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Journal of Neuroimmunology 308 (2017) 6–11

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Review article

Roles of regulatory T cells and IL-10 in virus-induced demyelination

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ARTICLE INFO

Article history:
Received 29 November 2016
Accepted 3 January 2017

Keywords:
Demyelination
Tregs
IL-10
Mouse hepatitis virus
Theiler's murine encephalomyelitis virus

ABSTRACT

Neurotropic viruses are important causes of morbidity and mortality in human populations. Some of these viruses preferentially infect oligodendrocytes in the white matter, causing either direct lysis of infected cells, or more commonly myelin damage as a consequence of the host immune response to the virus. Virus-induced demyelination has similarities to the human disease multiple sclerosis. To study this disease process in experimental animals, mice are infected, most commonly, with neurotropic strains of mouse hepatitis virus, a coronavirus or Theiler's murine encephalomyelitis, a picornavirus. While the diseases caused by these two viruses differ in some aspects, in both cases demyelination is a major consequence of the infection. As in autoimmune disease, therapeutic interventions that diminish an overactive immune response would be useful. However, unlike autoimmune disease, complete suppression would result in unchecked virus replication, generally leading to more severe disease. Here we discuss two approaches that dampen but do not fully suppress the host immune response. Regulatory T cells, especially those that are specific for antigens recognized by pathogenic T cells, and IL-10 are two anti-inflammatory/pro-resolution factors that demonstrate efficacy in experimental models of virus-induced demyelination and may be useful in patients infected with viruses that cause demyelination.

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Contents

Virus induced demyelination is observed in humans after infection with several neurotropic viruses, including JC virus (Progressive Multifocal Leukoencephalopathy, PML), measles virus (subacute sclerosing panencephalitis) and HIV (Stohlman and Hinton, 2001). Demyelination in mice can be induced experimentally by infection with pathogens such as a murine coronavirus (CoV, mouse hepatitis virus, MHV), Theiler's encephalomyelitis virus (TMEV) and Semliki Forest virus (Stohlman and Hinton, 2001). Demyelination may result from direct virus lysis of oligodendrocytes, the cell responsible for myelin production. This mechanism is likely operative in PML, which occurs in immunocompromised patients with low CD4 T cell numbers (Bowen et al., 2016). More commonly, demyelination occurs as a consequence of virus clearance mediated by the host immune response. This is illustrated in mice infected with MHV or TMEV, and is generally T cell-mediated. In mice infected with MHV or TMEV, mice that are irradiated or lack T cells or B cells ((RAG1$^{-/-}$ (recombination activation gene 1) or SCID (severe combined immunodeficiency)) develop extensive and widespread infection throughout the brain and spinal cord but little evidence of myelin destruction or of macrophage infiltration, which are the final scavenger cells for removing myelin debris (Houtman and Fleming, 1996; Tsunoda and Fujinami, 2010; Wang et al., 1990; Wu et

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http://dx.doi.org/10.1016/j.jneuroim.2017.01.001
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al., 2000). However, if T cells are infused into mice a few days after the infection is established, demyelination and extensive macrophage infiltration into the white matter are observed. Myelin destruction similarly occurs in the human disease, multiple sclerosis (MS). While MS is considered an autoimmune disease, virus infections are associated with disease initiation and exacerbation (Dendrou et al., 2015; Noseworthy et al., 2000). Although the details of MS remain murky, T cells that recognize myelin are considered a key components of the destructive immune response. Thus, in both virus-induced demyelination and autoimmune demyelination, either dampening or eliminating, respectively, the T cell response, would be expected to improve outcomes. Of course, the pathogenesis of virus-induced and autoimmune demyelination is fundamentally different. In the case of viral infections of the CNS, virus-specific T cells are essential for clearance so that complete suppression of the T cell response would be deleterious while in autoimmune disease, complete suppression of the myelin-specific T cell response is a reasonable goal. A second consideration in both diseases is that ideally only pathogenic T cells would be targeted. In many cases, therapeutic interventions that suppress demyelination in patients with MS are more broadly immunosuppressive than is desirable (Noseworthy et al., 2000; Ransohoff et al., 2015). While these approaches are sometimes effective in suppressing MS progression or exacerbation, they also make patients susceptible to developing opportunistic infections, including PML. Thus, a major goal of therapy in MS patients is to target the myelin-specific T cell response so that the general ability to control infections is not impaired. Similar considerations also occur in the context of virus-induced demyelination. Additionally, broad immunosuppression would allow no or less control of the ongoing virus infection, making it even more critical that suppression of the immune response be targeted. Consequently, recent research efforts have focused on increasing the specificity of immunosuppression, with the goal of increasing efficacy without causing generalized immunosuppression.

