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Current Concepts of Interleukin-16 (IL-16) in the Onset and Progression of Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system (CNS) of unknown etiology. MS is a leading cause of disability among young adults in the western hemisphere. Auto-reactive myelin and neuronal antigen-directed $CD4^+$ T cells mainly contribute to initiation and/or perpetuation of neuronal damage in MS. We present the current understanding of the molecular and cellular pathways by which autoagressive T cells mediate tissue damage in experimental allergic encephalomyelitis (EAE) and MS, specifically interleukin-16 (IL-16)-related pathways. IL-16 is a $CD4^+$ T cell-specific chemotactic factor. IL-16 regulates major biological properties of $CD4^+$ T cells including cell activation, CD25 expression, MHC class II expression and cross receptor desensitization of chemokine-induced chemoattraction. Various approaches in halting CNS homing, or modulating immune properties of autoimmune T cells have been taken to treat MS and EAE, an animal model, commonly used to study immune pathogenesis of MS. In this chapter we revisit immunopathogenetic mechanisms implicated in onset and progression of the disease. We focus on IL-16 mechanisms involved in regulation of relapsing MS and EAE.

Contents: 1. Introduction; 2. Regulation of neuroinflammation; 3. IL-16 and its role in neuroinflammation; 4. Conclusion.

KEYWORDS

Interleukin-16; Multiple Sclerosis; Experimental Autoimmune Encephalomyelitis; $CD4^+$ T cells; Therapy; Immune Pathogenesis

INTRODUCTION

Multiple Sclerosis: Clinical Course and Histopathology, Epidemiology, and Genetic factors

Clinical course and histopathology

Multiple sclerosis (MS) is a chronic paralytic disease caused by inflammation, demyelination and axonal degeneration of the central nervous system (CNS). The autoimmune nature of MS is strongly suggested by evidence of myelin-specific autoreactive T cells and antibodies. The onset and clinical course of MS are unpredictable. Clinical symptoms of MS are heterogeneous and primarily include loss of motor and sensory functions in the extremities and visual impairments. The symptoms develop abruptly and typically last several weeks, followed by spontaneous remissions, at least in earlier stages of the disease. In a majority of patients, the disease has a relapsing-remitting course. Each relapsing episode presents with more severe functional impairment. During remissions, recovery of motor functions is often incomplete, and after a decade or two of relapsing-remitting course, the disease becomes chronic progressive. Besides the relapsing-remitting form, the disease can also take either a primary, or a secondary progressive course. Therefore, clinical forms of disease vary among people suffering from MS. Over time, it is not uncommon to observe shifts from one to another clinical form in the same patient. It is believed that distinctive immunopathogenetic mechanisms regulate chronic inflammation and subsequent axonal and neuronal damage in the central nervous system (CNS) and may be responsible for clinical heterogeneity. Although the etiology of MS remains obscure, combinations of genetic susceptibility, environmental influences, and autoimmune mechanisms of tissue damage are believed to be fundamental for disease initiation and pathophysiology. Subsequently, most current MS therapies have non-specific immunosuppressive and anti-inflammatory effects [1]. Although those therapies provide partial and temporal relief of symptoms in some patients, there is a lack of noticeable effects in others. A better understanding of specific mechanisms of disease regulation is critical for development of new and specifically targeted therapies.

Inflammatory demyelination initiates axonal dysfunction and/or loss, especially in relapsing-remitting MS. Damage and subsequent loss of axonal cytoskeletal neurofilaments correlates directly with neurological disability in MS. Histopathology analysis of CNS lesions from MS autopsies revealed a close association between inflammation and nerve cell degeneration in all stages of MS [3]. As a consequence of neuroinflamation, damage and/or dysfunction of oligodendrocytes occurs in MS. Damage to oligodendrocytes precludes maintenance of homeostatic myelin-axonal signaling and their proper communication. Those changes are typically thought to be irreversible

and to have profound impacts on function of myelinated axons. The axonal medium chain of neurofilament (NF160) is essential for myelination-dependent outside-in signaling, which arises from oligodendrocytes and controls axonal caliber and conduction velocity of motor axons.

Epidemiology

World-wide, MS affects about 2.5 million people. Because the Centers for Disease Control and Prevention (CDC) does not require U.S. physicians to report new cases, and because symptoms can be completely invisible, the numbers can only be estimated [2]. Multiple sclerosis is the second most common neurological disorder leading to disability in young adults, surpassed only by trauma. MS affects the working population between 30-50 years of age. This is a progressive, debilitating, paralytic, disease with unknown etiology. MS patients require therapy, counseling and rehabilitation. Most often, with progression of the disease, they are unable to continue working [2,3]. Economic cost estimates include not only the disability and lost income from those with the disease, but also the cost of long-term medical care and support over the adult life span of patients with MS. The burden in terms of time and job loss for family care-givers adds significantly to the total cost. Identifying ways to treat the disease and/or decrease the disability would have an enormous economic impact.

MS is more prevalent in women than men. The ratio of female to male MS cases is 2-3:1, suggesting a role for hormonal factors in MS pathogenesis. A role for environmental factors, such as viral and microbial infections as immediate triggers of the disease in genetically susceptible individuals, has been proposed. Although epidemiological association of Epstein-Barr and human herpes virus 6 infections with MS has been established, our understanding of putative virus–induced mechanisms remains limited [4]. Epidemiology studies had also indicated a link between vitamin D deficiency and MS [5].

