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Involvement of the Janus Kinase/Signal Transducer and Activator of Transcription Signaling Pathway in Multiple Sclerosis and the Animal Model of Experimental Autoimmune Encephalomyelitis

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Multiple sclerosis (MS) and its animal model of experimental autoimmune encephalomyelitis (EAE) are characterized by focal inflammatory infiltrates into the central nervous system, demyelinating lesions, axonal damage, and abundant production of cytokines that activate immune cells and damage neurons and oligodendrocytes, including interleukin-12 (IL-12), IL-6, IL-17, IL-21, IL-23, granulocyte macrophage-colony stimulating factor, and interferon-gamma. The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway mediates the biological activities of these cytokines and is essential for the development and regulation of immune responses. Dysregulation of the JAK/STAT pathway contributes to numerous autoimmune diseases, including MS/EAE. The JAK/STAT pathway is aberrantly activated in MS/EAE because of excessive production of cytokines, loss of expression of negative regulators such as suppressors of cytokine signaling proteins, and significant enrichment of genes encoding components of the JAK/STAT pathway, including STAT3. Specific JAK/STAT inhibitors have been used in numerous preclinical models of MS and demonstrate beneficial effects on the clinical course of disease and attenuation of innate and adaptive immune responses. In addition, other drugs such as statins, glatiramer acetate, laquinimod, and fumarates have beneficial effects that involve inhibition of the JAK/STAT pathway. We conclude by discussing the feasibility of the JAK/STAT pathway as a target for neuroinflammatory diseases.

Introduction

Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating immune-mediated disease of the central nervous system (CNS; brain, spinal cord, optic nerves) of unknown etiology and heterogeneous clinical symptoms and course (Mayo and others 2012). A combination of immunologic, environmental, and genetic factors is thought to cause and/or contribute to MS. Symptoms are varied, ranging from numbness in limbs to severe disease, including paralysis or loss of vision. Further, cognitive impairment can occur. In approximately 85% of MS patients, disease is characterized by a relapsing-remitting (RR) stage, followed by a secondary progressive (SP) phase (Lopez-Diego and Weiner 2008). The RR stage involves activities of Th1 and Th17 cells that infiltrate the CNS, and the SP phase is triggered by inflammation caused by activation of the innate immune system (Weiner 2008). Hallmarks of MS are demyelination; inflammatory lesions; axonal damage; inappropriate activation of interferon-gamma (IFN-γ)-producing Th1 cells, and interleukin-17 (IL-17)-producing Th17 cells, as well as CD8+T-cells and B-cells; hyperactivation of innate immune cells such as macrophages/microglia, neutrophils, and dendritic cells (DCs); astrocyte activation; and exuberant production of cytokines/chemokines (Bhat and Steinman 2009; Ransohoff 2009; Disanto and others 2012). Existing FDA-approved drugs for MS patients such as IFN-β, Glatiramer Acetate (GA), Mitroxantrone, Natalizumab, and, most recently, Fingolimod, Tecfidera, and Aubagio are only partially effective (Lopez-Diego and Weiner 2008; Axtell and others 2010; Lalive and others 2011; Hauser and others 2013), indicating a clear need for new therapies.

Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE), which has been widely used as a model of MS, is induced by active immunization with CNS antigens or by adoptive transfer of CNS-reactive T-cells (Ponomarev and others

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2007; Bailey-Bucktrout and others 2008; Nair and others 2008; Linker and Lee 2009; Barr and others 2012). The pathogenesis of EAE is complex, with both IFN-γ-producing Th1 cells and IL-17-producing Th17 cells having pivotal roles in the development of neuroinflammation (Goverman 2009; Axtell and others 2010; Domingues and others 2010; Becher and Segal 2011). Th1 and Th17 cells also produce granulocyte macrophage-colony stimulating factor (GM-CSF), which is essential to induce EAE, and sustains neuroinflammation by recruitment of myeloid cells to the CNS (Kroenke and others 2010; Codarri and others 2011; El-Behi and others 2011; McGeachy 2011). In both EAE and MS, it is particularly important to limit the entry of Th1 and Th17 cells into the CNS and/or limit expansion of these cells once they have breached the blood–brain barrier. Th2 cells, which produce high levels of IL-4 and IL-10, are correlated with resolution of EAE, and CD25+Foxp3+T regulatory cells (Tregs) function as inhibitors of CNS inflammation (Goverman 2009; Kuchroo and others 2012). In addition, innate immune cells such as DCs, neutrophils, macrophages, and microglia have critical roles in EAE development (Bhat and Steinman 2009; Goverman 2009; Steinman 2010; Ajami and others 2011; Kuchroo and others 2012; Mayo and others 2012; Starossom and others 2012). Similar to MS, EAE is characterized by the heighted production of many proinflammatory cytokines and chemokines, including IL-12, IL-6, IL-17A, IL-17F, IL-21, IL-23, GM-CSF, IL-1, TNF, IFN-γ, CCL2, and CXCL10.