Once it became clear that T cell recognition of myelin antigen was key in the development of MS, efforts were made to block disease by manipulating the T cell response. One approach that was successful in an animal model of MS, experimental autoimmune encephalomyelitis (EAE) was administration of myelin-specific peptides that specifically blocked pathogenic T cells (Hohlfeld, 1997; Smilek et al., 1991). However, this approach requires that therapy be individualized, with a group of peptides administered that matched the targets recognized in each patient of the patients received. This approach is currently being used in clinical trials (Bittner and Wiendl, 2016). Other approaches, which are not specific for myelin-related T cells include treatment with agents such as natalizumab or fingelimod, which block T cell entry into the CNS, or egress from draining lymph nodes, respectively (Bittner and Wiendl, 2016; Ransohoff et al., 2015). These interventions also increase the likelihood of opportunistic infections.

Thus, virus-induced and autoimmune demyelination share common pathogenic mechanisms, although dampening the immune response in the former must not allow uncontrolled virus replication. Here, using two models of virus-induced demyelination, we discuss two interventions that suppress the immune response, without greatly impairing control of the virus by the host. We focus primarily on the role of regulatory CD4 T cells (Tregs) in suppressing excessive pro-inflammatory immune responses. Tregs have been extensively studied in several autoimmune and infectious diseases. They are generated in the thymus (natural or nTreg) or in some cases, in the periphery from non-Treg CD4 T cells (induced or iTreg) (Vignali et al., 2008). iTreg at mucosal sites have been studied most intensively (Campbell, 2015) and whether they have any role with the central nervous system (CNS) remains to be determined. Tregs can be identified by expression of the transcription factor Foxp3, which is required for their development and function (Fontenot et al., 2005). Tregs effect immunosuppression by expression of several factors, some of which are soluble (IL-10, IL-35, adenosine) while others are cell-associated (CTLA-4, CD39/CD73, CD25) (Vignali et al., 2008). Tregs have been and are being studied extensively in the context of EAE and in human studies (Bittner and Wiendl, 2016; Zozulya and Wiendl, 2008). While Tregs may be useful in patients, these Tregs are generally not specific for a myelin epitope. Further, in mice with experimental autoimmune encephalomyelitis (EAE), the inflammatory milieu in the affected brain diminished T cell function, *via* the action of TNF and IL-6 (Korn et al., 2007), although this appears to be less of an issue in the context of virus-induced demyelination (see below). IL-10, which is expressed by Tregs and Tr-1 cells also plays a role in diminishing myelin destruction in virus-infected mice and will be discussed briefly.