Genetic factors associated with MS

Genome wide association (GWA) studies, which included large cohorts of MS patients, supported the view of poly-geneic regulation of susceptibility to disease [6,7,8]. A large number of genes associated with immune functions show linkage to MS. For example, among the first to be determined were genes encoding human leukocyte antigens (HLA), primarily HLA-DR and HLA-DQ loci. However, distinct HLA haplotypes were associated with the disease in different cohorts of patients. For example, the HLA-DRB1*1501-DQB1*602 (HLA-DR15) haplotype in the major histocompatibility complex (MHC), which is the predominant genetic risk factor in Northern Europeans. Besides the linkage with HLA genes, polymorphisms in some other genetic loci, such as those encoding cytokines (tumor necrosis factor alpha - TNFα), cytokine receptors (IL-7R, IL-2RA), chemokines and their cognate receptors, such as macrophage chemoattractant protein-1 (MCP-1), and CC chemokine receptor-5 (CCR5), were found among MS patients. Genetic studies have made enormous progress in revealing risk alleles associated with MS. Functional studies to evaluate therapeutic potential of such genetic linkage are needed in order to successfully translate

accumulated genetic knowledge into MS therapy [9]. Recent data on epigenetic regulation of autoimmune diseases further add to the complexity of factors associated with MS.

REGULATION OF NEUROINFLAMMATION

Blood–Brain Barrier (BBB)

Central nervous system (CNS) is an immune privileged site. It is sequestered from immune surveillance by the semi-permeable blood brain barrier (BBB). Endothelial cells interconnected by tight and adherence junctions derive the BBB. Besides endothelial cells, resident glia including astrocytes and microglia, constitute an integral part of the functional BBB. Complex molecular interactions essential for maintenance of homeostatic properties of tight and adherent junctions have been identified [10]. Many of these molecules such as, claudins (claudin 3, 5 and 12), junctional adhesion molecule (JAM-A), endothelial cell-selective adhesion molecule (ESMA), zonula occludens proteins (ZO-1, ZO-2 and Zo-3), calcium-dependent serine protein kinases (CASK), cingulin, multi-PDZ protein 1 (MUPP1), VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1) and catenin are subject of regulation by cytokines. In MS, reduced expression of ZO-1 on blood vessels in MS plaques has been reported [11]. During MS, activation of endothelial cells and astrocytes leads to improper formation of tight junctions, production and local release of mediators of inflammation, and expression of adhesion molecules, which altogether contribute to an increase in permeability of the BBB. Conversely, *in vitro* studies indicated that treatment with IFN-β stabilizes tight junction-associated proteins and restores BBB restrictive capacity [12]. Increased permeability of the BBB is an initial step in a cascade process of molecular and cellular events leading to neuroinflammation [13]. Extravasation and homing of activated blood-born mononuclear cells, including autoreactive myelin-specific T cells, is greatly facilitated by the compromised function of the BBB in MS. Under physiologic conditions, formation of tight junctions between endothelial cells of brain blood vessels prevents diffusion of molecules and random trafficking of non-activated immune cells from the vascular into the CNS compartment [14,15]. If appropriately activated these circulating myelin-specific T cells acquire properties necessary for penetration through the BBB, and homing into brain and spinal cord parenchyma. Mononuclear cell trafficking and homing into the brain parenchyma is tightly regulated by intrathecally produced chemoattractant cytokines and chemokines. These chemoattractant factors engage their specific receptors, which are readily expressed by activated mononuclear cells, including T cells. As opposed to activated cells, naive lymphocytes and unstimulated monocytes do not have homing properties because they do not express appropriate adhesion molecules and chemokine receptors. Once they enter the CNS compartment, activated monocytes and lymphocytes produce and locally secrete inflammatory and immune cytokines and/or acquire direct cytotoxic properties. Locally released cytokines then stimulate microglia and astrocytes, which contribute to local inflammation by additional production of inflammatory cytokines, chemoattractant chemokines and other mediators of inflammation. Co-ordinate expression of adhesion molecules facilitates evasion of inflammatory cells through the BBB.

When activated myelin specific T cells express adhesion molecules, such as very late antigen-4 (VLA-4), which enable their attachment to endothelial cells of the BBB by binding to vascular cell adhesion molecule-1 (VCAM-1) [16]. Attachment is an initial step in extravasation of activated T cells and their homing to CNS. Therapies to prevent VLA-4/VCAM-1 interaction in a mouse model of relapsing-remitting EAE suggested complex and distinct roles of such interaction in disease initiation and in exacerbation [17].