Janus kinase/signal transducers and activators of transcription pathway

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway is the predominant signal transduction cascade utilized by numerous cytokines and is critical for initiating innate immunity, orchestrating adaptive immune systems, and ultimately constraining inflammatory and immune responses (O'Shea and Plenge 2012). Cytokines activate receptor-associated JAKs, which phosphorylate the receptor cytoplasmic domain on tyrosine residues, leading to recruitment of STATs. The JAKs then tyrosine phosphorylate STATs, promoting their activation. Once activated, STATs dimerize, translocate to the nucleus, and bind to regulatory elements to induce transcription of target genes (Fig. 1A,B). Over 60 cytokines and growth factors use the JAK/STAT pathway (O'Shea and Plenge 2012). There are 4 JAKs (JAK1, JAK2, JAK3, and TYK2) and a total of 7 STATs (STAT 1, 2, 3, 4, 5a, 5b, and 6). Various combinations of JAK/STAT usage result in differential gene expression, particularly depending on the STAT transcription factor(s) that is activated. Cytokines, through activation of the JAK/STAT pathway, are of paramount importance in regulating the development, differentiation, and function of T-cells and myeloid cells (Weaver and others 2007; Geissmann and others 2010b). Specifically, Th1 cell differentiation is induced by IL-12 through activation of JAK2/TYK2 and STAT4, Th2 differentiation is induced by IL-4 activation of JAK1/3 and STAT6, while Th17 cell differentiation requires IL-6 and IL-23, which signal through JAK1/2 and STAT3 (Fig. 2) (Harris and others 2007; Weaver and others 2007). STATs also regulate innate immune cells (Geissmann and others 2010a; Galli and others 2011). JAK1/2 and STAT1 activation mediate the effects of IFN-γ on macrophage function, JAK1/2 and STAT3 are involved in IL-6 family signaling, and GM-CSF signals through JAK2 and STAT5 to affect myeloid development. JAKs and STATs are essential mediators of almost all biological signaling events initiated by cytokines. As such, unrestrained activation of the JAK/STAT pathway is detrimental and has been associated with numerous immune-mediated and autoimmune diseases, including MS (O'Shea and Plenge 2012). Indeed, a number of STAT target genes, including *IL-23R, IL-17A, IL-17F, IL-21, IL-22, IL-6, IFN-γ, RORγt, T-bet, CXCR3*, and *HLA-DR*, are overexpressed in both MS and EAE, and have been implicated in contributing to disease pathogenesis. Activating mutations in STAT proteins are rare; thus, STAT hyperactivation is usually caused by an overabundance of cytokines, and/or dysregulation of endogenous negative

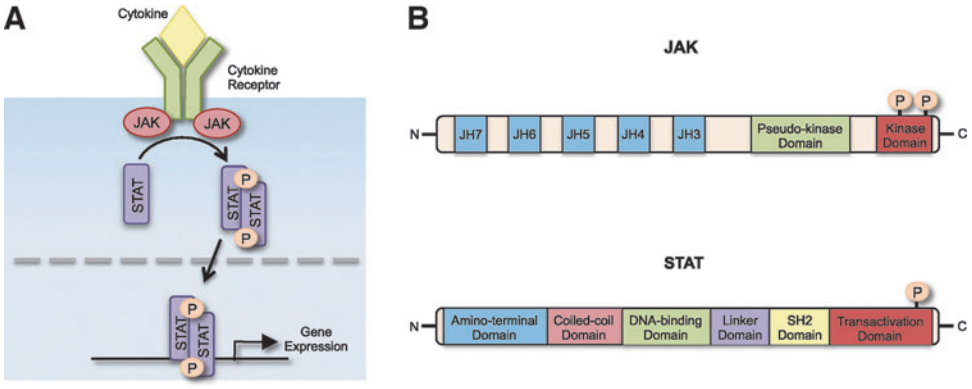

FIG. 1. The JAK/STAT signaling pathway and domain structure of JAK and STAT proteins. **(A)** Various cytokines bind to their corresponding cytokine receptor and activate receptor-associated JAKs, leading to recruitment of STATs. JAKs then tyrosine phosphorylate STATs, promoting their activation. STATs dimerize, translocate to the nucleus, and bind to regulatory elements to induce transcription of target genes. **(B)** JAK and STAT protein domain structure. JAK proteins contain 7 JH domains including the pseudo-kinase domain (JH2) and the kinase domain (JH1). Trans- and autophosphorylation of tyrosine residues in the C-terminal kinase domain lead to the recruitment and activation of STATs. STAT protein structure consists of an amino-terminal domain, a coiled-coil domain, a DNA-binding domain, a linker domain, an SH2 domain, and a transactivation domain. Phosphorylation in the C-terminal transactivation domain by JAKs leads to STAT activation and dimerization. JAK/STAT, Janus kinase/signal transducer and activator of transcription; JH, JAK homology.