1. MHV

MHV is a β-CoV within the *Coronaviridae* family (Masters and Perlman, 2013). Coronaviruses are positive-sense single-stranded RNA viruses with large genomes of approximately 31 kb. Coronaviruses, including various strains of MHV, are neurotropic, hepatotropic, pneumotropic or enterotropic, causing a variety of diseases in humans and domestic and companion animals. For the purpose of this review, we will focus on those MHV variants that cause acute and chronic demyelinating encephalomyelitis. Neurotropic strains of MHV tend to cause similar diseases in all strains of mice, except SJL mice, which encode a variant of the host cell receptor (CEACAM1b in lieu of CEACAM1a, Carcinoembryonic Antigen-related Cell Adhesion Molecule 1) (Ohtsuka et al., 1996; Williams et al., 1991). Studies of MHV generally use the neurotropic JHMV strain or its attenuated variant, J2.2-V-1 or the hepatotropic/neurotropic A59 strain (Bergmann et al., 2006; Cowley and Weiss, 2010; Lavi et al., 1984). As noted above, several studies indicated that myelin destruction in MHV-infected mice occurs during virus clearance. Thus, transfer of T cells to J2.2-V-1-infected RAG1$^{-/-}$ mice resulted in complete or partial virus clearance and demyelination within a few days of transfer, even though no demyelination was noted in the absence of transfer (Wu et al., 2000). Spleen cells from MHV-immunized mice were most effective in inducing demyelination. Transfer of splenic cells from naïve mice to infected mice could also induce demyelination, but only in a minority of mice, likely reflecting the presence of only a small number of virus-specific T cells in the naïve spleen (Houtman and Fleming, 1996). Additionally, while both virus-specific CD4 and CD8 T cells individually induced myelin destruction, transfer of CD4 T cells resulted in a rapidly fatal encephalomyelitis while CD8 T cell transfer caused extensive demyelination without clinical signs of encephalomyelitis (Wu et al., 2000). Lethal disease after CD4 T cell transfer was largely TNF driven because transfer of TNF$^{-/-}$ CD4 T cells resulted in greatly diminished morbidity, mortality and demyelination, with eventual virus clearance in many of the mice (unpublished data).

The largest fraction of the MHV-driven CD4 T cell response in C57BL/6 mice is directed against an epitope in the transmembrane (M) protein (M133) (Haring et al., 2001). Infection of mice with neurovirulent JHMV mutated in this epitope (rJ.M$_{Y135Q}$) increased survival from 0% to 100%, while reinsertion of an exogenous CD4 T cell epitope (LLO190) from *Listeria monocytogenes* resulted in increased mortality (Anghelina et al., 2006). These effects were not observed in BALB/c mice infected with rJ.M$_{Y135Q}$ since this epitope is not recognized in this strain of mouse or in RAG1$^{-/-}$ (recombination activation gene-1), which lack all T and B cell responses. Previous studies had shown that treatment of MHV infected mice with CD4 depleting antibody resulted in decreased survival (Williamson and Stohlman, 1990), so collectively these results suggested that dampening, but not eliminating the virus-specific CD4 T cell response would be protective. Thus, subsequent efforts were focused on diminishing the epitope M133-response.

1.1. Role of regulatory T cells

Initially, we focused on the possibility that Tregs would be useful in suppressing an excessive host immune response, thereby diminishing

disease. We transferred bulk populations of Tregs into MHV-infected C57BL/6 or RAG1$^{-/-}$ mice and found that transferred cells improved survival, diminished the number of inflammatory cells infiltrating into the CNS and decreased the amount of demyelination (Anghelina et al., 2009; Trandem et al., 2010). The transferred Tregs likely functioned in the draining lymph nodes (cervical lymph node, CLN) because they were not detected in the infected brain. Further, dendritic cell activation and cytokine expression in the lymph nodes and brain were decreased in mice that received conventional CD4 T cells (Tconv) and Tregs compared to those that received just Tconv. In addition, depletion of Tregs prior to infection resulted in increased demyelination at later times after infection (de Aquino et al., 2013). Other studies indicated that Tregs had additional effects in the draining lymph nodes, suppressing the outgrowth of self-reactive T cells that could lead to autoimmunity. Self-reactive T cells were detected in MHV-infected mice, but their presence did not lead to autoimmune demyelination (Cervantes-Barragan et al., 2012; Savarin et al., 2015). Both Foxp3 Tregs and IL-10-secreting Type 1 regulatory (Tr1) cells (see below) were required for suppression of self-reactive Tconv (Savarin et al., 2016). However, Tregs functioned specifically in the CLN, such that their depletion resulted in increased cellular infiltration and inflammatory cytokine production. Collectively, these results showed that bulk populations of Tregs, which presumably contained only small numbers of virus-specific Tregs, were able to ameliorate virus-induced demyelination as well as suppress self-reactive CD4 T cell numbers, largely by functioning in the CLN.