Immune Cells

Multiple sclerosis (MS) is an autoimmune organ specific disease. Clinical and histopathology data strongly supports the concept of autoimmune-mediated neuroinflammation and subsequent axonal and oligodendroglial damage in MS. The primary role in disease initiation and subsequent autoimmune damage in CNS has been attributed to myelin-specific $CD4^+$ T cells. These T cells are self-reactive immune cells, which escape deletion by mechanisms of central tolerance, and can be found within a pool of peripheral, circulating T cells. Mechanisms responsible for maintenance and conversely breaking of peripheral tolerance, including regulatory T cells (Treg) and expression of co-inhibitory receptors by T cells remained the focus of experimental studies over several decades. Identifying factors and molecular and cellular pathways critical to maintaining peripheral tolerance to myelin-specific T cells were expected to eventually lead to prevention of MS. Current concepts of tolerance in MS have been recently critically reevaluated [18,19]. Typically, within MS lesion, the presence of inflammation, demyelination and axonal degeneration, can be observed. Those histopathology changes develop because of CNS damage by autoagressive T cells and antibodies, which carry specificities for immunodominant epitopes of myelin proteins [20]. Different animal models of experimental autoimmune encephalomyelitis (EAE) have been extensively studied with attempts to understand the etiology and immunopathology underlying variations in clinical forms of multiple sclerosis (MS) [21]. Over two decades ago data from EAE experiments indicated that major effector mechanisms of autoimmune demyelination in the central nervous system (CNS) were governed by encephalitogenic $CD4^+$ T cells [22]. Since then many facets of molecular and cellular pathways of $CD4^+$ T cells pertinent to neuroinflammation have been revealed. Major effector mechanisms of autoimmune demyelination in the central nervous system (CNS) are mainly, but not exclusively, mediated by Th1 helper/inducer $CD4^+$ encephalitogenic T cells, followed by the phagocytosis of myelin debris by macrophages. Roles for cytotoxic $CD8^+$ T cell, B cell and NK cell mediated regulation of immune responses to myelin antigens have been proposed [23,24,25]. Development and progression of an autoimmune response to myelin antigens depends on a fine balance and interactions between the effector and regulatory T cells in the periphery. Once activated and clonally expanded, autoagressive myelin antigen specific $CD4^+$ effector T cells, Th1, Th17 and Th9, coordinate CNS inflammation and induce tissue damage. Conversely, $CD4^+CD25^+$ regulatory T cells (Treg) and Th2 $CD4^+$ T cells have roles in controlling and downregulating effector T cell mediated inflammation. $CD4^+$ T cell infiltration into the CNS precedes onset of clinical signs of disease. Similarly, relapses of disease are based

upon the reappearance of activated $CD4^+$ T effector cells into the CNS, which subsequently leads to demyelination and axonal damage [26,27,28,29].

Cytokines and Chemokines

Guidance cues provided by chemokines and chemoattractant cytokines regulate the influx and intrathecal accumulation of inflammatory cells. For example, the cytokines interferon-γ (IFN-γ) and interleukin-17 have a role in determining the localization of inflammation to the brain or spinal cord in EAE [30]. Activated T cells also produce and locally secrete immune and inflammatory cytokines, including interleukin (IL)-2, IFN-γ, TNF-α, IL-16 and IL-17. Infiltrating $CD4^+$ T cells, $CD8^+$T cells and B cells produce and secrete IL-16 within EAE and similarly within MS lesions [31,32]. Activated monocytes produce proinflammatory cytokines, including IL-12, IL-1, IL-6 and TNF-α. Cytokines are pleiotrophic factors exhibiting immunostimulatory, immunosuppressive or immunomodulatory properties. Within the CNS, locally released cytokines communicate within a local cytokine network by potentiating, inhibiting or modulating each other's effects. Cytokines play multiple roles in the pathogenesis of MS and EAE. Homing of encephalitogenic $CD4^+$ T cells through the blood-brain barrier is a process tightly regulated by the production of chemoattractant chemokines and expression of their cognate receptors by both, glial and inflammatory cells [33]. Timing and levels of produced chemokines and their receptors are primarily regulated by cytokines, produced by activated mononuclear cells and glia. These chemokine/receptor pairs, which are suggested as major regulators of inflammatory infiltration, include: CXC ligand (CXCL)10 (also known as interferon inducible protein 10kDa (IP-10)) and its receptor CXC chemokine receptor (CXCR)3; CC ligand (CCL)1 (macrophage chemoattractant protein (MCP)-1) and its receptor CC chemokine receptor (CCR)2; CCL3 (macrophage inflammatory protein (MIP)-1P) and its receptors CCR1 and CCR5; CCL4 (MIP-1α) and its receptor CCR5; and CCL5 (regulated upon activation, normal T-cell expressed and secreted (RANTES)) and its receptors CCR1, CCR3, and CCR5 [34] (Table 1). As an example, mice deficient for MCP-1 or for its specific receptor CCR2, developed less severe EAE compared to wild type controls [35,36,37]. Production of several chemokines and expression of their cognate receptors have been consistently found in MS lesions. These chemokine/receptor pairs suggested as major regulators of inflammatory infiltration include CXCL10 (IP-10)/CXCR3, CCL1 (MCP-1)/CCR2, CCL3 (MIP-1α)/CCR1/CCR5, CCL4 (MIP-1β)/CCR5 and CCL5 (RANTES)/CCR1/CCR3/CCR5 [34]. A similar role for chemokines in chemoattraction of macrophages and activated $CD4^+$ T cells has been documented in different models of EAE [38,39]. In EAE, activation of encephalitogenic $CD4^+$ T cells precedes the expression of appropriate chemokine receptors by T cells. Engagement of chemokine receptors by appropriate chemokines enable T cell homing through the blood-brain barrier, leading to inflammation. Activated $CD4^+$ Th1 cells produce cytokines including IL-2 and IFN-γ, which locally induce glial activation with consequent damage to oligodendrocytes.

