and Th17 cells in the peripheral lymphoid organs, thus directly contributing to the maintenance of self-tolerance.

Studies of the peripheral blood from MS patients showed an important role for Tregs in disease pathogenesis. The Hafler group reported defects in the function, but not the frequency, of $CD4^+CD25^{high}$ Treg populations in RR-MS patients.[50] Using a coculture assay with circulating $CD4^+CD25^{high}$ Tregs from relapsing–remitting MS (RR-MS) patients, they showed that these cells had a reduced capacity to suppress polyclonally activated $CD4^+CD25^-$ conventional T cells.[50] Hafler's group and others have shown that this functional alteration in Treg suppression in MS patients is due to a reduced expression of FoxP3 at both the mRNA and protein level.[51, 52] In addition, the Treg population in MS patients tends to have a lower proportion of newly generated cells from the thymus, as evidenced by a lower proportion of $CD31^+$ and $CD45RA^+$ cells, and a higher proportion of older, memory cells with the $CD45RO^+$ phenotype.[53, 54]

Furthermore, these dysfunctional Tregs in MS patients cannot carry out the same suppressive functions as those in healthy individuals. Extracellular adenosine triphosphate (ATP) acts as a pro-inflammatory cytokine, which can induce the secretion of IL-1β and IL-23, and lead to increased production of pathogenic Th17 cells.[55, 56] CD39 acts as an ectoenzyme and hydrolyzes ATP to adenosine monophosphate. Additionally, $CD39^+$ Tregs have been shown to catabolize extracellular ATP, resulting in decreased secretion of these inflammatory cytokines and a smaller Th17 population.[57] MS patients have decreased amounts of $CD39^+$ Tregs when compared with healthy controls.[55, 58] Recently, it has been shown that fingolimod is able to increase the proportion of $FoxP3^+$ $CD39^+$ Tregs within the $CD4^+$ T cell population in patients with RR-MS.[59]

These alterations in Treg function might also be explained by a production of pro-inflammatory cytokines. Studies by Dominquez-Villar *et al.* compared the Tregs in patients with untreated RR-MS with healthy controls, and found an increased proportion of IFN-γ producing Tregs in the patients, making these cells similar to pathogenic Th1 cells.[60] These cells still expressed FoxP3, while also expressing the transcription factor TBET and CXCR3, both of which are usually expressed on Th1 cells.[60] These Th1-like cells were also found to be less suppressive *ex vivo.* Many MS therapies have been shown to target the Treg population and to reverse the suppressive defects observed.[61–63] Interferon β-1a and glatiramer, in particular, have been shown to expand the native Treg population *in vivo* and to partially restore their suppressive function.[64, 65] The Hafler group also showed that treatment with IFN-β1a reduced the number of Tregs with the Th1-like phenotype.[60]

Despite the overwhelming evidence of the role of Tregs in the maintenance of self-tolerance in MS, their function in the CNS remains unclear. It has been previously shown that microglia can recruit Tregs into the CNS through the production of CCL22, which interacts with CCR4, expressed on the surface of Tregs.[66, 67] In both active and passive models of EAE, an accumulation of Tregs in the CNS correlates with disease recovery, and Tregs from these animals suppressed MOG-specific T cell responses by limiting IFN-γ production.[68, 69] Studies by Korn *et al.* reported that Tregs accumulated in the inflamed CNS, but lacked suppressive capabilities because of the presence of IL-6 and TNF-α.[70] Importantly, IL-6

signaling inhibits the conversion of conventional T cells into FoxP3$^+$ Tregs *in vivo*.[70, 71] These results suggest that Tregs have a role in suppressing inflammation in the CNS, which is dependent on the local inflammatory setting and the effector T cell populations.