At the time that these initial experiments were completed, there were few if any reports of virus-specific Tregs. One might expect that these cells would be more suppressive than bulk populations of Tregs, since they would be strongly activated by signaling through the T cell receptor. As a first step to investigating this possibility, brains and spinal cords harvested from J2.2-V-1-infected mice were examined for the presence of virus-specific Tregs (Zhao et al., 2011). We identified M133-specific CD4 T cells in the MHV-infected brain, using an MHC class II/peptide M133 tetramer. Further, we showed that these cells at the site of inflammation, the brain, expressed two pro-inflammatory cytokines, IFN-γ and TNF. Previous work had shown that Treg expression of T-bet was required for optimal trafficking and function of Tregs at sites of Th1 inflammation (Koch et al., 2009); our results now showed that these cells also expressed Th1 cytokines. These cells remained immunosuppressive, however, although subsequent experiments suggested that these cells might be slightly impaired in their immunosuppressive ability compared to cells not expressing these cytokines (data not shown). These cells were, at least in part, thymically derived because they could be detected in naïve Foxp3-GFP^{+} cells (Zhao et al., 2011). It should be noted that the number of virus-specific Tregs was very small. Approximately 10% of all CD4 T cells were Tregs and of the Tregs, 5–10% were specific for epitopes M133 plus S358, a second, less dominant CD4 T cell epitope recognized in infected C57BL/6 mice.

To further investigate the importance of M133-specific Tregs, we engineered mice that transgenically expressed a T cell receptor (TCR) specific for epitope M133 (Zhao et al., 2013). These mice contained a small number of M133-specific Tregs, which could be identified by GFP expression and were useful as tracers in infected C57BL/6 mice. Mice were crossed to CD45 and CD90 (Thy1) congenic mice, so that transferred Tregs and conventional CD4 T cells (Tconv) could be identified by CD45 and/or CD90 expression, while Tregs expressed GFP (Zhao et al., 2014). Using M133-specific Tregs and Tconv in a set of adoptive transfer experiments, we first showed that M133 Tregs but not bulk populations entered the brain, consistent with previous experiments in which only bulk populations of Tregs were analyzed (Trandem et al., 2010). Second, M133 Tregs, like M133 Tconv, proliferated in the draining lymph node, although with a one day lag compared to Tconv. M133 Treg proliferation preceded cell entry into the CNS. M133 Tconv and Treg proliferation occurred primarily in the CLN and deep CLN (DCLN) and not in other peripheral lymphoid tissue, because after treatment with FTY720, which inhibits cell egress from lymph nodes, dividing cells were detected only in these lymph nodes. The transferred Tregs functioned at several steps to dampen M133 Tconv accumulation in the brain (Fig. 1). M133 Tregs diminished the proliferation of Tconv in the DCLN and also decreased expression of activation markers on the cells. Notably, levels of CD69 and CD25, the two activation markers that were measured, were identical on nondividing cells, demonstrating that the transferred Tregs primarily affected cells after initial activation occurred. M133 Tregs also inhibited M133 Tconv egress from the DCLN, by decreasing expression of CXCR3, required for entry into the infected CNS, on these cells. Most importantly, transferred M133 Tregs, but not bulk Tregs enhanced survival and decreased the percentage of M133 and, to a lesser extent, S358 Tconv but not virus-specific (S510-specific) CD8 T cells in the infected brain. Virus clearance was not delayed in mice that received M133 Tregs. Microglia activation, as assessed by MHC class II expression, was decreased by M133 Treg transfer. These results suggested that M133 Tregs preferentially suppressed M133 proliferation and function. To confirm this, a set of experiments were performed *in vitro* that confirmed preferential suppressive effects of M133 Tregs on