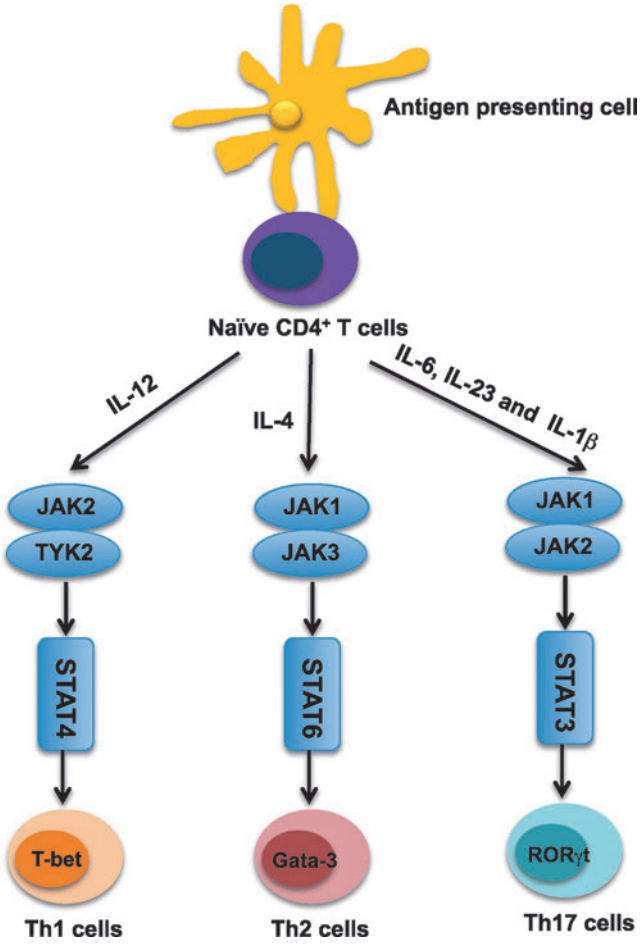

FIG. 2. JAK/STAT signaling is critical for the differentiation of CD4+T-cells. Naive CD4+Th cells differentiate into various distinct functional subsets depending on the cytokines they encounter. Specifically, Th1 cell differentiation is induced by IL-12 through activation of JAK2/TYK2 and STAT4, Th2 cell differentiation is induced by IL-4 activation of JAK1/3 and STAT6, while Th17 cell differentiation requires IL-6 and IL-23, which signal through JAK1/2 and STAT3, as well as IL-1β. IL, interleukin; Th, T helper.

regulators of JAKs, most notably, suppressors of cytokine signaling (SOCS) proteins. The SOCS family is composed of 8 members, CIS and SOCS1–7, and serve to restrict the duration of activation of cytokine-induced signaling by inhibiting JAK kinase activity after it has been turned on (Fig. 3A) (Yoshimura and others 2012). SOCS proteins contain an N-terminal variable region, a classical SH2 domain, and a C-terminal SOCS box (Fig. 3B). SOCS proteins are not constitutively expressed, but rather induced by cytokines, creating a negative feedback loop to prevent excessive activation of cytokine-induced JAK/STAT signaling. SOCS proteins bind to activated JAKs and to certain cytokine receptors via their SH2 domains, thereby suppressing further signaling events. In addition, the SOCS box interacts with components of the ubiquitin ligase machinery and mediates proteosomal degradation of associated proteins, most commonly, JAKs. SOCS1 and SOCS3 are unique among the SOCS proteins in terms of containing a 12 amino acid kinase-inhibitory region (KIR) (Fig. 3B), which acts as a pseudosubstrate for JAKs, conferring inhibition of JAK kinase activity. SOCS1 and SOCS3 in particular have critical functions in repressing innate and adaptive immunity, in part by inhibiting STAT activation induced by IFN-γ, IL-6, IL-12, IL-23, and GM-CSF, which are all implicated in MS and EAE pathogenesis (Yoshimura and others 2012; Kershaw and others 2013).

Dysregulation of the JAK/STAT Pathway in MS

Genome-wide association studies have shown that cytokines, their receptors, JAKs, STATs, and SOCS proteins are associated with human autoimmune diseases, especially pathways leading to aberrant STAT3 and STAT4 activation (Oksenberg and Baranzini 2010; Sawcer and others 2011; Vandenbroeck 2012). In MS, there is significant overexpression of immunologically relevant genes involved in Th cell differentiation and antigen presentation. These include cytokine and cytokine receptor genes such as *IL-7, IL-12, IL-2RA, IL-7R, IL-28RA, OSMR*, and *IL-22R* (Bronson and others 2010; Oksenberg and Baranzini 2010; Couturier and others 2011; Oksenberg and Hauser 2011; Zuvich and others 2011; Vandenbroeck and others 2012; Beecham and others 2013; IMSGC 2013). A TYK2 variant in MS patients is a protective allele that results in diminished TYK2 kinase activity, leading to a decrease in STAT1 activation (Couturier and others 2011). This promotes a deviation of Th cell differentiation to the Th2 phenotype, and is associated with a decreased risk of MS. STAT3 has been identified as an MS susceptibility gene (Baranzini and others 2009; Jakkula and others 2010; Oksenberg and Baranzini 2010), and independent replication supports the association between STAT3 and an increase in MS risk (Lill and others 2012). In addition, genes encoding components of the JAK/STAT pathway were recently demonstrated to be significantly enriched in MS patients (IMSGC 2013). T-cells and monocytes from MS patients during relapse have elevated levels of activated STAT3 compared with cells from patients in remission, which is correlated with low levels of SOCS3 (Frisullo and others 2006). This suggests an association of decreased SOCS3 expression, increased STAT3 activation, and MS relapse. Furthermore, high levels of activated STAT3 in T-cells from patients with clinically isolated syndrome predict conversion to clinically defined MS (Frisullo and others 2008). A SOCS1 variant was recently validated as a novel risk factor for MS (Vandenbroeck and others 2012), although the functional significance of this single-nucleotide polymorphism is not known. Interestingly, single-nucleotide polymorphisms related to various components of the NF-κB pathway have been identified in MS patients (Beecham and others 2013), suggesting overactivation of this pathway. This is relevant to the JAK/STAT pathway as there is considerable crosstalk between these two signaling cascades, and they function in a feed-forward loop to ensure continuous activation (McFarland and others 2013).