Table 1: Chemokine – Cognate Receptor Pairs associated with MS and EAE and regulation of chemokine receptor signaling by IL-16/CD4

Chemokine	Chemokine receptor	IL-16/CD4 cross-desensitization
CCL1 (MCP-1)	**CCR2**	
CCL3 (MIP-1α); CCL4 (MIP-1β)	**CCR5, CCR1**	CCR5
CCL5 (RANTES)	**CCR1, CCR3, CCR5**	CCR5
CXCL10 (IP-10)	**CXCR3**	CXCR3
CXCL12 (SDF-1)	**CXCR4, CXCR7**	CXCR4
Fractalkine	**CX3CR1**	

Glia

Astrocytes and microglia actively participate in neuroinflammation. They become activated by inflammatory (IL-1, IL-6 and TNF- α) and immune (IFN- γ) cytokines. Activated glial cells produce and locally release chemokines and cytokines, which become instrumental in orchestrating influx and homing patterns of activated autoimmune T cells and macrophages. In EAE, activation of myelin-specific $CD4^+$ T cells precedes the expression of appropriate chemokine receptors by those T cells. Engagement of chemokine receptors by appropriate chemokines enables T cell homing through the blood-brain barrier, leading to inflammation. Activated $CD4^+$ Th1 cells produce cytokines including IL-2 and IFN- γ, which locally induce glial activation with consequent damage to oligodendrocytes. Comparative analysis of chemokines and cytokines produced in acute and relapsing stages of EAE revealed significant differences [39]. Chemokines, including MCP-1, MIP-1α, and MIP-1β, are implicated in regulation of acute and relapsing disease. While the role of chemokines in regulation of mononuclear cell homing has been extensively studied, their suggested roles in other aspects of local immune regulation are largely unknown. As opposed to the majority of CC and CXC chemokines implicated in the immunopathology of EAE, which do not discriminate between cell phenotypes, IL-16 is a $CD4^+$T-cell-specific chemoattractant cytokine. IL-16 is a more effective chemoattractant for Th1 than for Th2 $CD4^+$ T cells. IL-16 binds specifically to the CD4 receptor, leading to positive T cell chemotaxis. Production of IL-16 is tightly regulated within the cytokine network in the CNS. Production of IL-16 by microglia can be induced by $IL\text{-}12^{p40}$ in vitro [40]. EAE studies demonstrated high levels of intrathecal IL-16, primarily produced by infiltrating T cells and by resident microglia during neuroinflammation [31,32,41].

IL-16 AND ITS ROLE IN NEUROINFLAMMATION

Interleukin (IL)-16 has been identified as a major source of T cell lymphocyte chemotactic activity and was originally named leukocyte chemoattractant factor (LCF) [42]. Precursor molecule, pro-IL-16 (80 kDa) is constitutively expressed in lymphocytes. T cell receptor (TCR) mediated or cytokine induced T cell activation leads to the enzymatic cleavage of pro-IL-16 by activated Caspase-3 and release of active IL-16 (14 kDa) [43].

Biological Properties

The precursor molecule, pro-IL-16 (80 kDa) is constitutively produced in T lymphocytes.

Activation and release of bioactive IL-16 (17 kDa) is distinctly regulated among T-cell subsets. In $CD4^+$ T cells, T-cell receptor (TCR) mediated or cytokine induced T-cell activation, leads to the enzymatic cleavage of pro-IL-16 by activated caspase-3 and release of active C-terminal portion of IL-16 (14-17 kDa), while secretion of IL-16 from $CD8^+$ T cells is caspase-3 independent. Cleavage of the C-terminal portion of pro-IL-16 is regulated by phosphorylation of pro-IL-16 on Ser144, which engages Erk1/2 kinase activity, while the secretion of bioactive IL-16 is regulated by MAP kinase. In quiescent $CD4^+$ T cells only pro-IL-16 (80 kDa) can be detected. Following $CD4^+$ T cell activation, an intermediate product of cleavage (50-60 kDa) and bioactive IL-16 (14-17 kDa) are readily observed. Bioactive IL-16 is secreted from activated cells. Secreted IL-16 multimerizes into homotetramers composed of 14-17 kDa chains, necessary for the binding to CD4 receptor and subsequent signaling [44]. Binding of IL-16 to the CD4 receptor is followed by autophosphorylation of $p56^{lck}$ and rises in intracellular Ca^{++}. Production of IL-16 is described in $CD4^+$ and $CD8^+$ T cells, B cells, dendritic cells and eosinophils. A role for IL-16 in the cross talk between dendritic cells and T cells has been proposed [45]. IL-16 induces chemoattraction of $CD4^+$ T cells by binding to CD4 receptor and signaling that involves p56lck, with the requirement for SH2/SH3 recruitment domains, which means involvement of other intracellular signaling proteins. The minimal C-terminal peptide RRKS (corresponding to Arg^{106} to Ser^{109}) was shown to be critical for mediating chemoattracting activity of mature IL-16. While the secreted C-terminal domain of IL-16 achieves its pleiotropic effects on $CD4^+$ cells through CD4 receptor-initiated signaling pathways, the residual N-terminal domain translocate to the nucleus, where it induces G0/G1 cell cycle arrest. Nuclear translocation of the N-terminal portion of IL-16 is enabled by the CcN motif, which contains a nuclear localization sequence (NLS), protein kinase CK2 substrate site and a cdc2 kinase substrate site. Other known effects of IL-16 on $CD4^+$ T cells include induction of CD25 (IL-2Rα) expression, induction of cytokine synthesis, modulation of T cell proliferation and chemotaxis. $CD4^+$ monocytes can be also chemoattracted by IL-16. In vitro treatment with IL-16 induces MHC class II expression by mononuclear cells. Thus, IL-16 is postulated to be a proinflammatory and immunoregulatory molecule with an important role in recruitment and activation of $CD4^+$ T cells at the site of inflammation [46]. At the site of inflammation, IL-16 can modulate T cell chemoattraction by other T cell chemoattractant chemokines. Depending on the specifics of tissue and type of inflammation IL-16 may either contribute to chemoattraction by other chemokines such as RANTES [47] or modulate their activity, such as CCL4 [48]. Taken together, biological properties of IL-16 including $CD4^+$ T cell chemoattraction, modulation of chemokine-induced T cell chemoattraction, T cell activation and chemokine production [49] make this cytokine of interest for studies of immune regulation of EAE and MS (Figure 1).