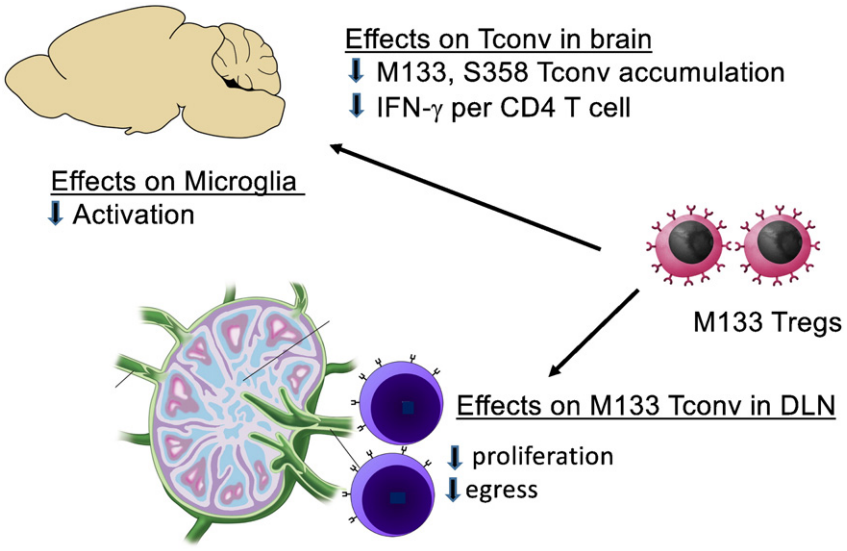

Fig. 1. M133 Tregs suppresses immune response in draining lymph nodes and brain. M133 Tregs inhibit M133 Tconv proliferation in and egress from cervical and deep cervical lymph nodes (DLN). M133 Tregs also decrease accumulation of M133 and S358 Tconv in the brain as well as decreasing microglia activation (Zhao et al., 2014).

M133 Tconv > S358 Tconv > S510 CD8 T cells. Together, these results indicated that MHV-specific Tregs were present in the draining lymph nodes and brain and functioned at both sites to suppress an excessive pro-inflammatory T cell response. Treg-mediated suppression of Tconv responding to the cognate epitope was most potent.

1.2. Role of IL-10

In addition to Tregs, Tr1 and IL-10-expressing CD8 T cells also play a role in controlling the pro-inflammatory T cell response. Initial studies showed that the complete absence of IL-10 increased morbidity and mortality in J2.2-V-1 infected mice. Inflammatory cell infiltration and pro-inflammatory cytokine production were increased in infected mice and likely contributed to increased disease severity (Lin et al., 1998). Next, the source for IL-10 in infected mice was examined by flow cytometry (Puntambekar et al., 2011; Trandem et al., 2011b). At the peak of infection, both CD4 and CD8 T cells expressed IL-10, although expression by CD8 T cells was relatively transitory and decreased as inflammation subsided. IL-10 expression by CD4 T cells persisted even after mice recovered from acute disease. IL-10 was produced by the most activated virus-specific CD8 T cells and was postulated to serve as an autocrine "break" on a potentially hyperinflammatory T cell response (Trandem et al., 2011b). Consistent with this notion, virus was cleared more rapidly from infected brains in the absence of IL-10. Further support for the transitory nature of IL-10 production by these cells was the loss of IL-10 expression after adoptive transfer of IL-10-expressing CD8 T cells into infected hosts. Finally, transfer of IL-$10^{+/+}$, but not IL-$10^{-/-}$ CD8 T cells modestly reduced demyelination after transfer into J2.2-V-1-infected IL-$10^{-/-}$ mice. These results showed an important role for CD8 T cell-expressed IL-10 in diminishing morbidity, mortality and demyelination and also suggested that IL-10 may be most important during the early stages of the infection. To address this more precisely, a recombinant version of J2.2-V-1 that expressed IL-10 (rJ2.2-IL-10) was developed and used to infected C57BL/6 and IL-$10^{-/-}$ mice (Trandem et al., 2011a). Viral expression of IL-10 was maximal at day 2 after infection of C57BL/6 mice but returned to baseline by day 14. Viral expression of IL-10 delayed virus clearance modestly, but also substantially decreased the level of demyelination. Levels of cellular infiltrates, microglial activation and proinflammatory cytokine and chemokine expression were diminished in mice infected with rJ2.2-IL-10 compared to control virus. Infection of IL-$10^{-/-}$ mice with rJ2.2-IL-10 but not control virus resulted in decreased mortality, weight loss, clinical disease and demyelination with delayed kinetics of virus clearance. To extend these experiments, IL-10 was neutralized at early and late times after infection of C57BL/6 mice. IL-10 neutralization from days 1–11 but not days 5–11 after infection resulted in increased demyelination, suggesting that IL-10 was required at early times after infection, even though demyelination was not evident until later in the disease course. In addition to effects on the T cell and macrophage response, IL-10 has also been implicated in tissue repair in the CNS. Thus, in the absence of IL-10, astrocyte activation and glial scar formation, which limit ongoing demyelination, were hindered resulting in more extensive myelin destruction (Puntambekar et al., 2015). Together these results show that IL-10 is not only anti-inflammatory, modulating a potentially harmful host response and only modestly delaying virus clearance, but also affects other aspects of the resolving phase of inflammation after virus is cleared.