Statins have been intensely studied for immunomodulatory and anti-inflammatory properties (Zamvil and Steinman 2002; Weber and others 2007b). Simvastatin has been shown to induce SOCS3 expression in monocytes from MS patients with RR disease, which was associated with diminished STAT1 and STAT3 activation (Zhang and others 2008). This also decreased the production of IL-6 and IL-23 by monocytes, leading to diminished IL-17 production by

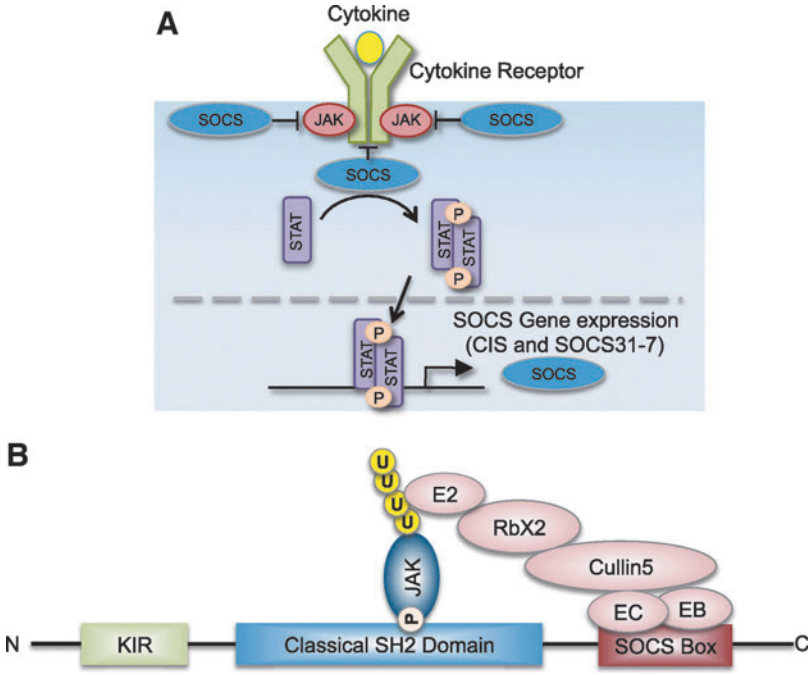

FIG. 3. Induction of SOCS expression, and domain structure of SOCS3. **(A)** SOCS proteins (CIS and SOCS1–7) are not constitutively expressed; rather, they are induced by cytokines, creating a negative feedback loop to prevent excessive activation of cytokine-induced JAK/STAT signaling. **(B)** SOCS proteins contain an N-terminal variable region, a classical SH2 domain, and a C-terminal SOCS box. The SOCS box interacts with components of the ubiquitin ligase machinery and mediates proteosomal degradation of associated proteins, most commonly, JAKs. SOCS1 and SOCS3 are unique among the SOCS proteins as they contain a KIR, which acts as a pseudosubstrate for JAKs, conferring inhibition of JAK kinase activity. KIR, kinase-inhibitory region; SOCS, suppressors of cytokine signaling.

T-cells. Furthermore, simvastatin directly inhibits Th17 cell differentiation in RR MS patients (Zhang and others 2011). This is accomplished by inhibition of IRF-4 expression, which is a key transcription factor for human Th17 cell differentiation, leading to decreased IL-17A, IL-17F, IL-21, and IL-22 production (Zhang and others 2011). Lastly, simvastatin directly targets DCs from MS patients, which causes an induction of SOCS1 and SOCS3 expression, and decreased STAT1 and STAT3 activation (Zhang and others 2013). This in turn decreased expression of IL-1, IL-23, TGF-β, IL-21, and IL-12 from DCs, providing an inhibitory cytokine environment for Th1 and Th17 cell differentiation (Zhang and others 2013). These studies collectively suggest that SOCS proteins may represent therapeutic targets in MS (Ramgolam and Markovic-Plese 2011). Indeed, a SOCS1 mimetic has efficacy in various EAE models, which is described in more detail below.