Type 1 regulatory T cells in MS and EAE

The highly immunosuppressive subset of iTregs, Tr1 cells, is believed to play a significant role in the maintenance of immunological tolerance.[72, 73] Tr1 cells are a subset of regulatory cells in which FoxP3 and CD25 are not expressed. These cells can be generated in the absence of nTregs, suggesting that Tr1 cells might be developmentally distinct.[74, 75] These cells are characterized by their secretion of high amounts of IL-10 and the expression of the newly defined cell surface markers, CD49b and LAG-3, which can be found on both human and murine cells.[76] Tr1 cells have a low proliferative capability, but can expand in the presence of IL-2 and IL-15 because of high expression of these receptors after activation.[77] The main mechanisms of Tr1-mediated suppression are secretion of a high level of IL-10 and the killing of APC by granzyme B.[78, 79]

Recently, the immunomodulatory cytokine, IL-27, has been identified as a differentiation factor for the generation of both human and murine IL-10-producing Tr1 cells.[80–82] T cell activation in the presence of IL-27 induces the transcription factors c-Maf and the aryl hydrocarbon receptor. Activation of these transcription factors is crucial for the differentiation and secretion of IL-10 from developing Tr1 cells.[83–85] Studies by Gandhi *et al.* have shown that aryl hydrocarbon receptor plays an important role in Tr1 differentiation in humans, and suggests that this could be a possible mechanism to target the generation of iTregs in autoimmune disease.[86] Tr1 cells have also been shown to play a suppressive role in MS and EAE. In EAE, the transfer of *in vitro* generated OVA-specific Tr1 cells prevented the development of neurological symptoms when OVA peptide was injected intracranially.[75] In EAE, the *in vivo* induction of Tr1 cells was achieved using soluble myelin basic protein (MBP) p87–99, which reversed ongoing disease in rats immunized with MBP.[87]

In MS patients, Astier *et al.* showed that Tr1 cells isolated from these patients had impaired IL-10 production when compared with healthy controls. Although Tr1 cells were impaired, the levels of IFN-γ production remained consistent.[88, 89] Additionally, Martinez-Forero *et al.* found that IL-10-mediated suppressive effects of Tr1 cells were reduced in *ex vivo* samples isolated from MS patients.[90] Taken together, these studies suggest that Tr1 cells might play a protective role in MS.[88]

DCs and IL-27 in immune tolerance induction

DC prime T cells for inflammatory responses, but these cells can have a dual role and also promote the development of tolerance. Evidence supporting this dual role comes from studies in which DC were completely ablated, which resulted in fatal autoimmunity.[91] To further support their tolerogenic role, Yamazaki *et al.* showed that DC can induce expansion of the CD25$^+$ CD4$^+$ T cell population;[92] in addition, Darrassee-Jeze *et al.* showed that a decrease in DC leads to a decrease in Tregs.[93] iDC, which have low expression of major

histocompatibility class II and costimulatory molecules, are able to induce tolerance through T cell anergy.[93]

One of the main mechanisms by which DC contribute to tolerance induction is through the induction of Tregs. This process is dependent on TGF-β, and absence of this cytokine has been shown to reduce the ability of DC to stimulate a pathogenic response from T cells.[94] In EAE, the absence of TGF-β resulted in more severe EAE in mice. In addition, DC were able to stimulate the expansion of $Foxp3^+$ Tregs in the presence of TGF-β and retinoic acid. When these TGF-β-induced Tregs were expanded and separated, they were able to suppress EAE.[95]

DC also exert some of their tolerogenic effect through the immunomodulatory cytokine, IL-27, a member of the IL-12 family of cytokines. IL-27 is composed of EBV-induced molecule 3 (EBI3), an IL-12p40 homolog, and p28, an IL-12p35 homolog, which non-covalently associate with EBI3, with its main sources of IL-27 being DC and macrophages.[96] The IL-27 receptor is composed of WSX1 and gp130 (a part of the IL-6 receptor complex)[97,98], and signaling through IL-27 receptors results in phosphorylation of Jak and Stat proteins, including Jak1, Jak2, Tyk2, Stat1, Stat3, Stat4 and Stat5.[99–102]