2. TMEV

TMEV is a single stranded RNA cardiovirus in the *Picornaviridae* family. Isolated in the 1930's by Max Theiler from a mouse with paralysis, it causes acute encephalitis as well as acute and chronic demyelinating encephalomyelitis in mice. One group of viruses, represented by the GDVII strain, primarily infects neurons and cause acute encephalomyelitis without evidence of virus persistence in survivors (Tsunoda and Fujinami, 2010). A second group, characterized by the DA and BeAn strains causes chronic demyelinating encephalomyelitis, with virus persistence largely in macrophages (Lipton et al., 1995). Disease outcomes are mouse strain-dependent (Clatch et al., 1985). Thus some strains, such as C57BL/6 mice, are resistant to TMEV demyelinating disease, while others, such as the SJL strain, develop a biphasic disease and become persistently infected. The host immune response is believed to play a major role in these different outcomes. C57BL/6 mice develop a robust and effective virus-specific CD8 T cell response, which effectively clear the infection while the CD8 T cell response in SJL is weak (reviewed in Tsunoda and Fujinami, 2010). SJL develop a predominant virus-specific CD4 T cell response that contributes to myelin destruction. Unlike MHV-infected mice, infection of TMEV-infected SJL mice is characterized by epitope spreading. Initially the T cell response is directed at T cell epitopes encoded by the virus, but by 2–3 months after infection, self-reactive T cells that recognized an epitope from a component of myelin, proteolipid protein, were recognized in infected mice and were postulated to contribute to disease severity (Miller et al., 1997). While autoimmune T cells are generally believed to undergo priming in the CLN or DCLN, other studies suggest that priming occurs in the CNS (McMahon et al., 2005). Like MHV, the demyelination is T cell mediated, with CD4 and CD8 T cells making different contributions to virus clearance, demyelination and the development of clinical disease in both resistant and susceptible strains of mice (Murray et al., 1998). Together, these results suggest that tempering of the host immune response would be useful in TMEV-infected as it was in MHV-infected mice. Notably, only a few studies addressed the role of Tregs or IL-10 in TMEV pathogenesis, as described below.