IL-16 in Regulation of Autoimmune Diseases

Interleukin 16 (IL-16) was discovered almost two decades ago, when it was identified as a $CD4^+$ T cell chemoattractant. This cytokine binds specifically to the CD4 molecule, which is predominantly expressed by mononuclear cells involved in regulation of innate and adoptive immune responses.

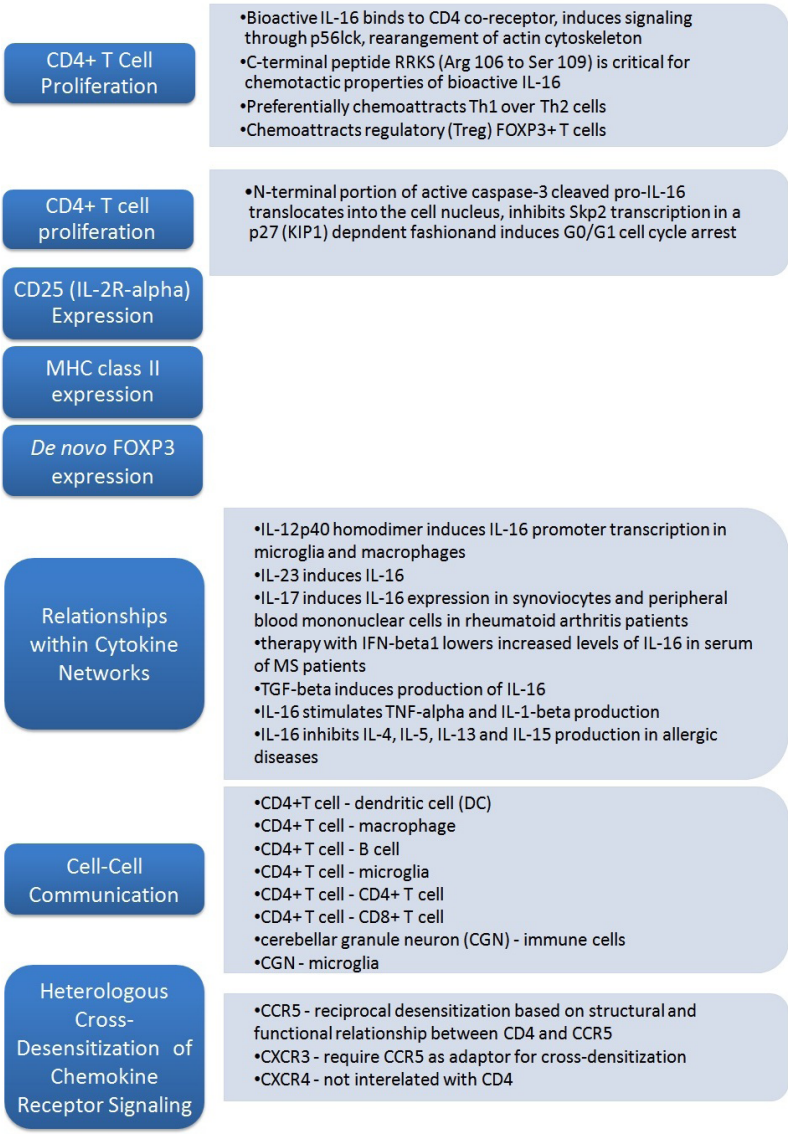

Figure 1: Biological properties of IL-16 pertinent to MS regulation

These cells include immune cells, all $CD4^+$ T cells, subsets of $CD8^+$ T, NK and NKT cells; antigen presenting cells (APC), macrophages and dendritic cells; eosinophils and mast cells. Concordant with their CD4 expression, these lymphoid cells contain pro-IL-16 and have the potential to locally release bioactive IL-16, upon cell activation. Besides T cells and APC, B cells are identified as additional sources of IL-16 in lymphoid tissues. Based on its demonstrated potential in recruiting $CD4^+$ cells, IL-16 is considered an inflammatory cytokine. Other biological properties of IL-16 include the regulation of T cell activation, cell cycle progression, cytokine and chemokine production, and modulation of responsiveness to some chemokines. An extensive body of experimental data demonstrating important role of IL-16 in regulation of immune responses prompted investigations of the role of this cytokine in immune mediated diseases. IL-16 is a pleiotrophic cytokine implicated in the regulation of multiple sclerosis and other autoimmune diseases [50]. IL-16 selectively attracts $CD4^+$ cells and bind specifically to the CD4 receptor. Peripheral immune cells, which express the CD4 molecule on their surface, include $CD4^+$ T cells, a subset of monocytes, eosinophils and dendritic cells. There is evidence for the role of IL-16 in regulation of inflammation in rheumatoid arthritis [51], Graves' disease [52], atopic dermatitis [53], and SLE [54]. Production of IL-16 along with MIP-1α and β, and IP-10 has been shown in PLP-specific $CD8^+$ T cells [55]. The role of IL-16 in regulation of diabetes mellitus type I has been suggested in rat and mouse models. In NOD mice, therapy with neutralizing IL-16 antibody successfully protected mice from the development of diabetes [56]. We demonstrated that IL-16 production was associated with infiltrating mononuclear cells in the pancreas of BB/W spontaneously diabetic rats [57].