Initially, IL-27 was thought only to promote Th1 cell differentiation[99], but studies have shown that this cytokine plays a crucial role in limiting Th17 cell differentiation by suppressing RORγt, a key transcription factor for Th17 cells.[103] Mice deficient in IL-27 signaling, either EBI3 or WSX, have increased IL-17 and are more susceptible to EAE.[103, 104] Data from our laboratory show that IL-27 has a suppressive effect on encephalitogenic Th17 cells and the effector phase of EAE.[105] The suppression of Th17 cells and EAE by IL-27 is associated with IL-27-induced production of IL-10 in T cells, including Th1 but not Th17 cells.[81] IL-27 induction of the IL-10-producing immunosuppressive Tr1 cell subset is STAT1- and STAT3-dependent, and induces the transcription factor, c-maf, which can in turn activate IL-21 production by T cells.[99] Along with IL-21, IL-27 can also induce upregulation of IL-21 receptor, which can act in an autocrine manner to promote Tr1 cell growth and differentiation.[82] Xu *et al.* showed that in the absence of IL-21 signaling in T cells, IL-27 driven generation of Tr1 cells and IL-10 cytokine production is inhibited.[106] However, IL-27 alone can increase IL-10 production for only a limited period of time. Studies by Awasthi *et al.* showed that simultaneous stimulation with IL-27 and TGF-β caused a long-lasting production of IL-10 by T cells.[80]

Because of the significant immunomodulatory properties of IL-27, it is thought to have a therapeutic potential in MS. Fitzgerald *et al.* showed that exogenous IL-27 could reduce the severity of EAE when delivered by subcutaneous osmotic pump. IL-27 reduced infiltration of Th17 cells into the CNS and inhibited IL-17A production by myelin-specific T cells.[105] A recent study by Mascanfioni *et al.* showed that IL-27 signaling in DC upregulates CD39 expression and limits EAE severity by reducing the extracellular levels of ATP. They showed that these IL-27-conditioned DC can suppress EAE when administered i.v. in a chronic EAE model.[107] These studies show the variety of potential therapeutic uses for IL-27.

Mediators of tolerance

I.v. tolerance

In EAE, antigen-specific tolerance can be achieved by i.v. administration of encephalitogenic antigens.[108] Treatment of $CD4^+$ T cells with antigen has been shown to reduce the production of cytokines and decrease the extent of their antigen-specific proliferation.[108, 109] This is augmented by multiple treatments with antigen, especially for memory T cells, which might not be fully suppressed on interaction with only one round of antigen.[110] Induction of i.v. tolerance is associated with the disappearance of transgene-positive T cells from peripheral lymphoid tissues.[110] In an adoptive transfer model of EAE, multiple i.v. injections of MBP to recipient mice after transfer of MBP-specific TCR transgenic T cells prevented disease.[111] Administering antigen i.v. is more effective in inducing tolerance after disease onset when compared with mucosal routes.[112, 113]

Antigen-specific tolerance is produced by a multifactorial mechanism. Mechanisms of tolerance induction include clonal deletion, anergy, induction of regulatory T cell populations and upregulation of CTLA-4.[12–14,108,114,115] Induction of i.v. tolerance is associated with the disappearance of transgene-positive T cells from peripheral lymphoid tissues. Our group has shown that i.v. injection of the myelin autoantigen suppressed antigen-specific Th1 and Th17 responses, and induced nTregs and IL-10-producing Tr1 cells (Figure 1).[116,117] Li *et al.* discovered that i.v. tolerized mice had increased proportions of tolerogenic DC in their spleens and CNS. When these DC were transferred into recipient mice with ongoing EAE, they were able to suppress disease by inhibiting $MOG_{35–55}$-specific T cell proliferation, and by inducing nTregs and the regulatory cytokines, IL-10 and IL-27.[117]

I.v. tolerance is currently being explored as a potential therapeutic for the treatment of MS. Common methods of introducing antigen i.v. include using a soluble peptide or linking the peptide to splenocytes or microparticles. However, the use of soluble peptide i.v. carries the risk of an anaphylactic reaction, which has been shown to develop in several strains of mice.[118] The administration of antigen-coupled splenocytes or microparticles is not known to cause these types of reaction and thus provides a safer method of peptide delivery.