2.1. Role of regulatory T cells

As described above, Tregs, by suppressing the immune response, may ameliorate immunopathology but also delay virus clearance. Higher numbers of Tregs are present in the brains of susceptible SJL compared to resistant C57BL/6 mice. Treatment of SJL mice with anti-CD25 antibody, which depletes or inactivates Tregs (Kohm et al., 2006), decreased clinical disease, enhanced the kinetics of virus clearance and augmented TMEV-specific CD8 T and CD4 T cell responses (Richards et al., 2011). In a complementary approach, Tregs were developed *in vitro* (induced Tregs, iTregs) and delivered to TMEV-infected SJL mice during either the acute or chronic phase of the infection. Treatment during the acute phase resulted in decreased virus clearance, decreased inflammation, greater weight loss and worsened clinical disease, while treatment during the chronic phase resulted in diminished demyelination. In both the acute and chronic phases of the disease, the iTegs functioned by enhancing IL-10 production (Martinez et al., 2014). In contrast, Tregs had only a small role in the immune response in resistant (C57BL/6) mice infected with TMEV. In these mice, Treg depletion resulted in increased recruitment of T cells at early times after infection, with no effects on the kinetics of virus clearance or disease outcome (Prajeeth et al., 2014). Together, these results indicate that Treg effects in TMEV-infected SJL mice were complex, since their presence at early times after infection inhibited virus clearance, which resulted in more severe demyelination and clinical disease during the chronic phase. However, treatment with exogenous Tregs during the chronic phase reduced inflammation and demyelination, suggesting that use of exogenous Tregs in this setting requires careful consideration of the phase of the infection.

2.2. Role of IL-10

Several studies show a correlation between IL-10 expression and improved outcomes in TMEV-infected mice. Thus, treatment of TMEV-infected SJL mice with IFN-β or poly I:C diminished demyelination and decreased the number of autoreactive CD4 T cells. Concomitant with these changes was an increase in IL-10 expression within the CNS

(Olson and Miller, 2009), consistent with a change from a Th1 to Th2 response. In another study, a TMEV variant with low pathogenesis was shown to differ from wild type TMEV by a single amino acid change in a CD4 T cell epitope. This mutation resulted in a change from a Th1 to a Th2 response, such that cells responsive to the epitope expressed IL-4 rather than IFN-γ and was driven by antigen presenting cell (APC) expression of IL-10 instead of IL-12 in infected mice (Palma et al., 2002). In another study, IL-10 produced largely by T cells was shown to be elevated in TMEV-infected SJL compared to C57BL/6 mice, and postulated to contribute to delayed virus clearance, much like Tregs did, as described above. IL-10 expression remained elevated even when Treg numbers decreased, suggesting that IL-10 was expressed later during the infection by a non-Treg T cell (Herder et al., 2012). Collectively, these results are consistent with the notion that IL-10 had similar effects as Tregs in TMEV-infected mice, decreasing the kinetics of virus clearance at early times after infection and ameliorating disease at later times. However, studies of IL-10 in the context of TMEV are all correlative and whether IL-10 actually has these postulated roles will require additional work.

3. Summary and future directions

Dampening of the immune response has been a mainstay of treatment of autoimmune disease. Moreover, studies of the pathogenesis of many viral infections, including those that result in myelin destruction, demonstrate an important role for the host immune response in tissue damage. In this review, we focused on two well studied examples of virus-induced demyelination. In both cases, myelin is damaged or destroyed during the process of virus clearance. Treatment with anti-inflammatory/pro-resolution factors such as regulatory T cells or IL-10 ameliorates host tissue damage but as especially evident in TMEV infections, may diminish virus clearance, resulting in more virus-induced tissue damage as well as additional targets for the host response. Of all the therapeutic modalities described above, virus-specific Tregs appear to be most useful since they preferentially down modulate virus-specific immune responses that may be pathogenic. However, adaptation of virus-specific Tregs to human disease will require substantial amounts of work. Approaches that increased Tregs in infected patients, especially those that targeted virus-specific epitopes, would be required to maximize effects on deleterious immune responses without significantly inhibiting virus clearance. Efforts at engineering such Tregs would also be useful in the setting of autoimmune disease (Berdien et al., 2014; Doudna and Charpentier, 2014; Provasi et al., 2012).

Acknowledgements

Supported in part by grants from the NIH (NS36592) and National Multiple Sclerosis Society (RG5340-A-7). We thank Dr. Jincun Zhao for critical reading of this review.

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