Dysregulation of the JAK/STAT Pathway in EAE

Activation of the JAK/STAT pathway, particularly STAT1, STAT3, and STAT4 activation, is observed in various models of EAE, including active induction of classical and atypical EAE, as well as Th1 and Th17 cell-adoptive transfer models of EAE (Zaheer and others 2007; Chen and others 2009; Jia and others 2011; Qin and others 2012b; Egwuagu and Larkin 2013; Liu and others 2014). Mice with targeted deletion of STAT proteins have been used to determine the functional role of different STAT members in EAE. Given the importance of STAT3 in differentiation of Th17 cells, several groups have examined mice with targeted deletion of STAT3 in T-cells for susceptibility to EAE. Loss of STAT3 in T-cells renders mice resistant to EAE disease (Harris and others 2007; Liu and others 2008). These studies demonstrate that STAT3, by regulating the expression of the transcription factor RORγt and the IL-23R, is essential for the development of Th17 cells. As such, STAT3 knock-out mice cannot generate Th17 cells and thus are protected from developing EAE (Harris and others 2007; Liu and others 2008). In addition, T-cells from these mice are defective in expression and activation of the integrins α4β1 and α4β7 and cannot traffic into the CNS (Liu and others 2008). STAT4-deficient mice are defective in the differentiation of Th1 cells and are resistant to EAE induction (Chitnis and others 2001). STAT4 knockout mice have been shown to have a predominantly Th2 phenotype, which is associated with high levels of IL-4 and IL-5. As such, the resistance to EAE may be because of the protective effects of Th2-derived cytokines. Interestingly, mice lacking STAT1 are highly susceptible to EAE (Bettelli and others 2004). It is possible that in the absence of STAT1, STAT3 and/or STAT4 signaling may compensate to drive Th1 cell responses as these mice are characterized by IFN-γ–producing Th1 cells. It has been suggested that mice lacking STAT1 cannot benefit from the protective effect of IFN-γ and thus have severe disease. IL-6 has a deleterious role in EAE by activation of STAT3, which is pivotal for induction of pathogenic Th17 cells, and trafficking of Th1 and Th17 cells into the CNS (Yang and others 2007; Jin and others 2009; Quintana and others 2009; Scheller and others 2011). Thus, IL-6-deficient mice have been shown to be resistant to EAE induction because of a lack of development of Th17 cells (Quintana and others 2009).

The JAK/STAT pathway also regulates the innate immune response in EAE. Macrophages, microglia, and DCs can promote both injury and repair, and have detrimental and protective roles in diseases such as EAE and MS (Block and others 2007; Gensel and others 2009; Mildner and others 2009; Rivest 2009; Shechter and others 2009; Zhu and others 2011; Yogev and others 2012). These divergent functions are dictated by their microenvironment, which can promote a spectrum of macrophage/microglia phenotypes (Fig. 4). Polarized macrophages are termed proinflammatory, classically activated M1, and anti-inflammatory, alternatively activated M2, which represent 2 extremes of the macrophage continuum (Gordon 2003; Mantovani and

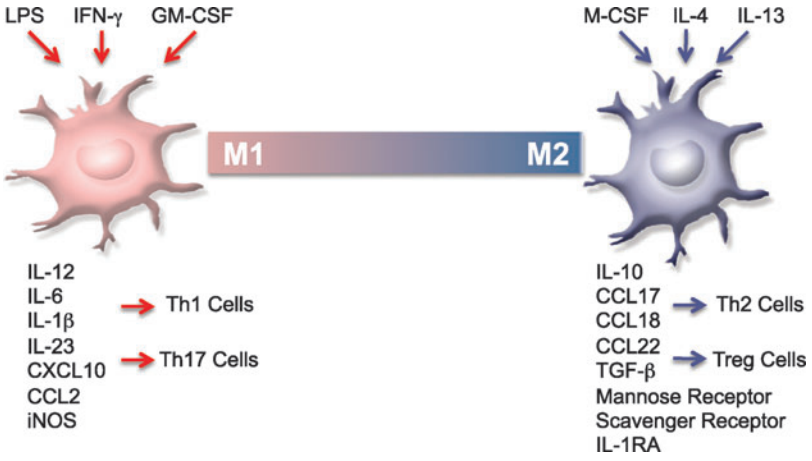

FIG. 4. M1 and M2 macrophage polarization. Polarized macrophages are termed proinflammatory, classically activated M1, and anti-inflammatory, alternatively activated M2. Macrophages are polarized to the M1 phenotype by LPS, IFN-γ, and GM-CSF, produce high levels of IL-12, IL-6, IL-1β, IL-23, CXCL10, and CCL2, increase levels of reactive oxygen species, and participate in the induction of Th1 and Th17 responses. M-CSF, IL-13, and IL-4 induce M2 macrophages, which upregulate scavenger and mannose receptors, the IL-1 receptor antagonist, and express high levels of IL-10, CCL17, CCL18, CCL22, and TGF-β. M2 macrophages can also be induced by GA and Laquinimod treatment. GM-CSF, granulocyte macrophage-colony stimulating factor; IFN, interferon.