Antigens of interest can be coupled to splenocytes using 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide.[119] Infusion of these antigen-linked splenocyctes has been shown to induce the rapid production of IL-10 and TGF-β, leading to anergy of pathogenic T cells and the induction of Tregs.[119, 120] It is thought that antigen-linked splenocyctes undergo rapid apoptosis after i.v. infusion, and this apoptotic debris is taken up by marginal zone macrophages, leading to the production of IL-10.[121] Additionally, the antigen-linked splenocyctes might interact directly with autoreactive T cells, leading to anergy.

Recently, Lutterotti *et al.* examined these principles of tolerance in a translational phase 1 trial in human subjects.[122] They studied nine patients with RR-MS or secondary progressive MS who were reactive to one of seven myelin proteins. For this trial, they administered a one-dose cocktail of myelin proteins, chemically coupled to the patients' own peripheral

blood mononuclear cells. They studied escalating doses from 1×10^3 and 3×10^9 peripheral blood mononuclear cells, and patients were observed over a 3-month period post-infusion. The primary purpose of the study was to establish feasibility, safety and tolerability of this regimen, which, overall, proved to be feasible, with generally mild side-effects. At higher doses, the study authors noted a decrease in T cell response to the myelin proteins indicative of successful tolerization.

The production and storage of leukocytes is, however, expensive and problematic. In a recent study, Getts *et al.* developed a new method of i.v. tolerance induction by covalently coupling myelin-specific antigens to biodegradable microparticles.[119] These microparticles were made of a relatively inexpensive polystyrene or poly(lactide-*co*-glycolide) measuring 500 nm in diameter. The antigen-linked particles were then i.v. injected into mice suffering from relapsing EAE, leading to significant amelioration of clinical disease.[119] These antigen-coupled microparticles suppressed Th1 and Th17 cell proliferation, and the primary mechanism of tolerance induction observed was T cell anergy. Hunter *et al.* continued this line of research, and found that i.v. administration of antigen-linked nanoparticles led to amelioration of disease.[123] These studies highlight the potential therapeutic uses of antigen-specific i.v. tolerance for the treatment of MS.

Galectin-1

The role of lectin-binding proteins and tolerance induction in autoimmunity is an emerging field. It has been observed that galectin-1, a member of the β-galactosidase binding protein family, plays an important immunoregulatory role in EAE.[124, 125] Galectin-1 is synthesized and secreted by nTregs and iTregs, as well as by activated B cells and T cells, inflammatory macrophages, and decidual natural killer cells, and it is upregulated on TCR activation.[126, 127] Mice deficient in galectin-1 have augmented Th1 and Th17 responses, and are more susceptible to autoimmune diseases and immune-mediated fetal rejection when compared with wild-type mice.[125]

Ilarregui *et al.* showed that galectin-1 significantly increased the development of Tr1 cells *in vitro* through the generation of tolerogenic DC.[124] These tolerogenic DC produce IL-27, which acts on T cells and promotes their IL-10 production. Additionally, treatment of naïve T cells with recombinant mouse galectin-1 induced Tr1 cells, which were able to suppress Th1- and Th17-mediated inflammation.[124] Galectin-1 is upregulated in Treg cells on TCR activation, and blockade of galectin-1 binding reduces inhibitory activity of human and mouse Tregs.[128–130]

Conclusions

MS is a complex disease with the involvement of multiple pathways that can lead to CNS inflammation. Studies on the primary pathogenic (Th1/Th17) and the tolerogenic (Treg/Tr1/IL-27-producing) mechanisms used to combat these pathogenic responses have considerably enhanced our knowledge of disease pathogenesis. By continuing to study their underlying mechanisms, we can further understand the complex balance these cells must maintain to preserve homeostasis in the healthy individual.

There is evidence that the currently established medications for MS work, at least in part, by using these tolerogenic mechanisms. Recent findings have shown the potential therapeutic uses of tolerogenic cells and antigen-coupled nanoparticles to treat EAE and MS. By continuing to explore this field of research, we should be able to develop improved therapeutic interventions that better target the underlying dysfunction, potentially resulting in more effective therapy and with fewer adverse events. In short, induction of these tolerogenic immune cell subsets can help to direct the future of MS therapeutics.