IL-16 in EAE and MS

Within MS, similar to EAE lesions, major cellular sources of intrathecally produced IL-16 include $CD4^+$ and $CD8^+$ T cells, B cells and activated resident microglia [31,32,58]. Through its CD4 receptor-specific effects, IL-16 induces positive chemoattraction of $CD4^+$ T cells. $CD4^+$ T cells are implicated in the pathogenesis of MS and EAE as Th1 $CD4^+$ myelin-specific autoimmune cells can initiate inflammation, axonal and ligodendroglial damage and demyelination. Conversely, regulatory $CD4^+$Treg cells can mediate protection from the damaging effects of encephalitogenic cells. Compared to $CD4^+$ T cell chemoattractant chemokines, IL-16 has a unique potential to specifically chemoattract $CD4^+$ T cells regardless of their activation stage. As opposed to the CD4 selective binding by IL-16, chemokines are non-selective for cell type that they chemoattract. Chemokines specifically bind to corresponding chemokine receptors. Chemokine receptors are readily expressed by different types of activated cells, including T cells ($CD4^+$ and $CD8^+$), B cells, macrophages and dendritic cells. For example, chemokines known to regulate inflammation in EAE through their chemoattractant properties, such as CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), and RANTES are not exclusive for $CD4^+$ T cells. The fact that IL-16 chemoattraction is not related to the $CD4^+$ T cell activation state was especially interesting because such non-selectivity might provide recruitment from the pool of MOG-specific long-term TH1 memory (peripheral

memory) cells [59], which do not readily express activation markers and activation induced chemokine receptors. The existence of peripheral memory cells to MOG_{35-55} is suggested by our finding of strong lymph node and splenic T cell proliferation to MOG_{35-55} in relapsing H-$2^{b/s}$ mice [60]. $CD4^+$ T cell infiltration and TH1 $CD4^+$ T cell effector mechanisms play important roles in immune regulation of onset and relapses of EAE. Homing of encephalitogenic $CD4^+$ T cells through the blood-brain barrier is a process tightly regulated by the production of chemoattractant chemokines and expression of their cognate receptors by both glial and inflammatory cells. Timing and levels of produced chemokines and their receptors are primarily regulated by cytokines, produced by activated mononuclear cells and glia. Thus far major therapeutic efforts to prevent and/or cure autoimmune mediated damage of brain and spinal cord in EAE have been directed towards preventing $CD4^+$ T cell infiltration. Understanding regulation of $CD4^+$ T cell infiltration in response to MOG_{35-55} is of great importance for multiple sclerosis (MS). Strong immune responses to MOG_{35-55} by $CD4^+$ TH1 cells are found in patients with MS. The encephalitogenic epitope of myelin oligodendrocyte glycoprotein (MOG) p35-55 is highly conserved among species, including mouse and human. Studies of mechanisms regulating relapsing disease in response to MOG_{35-55} have been hampered by the lack of appropriate animal models [21]. We developed such a model in (B6 x SJL)F1 (H-$2^{b/s}$) mice [60]. We found that H-$2^{b/s}$ mice develop an immune response to myelin oligodendrocyte peptide (MOG_{35-55}) similarly as H-2^b mice, which causes distinct clinical EAE, immunopathology of relapsing lesions and T cell responses to MOG_{35-55}. H-$2^{b/s}$ mice readily developed 2 to 4 relapses with severity score 3 times higher than H-2^b over an 80 day period. In comparison, only few of H-2^b mice relapsed and with only one relapse cycle, while the remaining mice recovered. During the relapses in H-$2^{b/s}$ mice, phenotypes of CNS infiltrates were skewed towards $CD4^+$ T cells and $B220^+$ B cells compared to $Mac\text{-}3^+$ macrophages. Quantitative analysis of infiltrating cells revealed significantly higher numbers of $CD4^+$ T cells, and significantly lower numbers of macrophage/microglia in infiltrates of relapsing H-$2^{b/s}$ compared to H-2^b mice. Our data suggested enhanced regulation of $CD4^+$ T cell trafficking compared to macrophages during the relapsing stages of disease. We hypothesized that $CD4^+$ T cell- mediated mechanisms play a major role in relapsing disease in H-$2^{b/s}$ mice. Thus preventing $CD4^+$ T cell infiltration should protect mice from relapsing. Therefore, we examined several chemoattractant factors that regulate trafficking of $CD4^+$ T cells. While it was difficult to find a chemokine exclusive for $CD4^+$ T cells, we focused on IL-16, a cytokine which binds specifically to the CD4 receptor and regulates $CD4^+$ cell migration. Moreover, IL-16 is produced by T cells and B cells, which in H-$2^{b/s}$ mice represented the majority of infiltrating cells. Some macrophages can also express CD4 although they do not produce IL-16.