others 2005; Cassol and others 2010). Macrophages are polarized to the M1 phenotype by LPS, IFN-γ, and GM-CSF (Mantovani and others 2007; van der Does and others 2010; Krausgruber and others 2011); produce high levels of IL-12, IL-6, IL-1β, IL-23, CXCL10, and CCL2, increased levels of reactive oxygen species, and low levels of IL-10; and participate in the induction of Th1 and Th17 responses (Mantovani and others 2005; Cassol and others 2010; Krausgruber and others 2011). We have recently shown that the absence of SOCS3 in macrophages leads to a ''heightened'' M1 phenotype associated with excessive STAT activation (Qin and others 2012a,b). Furthermore, mice with targeted deletion of SOCS3 in myeloid cells develop a severe, nonresolving atypical form of EAE, which is associated with lesions in the cerebellum, rather than the spinal cord (Qin and others 2012b). These mice have elevated levels of M1 macrophages; exhibit hyperactivation of STAT1, STAT3, and STAT4; and have elevated numbers of inflammatory cells in the cerebellum, including macrophages and neutrophils, and a prominent Th1 and Th17 cell infiltrate (Qin and others 2012b). IL-4, IL-13, and M-CSF induce M2 macrophages, which upregulate scavenger and mannose receptors, the IL-1 receptor antagonist, and express high levels of IL-10, CCL17, CCL18, and CCL22 (Gordon 2003). M2 macrophages resolve inflammation and promote Th2 responses (Wang and others 2010). M2 macrophages are protective in multiple models of EAE. Weber and others (2007a) demonstrated that M2 macrophages are induced by GA treatment, characterized by increased secretion of IL-10 and TGF-β and diminished STAT1 activation, and inhibit EAE disease upon adoptive transfer by suppressing Th17 development and promoting Th2 and Tregs. Laquinimod, an agent under evaluation for RR MS, is protective in EAE models by induction of M2 macrophages, also characterized by low STAT1 activation (Schulze-Topphoff and others 2012; Thone and others 2012). Fumurates, which were approved by the FDA in 2013 for treatment of RR MS patients, protect mice from EAE by the generation of anti-inflammatory type II DCs, which induce Th2 cells (Ghoreschi and others 2011a). The type II DCs have impaired STAT1 phosphorylation. Thus, agents that are protective in EAE models and in MS (GA, laquinimod, fumarates) promote protective M2 macrophage and type II DC phenotypes, which are associated with diminished STAT1/3 activation, decreased production of IL-6, IL-12, and IL-23, and elevated secretion of IL-10. We have also shown that M2 macrophages are protective in the atypical model of EAE associated with SOCS3 deficiency in myeloid cells (Qin and others 2012b). Adoptive transfer of M2 macrophages diminished the inflammatory infiltrate observed in the cerebellum, reduced Th1 and Th17 cells while enhancing expression of Th2 and Tregs, and inhibited STAT activation in the cerebellum (Qin and others 2012b).

As mentioned previously, SOCS1 and SOCS3 are critically involved in regulating innate and adaptive immune responses (Yoshimura and others 2012). In EAE models, expression of both SOCS1 and SOCS3 is detected predominantly in macrophages during the early stages of disease, as well as in active disease (Stark and Cross 2006; Berard and others 2010). In a study comparing RR EAE to chronic (CH) EAE, it was noted that the number of SOCS1-expressing macrophages at the peak of RR disease was significantly higher than in CH-EAE (Berard and others 2010). This correlated with diminished expression of iNOS, whose expression is regulated by SOCS1, in RR-EAE mice compared with CH-EAE mice. The authors speculate that the inhibition of iNOS may promote remission in the RR-EAE model, and that SOCS1 expression by macrophages may contribute to induction of remissions. SOCS3 expression in DCs is also protective in EAE (Li and others 2006). Adoptive transfer of SOCS3-expressing DCs at EAE induction or at disease onset reduces the clinical severity of EAE compared with control DCs. This was associated with a limited differentiation of Th1 and Th17 cells and a robust induction of Th2 cells, which provide protection in EAE. As discussed previously, IL-6 and IL-23 activation of STAT3 is critical for Th17 cell differentiation (Basu and others 2013). Deficiency of SOCS3 in T-cells leads to preferential polarization to the Th17 phenotype, because of heightened STAT3 phosphorylation and subsequent IL-17A and IL-17F expression (Chen and others 2006). TGF-β is also required for Th17 cell differentiation (Basu and others 2013). We have shown that TGF-β inhibits SOCS3 expression, leading to enhancement of STAT3 activation and Th17 cell polarization (Qin and others 2009). These results indicate that SOCS3 expression is critical to constrain the differentiation of Th17 cells.