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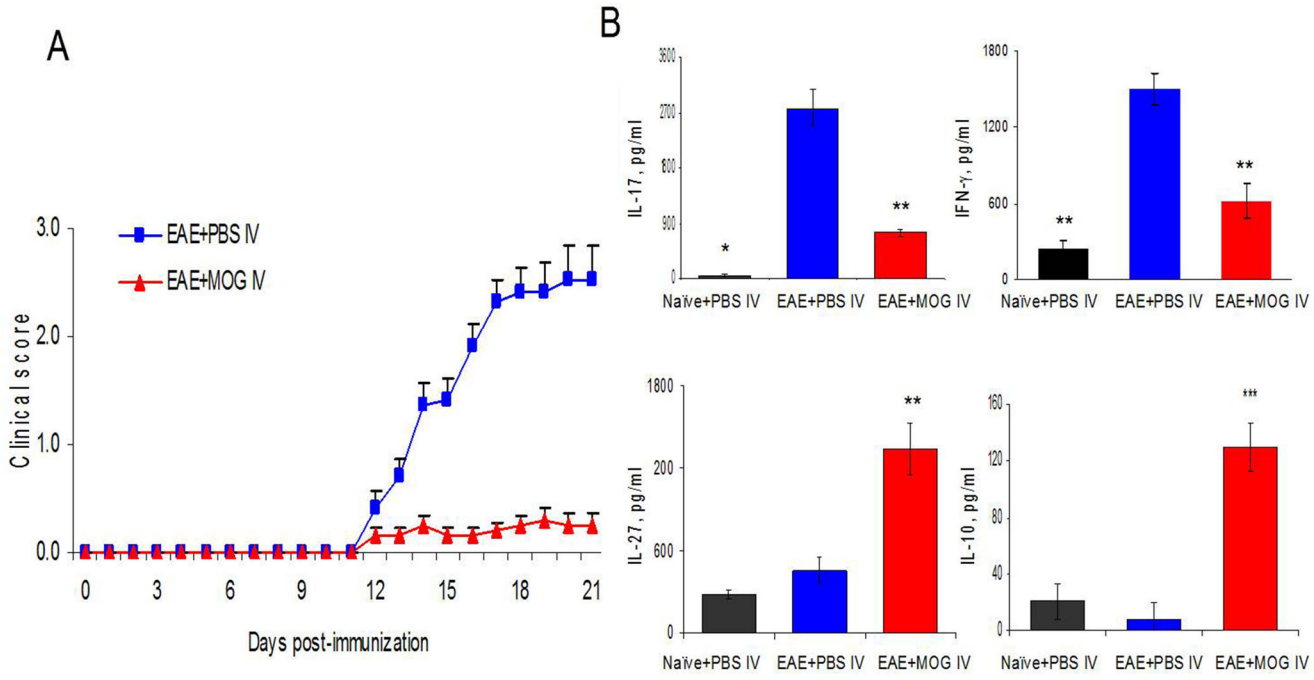

Figure 1.

Myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) i.v. inhibited the development of experimental autoimmune encephalomyelitis (EAE). EAE was induced in C57BL/6 mice by immunization with $MOG_{35–55}$/CFA, and pertussis toxin was given on days 0 and 2. At days 0, 3 and 6 p.i., mice were i.v. injected with 200 μg $MOG_{35–55}$ to induce tolerance with the same volume of phosphate-buffered saline (PBS) to serve as control. (a) Daily clinical scores of each mouse group (10 mice each group; $P < 0.01$). (b) Splenocytes in duplicate from mice in (a) were isolated at day 21 p.i., and cultured with 10 μg/mL $MOG_{35–55}$ for 72 h. Production of interleukin (IL)-17, interferon (IFN)-γ, IL-27p28 and IL-10 in supernatants was analyzed by enzyme-linked immunosorbent assay. Data were pooled from two independent experiments and presented as mean value ± SEM ($n = 10$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.