We initially compared the regulation of IL-16 between relapsing-remitting H-$2^{b/s}$ mice (with high numbers of infiltrating $CD4^+$ T cells) and low relapsing H-2^b mice (with high numbers of macrophages in relapsing lesions). We found the highest production of pro-IL-16 (80 kDa) and 17 kDa mature IL-16 in spinal cord of H-$2^{b/s}$ relapsing mice. Differences in IL-16 production between

the two strains were observed at all stages of disease. In acute EAE, IL-16 was not detectable in H-2^b but was found in appreciable levels in H-$2^{b/s}$ mice. This finding correlated with the different representation of phenotypes of infiltrating cells found in acute disease. The majority of cells in acute lesions of H-2^b mice were macrophages, which do not readily produce IL-16. In H-$2^{b/s}$ mice with acute lesions, infiltration of $CD4^+$ T cells exceeded that of macrophages. T cells constitutively contained pro-IL-16 and upon cleavage with activated Caspase-3, they secreted the active 17-kDa forms. The highest production of pro-IL-16 and its active form was observed in relapsing F1 mice, which correlated well to levels of $CD4^+$ T cell infiltration. In relapsing H-2^b mice, lower levels of IL-16 were observed, corresponding to lower numbers of infiltrating $CD4^+$ T cells. During remission, IL-16 levels significantly dropped in both strains of mice. This was consistent with the clearance of infiltrating cells from spinal cord during remission [61]. It is very interesting that in F1 mice with chronic disease (more than three months after onset), appreciable levels of IL-16 were found. These data suggested that IL-16 production closely correlated with levels of $CD4^+$ T cell infiltration in acute, relapsing and chronic stages of EAE in H-$2^{b/s}$ mice with more severe disease (Figure 2).

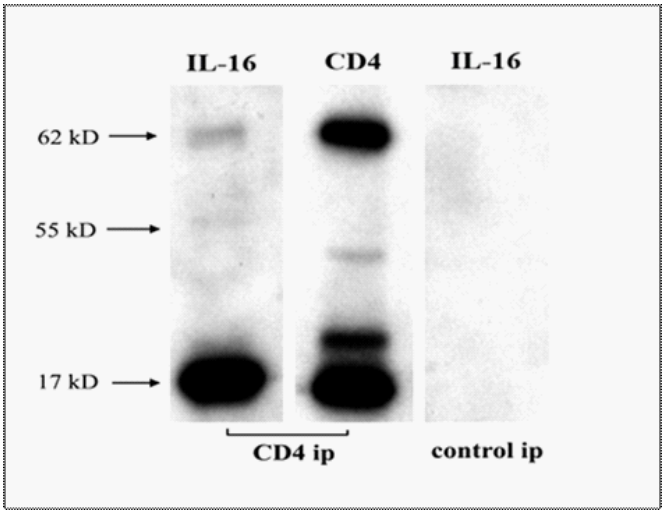

Figure 2: Co-immunoprecipitation of IL-16 with CD4 in CNS of EAE mice

IL-16 was co-immuno-precipitated with CD4 from CNS of mice with relapsing disease. CD4 was immunoprecipitated using an anti-CD4 specific antibody, from total proteins isolated from spinal cord and subjected to western blotting using anti-IL-16 antibody. The membrane was then stripped and reblotted with anti-CD4 antibody. In a separate, similarly performed experiment, immunoprecipitation was done with control antibody, which was isotype matched with anti-CD4 specific antibody. IL-16 specific immunoreactivity was not observed with control immunoprecipitates. Blot shows results from spinal cord of (B6 x SJL) F1 mouse, at third relapse, 68 dpi, with clinical grade of 4 [31].

We then proceeded to identify the cellular sources of IL-16 activity in relapsing lesions. We

analyzed infiltrating cells for IL-16 production because pro-IL-16 is constitutively produced by T and B-lymphocytes. IL-16 positive immunostaining co-localized with $CD3^+$ $CD4^+$ T cells, and $B220^+$ B cells in relapsing H-$2^{b/s}$ mice [60]. IL-16 specific immunoreactivity was mostly confined to $CD8^+$ T cells in H-2^b mice. These findings provided the foundation for design of anti-IL-16 therapy of EAE mice. Treatment with neutralizing anti-IL-16 antibody improved clinical signs of relapsing-remitting EAE in H-$2^{b/s}$ mice. Mice with clinical signs of EAE were treated by intraperitoneal injection of either neutralizing mouse anti-human IL-16, or polyclonal anti-IL-16 antibody [43]. The high degree of sequence homology (>80%) between the human and murine IL-16 allows for antibody cross-specificity. Anti-human IL-16 mAb could neutralize the bioactivity of murine IL-16, and block chemoattractant activity of $CD4^+$ T cells induced by murine IL-16 [62]. The three different treatment schedules were injections with IL-16 neutralizing antibody, a single dose administered after the onset of disease (group 1), group two – two doses administered following acute and relapsing disease (group 2) and a single dose following the first relapse (group 3). Mice in group 2 (two injections) were treated only with mouse monoclonal antibody. Polyclonal antibody was tested in mice which received single injection and results were similar to those obtained with monoclonal antibody treatment. Each anti-IL-16 treated group was compared to its own two control EAE groups, non-treated and isotype-matched IgG treated, within the same experiment. Following the treatment with neutralizing anti-IL-16 antibody, we observed significant differences in the clinical course between mice treated with neutralizing anti-IL-16 antibody and controls in all three examined treatment paradigms [58]. An improvement in histopathology of CNS was observed in anti-IL-16 treated mice. In spinal cords of mice treated with anti-IL-16, demyelinating areas were smaller when compared to non-treated and IgG-control groups. Analysis of 1μ semi-thin sections enabled better visualization of demyelinating and degenerating axons. Reduction of mononuclear cell infiltration was accompanied by less demyelination and overall better preservation of axons in anti-IL-16-treated mice compared to non-treated and IgG control groups. EM analysis further revealed differences between relapsing non-treated and antibody-treated mice. Control EAE mice showed changes in histopathology typical of EAE, consistent with our previous reports [60]. Presence of degenerated fibers with myelin sheaths at various stages of degeneration or demyelination adjacent to infiltrating macrophages were prominent in non-treated EAE mice. In mice treated with anti-IL-16 antibody, myelinated axons had more preserved morphology and were surrounded by myelin sheaths of various diameters. By semi quantitative analysis, we found approximately five-fold decrease in relative numbers of $CD4^+$ T cells, two-fold decrease in $B220^+$ cells and modest decrease of $Mac3^+$ cells. Reduction of infiltrating B cells following anti-IL-16 treatment may be due to indirect effects of therapy on regulation of chemokine production by infiltrating or glial cells. Although a subset of circulating monocytes expresses CD4R, the relatively modest effects of IL-16 neutralization on $Mac3^+$ macrophage/microglia infiltration may be the result of their predominantly $CD4^-$ phenotype in CNS. Taken together, anti-IL-16 treatment significantly improved relapsing-remitting EAE, diminished demyelination and impeded infiltration by $CD4^+$ T cells. Data also