Therapeutic intervention of the JAK/STAT pathway in EAE

The JAK/STAT pathway has received significant attention as a therapeutic target in inflammation, autoimmune diseases, solid and liquid tumors, and transplant rejection (Opar 2010; O'Shea and Plenge 2012; Seavey and Dobrzanski 2012). A variety of JAK inhibitors have been developed with varying degrees of specificity for JAK1, JAK2, JAK3, and TYK2, and have demonstrated clinical efficacy in rheumatoid arthritis and other inflammatory disorders (Fridman and others 2010; Opar 2010; Ghoreschi and others 2011b; Stump and others 2011; O'Shea and Plenge 2012; O'Shea and others 2013a). Two JAK inhibitors have been approved by the FDA: Ruxolitinib, a JAK1/JAK2 inhibitor, was approved in 2011 for patients with myelofibrosis and polycythaemia vera, and Tofacitinab, a JAK3/JAK1 inhibitor, was approved in 2012 for treatment of patients with rheumatoid arthritis (O'Shea and others 2013a,b). JAK inhibitors interrupt signaling downstream of multiple cytokines, representing a useful approach for MS, which is characterized by a ''cytokine storm'' in the periphery and CNS. Many of the key immunoregulatory cytokines involved in EAE and MS, including IL-6, IL-12, IL-23, IFN-γ, and GM-CSF, require activation of JAK1, JAK2, or both for subsequent activation of STATs, and ultimate biological responses (O'Shea and Plenge 2012). As such, a number of studies have examined direct inhibition of the JAK/STAT pathway in EAE. Bright and others (1999) previously demonstrated that tyrphostin B42, a JAK2 inhibitor, reduced severity of EAE. This was accomplished by inhibiting IL-12-induced activation of JAK2 in T-cells, leading to a decrease in Th1 cell polarization. *In vivo*, tyrphostin B42 decreased Th1 cell development, thereby reducing the incidence and severity of EAE. We have recently demonstrated that AZD1480, a JAK1/JAK2 inhibitor, has striking clinical efficacy in multiple models of EAE (Liu and others 2014). *In vitro*, AZD1480 inhibits the differentiation of both human and murine Th1 cells by inhibiting STAT1 and STAT4 activation, and decreasing levels of IFN-γ and T-bet, the transcription factor critical for Th1 cell polarization. AZD1480 also inhibited the differentiation of human and murine Th17 cells by inhibiting STAT3 activation, and downstream STAT3 target genes such as *IL-17A*, *RORγt*, *IL-22*, and *IL-23R*. AZD1480 also influenced macrophages and DCs by inhibiting STAT1, STAT3, and STAT5 activation, and expression of genes such as *iNOS*, *Class II MHC*, and *CD40* (Liu and others 2014). Our results indicate that AZD1480 does not promote the M2 macrophage phenotype but exerts an inhibitory effect on M1 polarization. *In vivo,* AZD1480 had a significant protective effect in 5 EAE models: classical EAE, atypical EAE, RR EAE, Th1 cell-mediated EAE, and Th17 cell-mediated EAE. The beneficial immunomodulatory effects of AZD1480 were associated with deactivation of myeloid cells, diminished polarization of Th1 and Th17 cells, decreased expression of proinflammatory cytokines/chemokines, and reduced infiltration of immune cells (Liu and others 2014). Importantly, AZD1480 treatment was administered at the onset of disease and in a therapeutic manner after the appearance of clinical symptoms, with potent clinical efficacy.

Studies on other pharmacologic inhibitors have implicated the JAK/STAT axis in regulating clinical manifestations of EAE. Peroxisome proliferator activated receptor-γ (PPARγ) and COX2 inhibitors suppress EAE severity, in part, by inhibiting IL-12-induced activation of the JAK/STAT pathway, and subsequent suppression of Th1 cell differentiation (Natarajan and Bright 2002; Muthian and others 2006). This was accomplished by inhibiting STAT3 and STAT4 activation in T-cells. The protective effect of GA in EAE is in part caused by inhibition of STAT3 phosphorylation in T-cells, leading to inhibition of RORγt expression, and suppression of Th17 cell differentiation (Chen and others 2009). Several herbal compounds, including plumbagin (PL), berberine, and quercetin, exert protective effects in EAE models by inhibiting STAT activation and Th1 and Th17 cell differentiation (Muthian and Bright 2004; Qin and others 2010; Jia and others 2011). Furthermore, both PL and berberine inhibited NF-κB activation in antigen-presenting cells, which diminished expression of IL-6 and iNOS by these cells. The lack of IL-6 likely contributes to the decrease in Th17 cell differentiation, while reductions in iNOS may promote the development of Tregs (Lee and others 2011), which aid in resolution of disease.

Lastly, there is much interest in the use of SOCS mimetics in neuroinflammatory diseases. SOCS1 and SOCS3 contain a KIR domain that binds to tyrosine-phosphorylated JAKs and inhibits their kinase activity (Fig. 3B). SOCS1 mimetics have been made that bind to the JAK2 autophosphorylation site, preventing activation of STAT1 and STAT3. The SOCS1 mimetic (Tkip) inhibits IFN-γ and IL-6 activation of STAT proteins *in vitro*, thereby suppressing downstream gene expression (Flowers and others 2004). The SOCS1 mimetic has been tested in several EAE models. Administration of Tkip before EAE induction in NZW mice prevents acute EAE, while in SJL/J mice, administration blocks the acute and relapse phases of EAE, even when given after establishment of disease (Mujtaba and others 2005). In C57BL/6 mice with CH EAE, Tkip reduced disease severity (Berard and others 2010). A more detailed analysis demonstrated that Tkip inhibits expansion of Th17 cells in EAE by blocking IL-23 activation of STAT3 (Jager and others 2011). The therapeutic efficacy of Tkip in EAE was associated with a reduction in cellular infiltration into the CNS. These findings collectively indicate that the SOCS1 mimetic can attenuate neuroinflammatory responses in the CNS and may have therapeutic value in MS patients.

STAT inhibitors

There is also interest in the development of STAT inhibitors, particularly inhibitors of STAT3 and STAT5, as their aberrant activation is associated with a wide range of cancers (Brantley and Benveniste 2008; Yu and others 2009; Walker and others 2013). In addition, STAT3 mediates many of the inflammatory responses associated with signaling by the IL-6 family of cytokines and has relevance as a target in numerous autoimmune diseases, including MS. Thus, STAT transcription factors appear to be targets with a high therapeutic index. However, the ability to target STATs has lagged far behind the great progress in targeting JAKs. Although STATs lack enzymatic activity, they do contain clearly defined functional domains that can serve as targets (Fig. 1B). A number of strategies have been employed, including (1) creating phosphopeptide mimetic prodrugs that

target the SH2 domain, thereby preventing dimerization of STATs (McMurray and others 2012); (2) targeting of the N-terminal domain of STATs to modulate JAK/STAT signaling (Timofeeva and Tarasova 2012); (3) development of decoy oligonucleotides that bind activated STATs, thereby interfering with DNA binding and transcriptional activity (Sen and Grandis 2012); and (4) screening chemical libraries for STAT modulators (Nelson and others 2011; Walker and Frank 2012). A number of STAT3 inhibitors have been described and tested successfully in preclinical models (Nelson and others 2011; Zhang and others 2012; Miklossy and others 2013). More importantly, a STAT3 decoy oligonucleotide has been tested in a phase 0 clinical trial in patients with head and neck cancers. The results demonstrate evidence of inhibition of STAT3 target genes in the tumor and are the first to document the efficacy of STAT3 inhibitors in humans (Sen and others 2012). In addition, a small molecule inhibitor of STAT3 is being tested in a clinical trial in patients with chronic lymphocytic leukemia (Frank 2012). Of relevance to neuroinflammation, a STAT3 inhibitor (ORLL-NIH001) has been tested in experimental autoimmune uveitis, a model of human posterior uveitis (Yu and others 2012). Treatment with ORLL-NIH001 attenuated disease severity by inhibiting the inflammatory properties of T-cells, inhibiting entry of T-cells into the retina, and reducing expression of CCR6 and CXCR3 (Yu and others 2012). In addition, ORLL-NIH001 inhibited the expansion of human Th17 cells *in vitro*. These encouraging findings bode well for the future development and use of STAT3 inhibitors in MS patients.

Conclusions

The JAK/STAT pathway is one of the most critical signal transduction systems utilized by cells of the innate and adaptive immune systems to initiate and regulate immune responses. Aberrant activation of this pathway promotes dysregulation of innate and adaptive immunity, including activation of pathogenic Th1 and Th17 cells, activation of macrophages, neutrophils, and DCs, and excessive production of proinflammatory cytokines, all of which contribute to the pathogenesis of MS. There have been remarkable advances in the development of specific JAK inhibitors that show great promise in the treatment of autoimmune diseases, as well as a variety of cancers (O'Shea and others 2013a,b). Common adverse effects associated with the JAK inhibitors include bacterial, fungal, and viral infections, but opportunistic infections are uncommon. Presumably because of interference with EPO signaling and signaling by colony stimulating factors via JAK2 inhibition, anemia and neutropenia can also occur. Hypercholesterolemia is observed with the use of JAK inhibitors, which may be due to blockade of IL-6 signaling. Because of the relatively short half-life of the JAK inhibitors, the drug can be stopped if adverse effects such as infections become severe. Another possibility may be to administer JAK inhibitors in a pulsatile manner.

Given the profound role of cytokines in autoimmunity, JAK inhibitors have great potential utility. As JAK inhibitors can interrupt the signaling of numerous cytokines, we believe that they may be useful for the treatment of MS as simultaneous inhibition of cytokine signaling involved in activation of innate and adaptive immune responses may break the cycle of inflammation characteristic of MS. Other diseases such as Parkinson's disease, spinal cord injury, and Alzheimer's disease, which have a prominent neuroinflammatory component, may also benefit from JAK inhibitors. In this regard, preliminary findings from our laboratory using a JAK1/2 inhibitor in a rat model of Parkinson's disease demonstrate a reduction in macrophage/microglial activation, and sparing of dopaminergic neurons (unpublished data). These findings collectively suggest that the JAK/STAT axis may serve as a therapeutic target for neuroinflammatory and neurodegenerative diseases.

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Author Disclosure Statement

The authors declare no conflicts of interest.

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