suggest that effectiveness of anti-IL-16 therapy depends on timing and duration of treatments. Our data suggest that in EAE, the role of IL-16 included more than just regulation of $CD4^+$ T cell chemoattraction. We demonstrated an important role for IL-16 in regulation of autoimmune inflammation and subsequent axonal damage using a mouse model of MS [31,32,58]. Similar roles of IL-16 in MS are largely unexplored. To facilitate an understanding of the potential significance of IL-16 for human disease, we examined regulation of IL-16 and correlation of IL-16 production with $CD4^+$ Th1 infiltration and inflammation-related changes of axonal cytoskeleton in MS lesions. We measured relative levels of IL-16, active caspase-3, T-bet, Stat-1 (Tyr^{701}), and phosphorylated medium and heavy chains of neurofilament [NF(M+H)], in brain and spinal cord lesions from MS autopsies, using western blot analysis. We examined samples from 39 MS cases, which included acute, subacute and chronic lesions, as well as adjacent, normal-appearing white and grey matter. All samples were taken from patients with relapsing remitting clinical disease. We employed two-color immunostaining and confocal microscopy to identify phenotypes of IL-16-containing cells in frozen tissue sections from MS lesions. We observed that IL-16 immunoreactivity was confined to T-bet^+ and active-$caspase\text{-}3^+$ infiltrating cells. In addition to $CD4^+$ Th1 cells, IL-16 immunoreactivity was observed in $CD8^+$ T cell, B cells and occasionally in microglia. We measured a marked increase in levels of pro and secreted IL-16 (80 kD and 22 kD, respectively) in MS lesions compared to controls. Levels of IL-16 peaked in acute, diminished in subacute, and were elevated again in chronic active lesions. Compared to lesions, lower but still appreciable IL-16 levels were measured in normal appearing white matter adjacent to active lesions. Production of bioactive IL-16 correlated with increased levels of active-caspase-3, T-bet and phosphorylated STAT1 in MS lesions. Altogether, our data suggests that IL-16 production occurs in MS lesions. We observed correlations between increased levels of secreted IL-16, $CD4^+$ Th1 cell inflammation, and phosphorylation of axonal cytoskeleton in MS lesions. These data suggest a possible role for IL-16 in the regulation of inflammation and of subsequent changes in the axonal cytoskeleton in MS [63], supported by recent observation that IL-16 is a specific cell signaling factor, associated with cathepsin processing during MS relapses [63].

Overall, evidence from EAE and MS studies suggest that accumulation of intrathecal IL-16 occurs in both relapsing-remitting EAE and in MS. Moreover, regulation of IL-16 corresponds to Th1 inflammation and related damage of axonal cytoskeletons. In EAE, neutralization of IL-16 activity leads to improvement of clinical disease, with diminished inflammation, demyelination and axonal damage. In MS lesions, we demonstrated correlation between intrathecal production of IL-16, Th1 inflammation and inflammation-related changes of axonal cytoskeletons. Taken together, our studies of IL-16 regulation and function in EAE and MS support the view that this cytokine has an important role in regulation of neuroinflammation and axonal damage in MS.

CONCLUSION

Multiple sclerosis is a chronic, inflammatory demyelinating and neurodegenerative disease. Infiltration of CNS by autoimmune $CD4^+$ Th1 cells, specific for immunodominant epitopes of myelin

specific antigens have an important role in demyelination, axonal degeneration, and neuronal and oligodendroglial damage and dysfunction. The cytokine IL-16 is a major regulator of $CD4^+$ T cell influx into CNS in relapsing-remitting EAE and MS. Locally produced IL-16 corresponds with the degree of $CD4^+$ T cell inflammation, demyelination and axonal degeneration in relapsing MS and EAE lesions. Neutralization of IL-16 led to reversal of paralysis and improved histopathology in relapsing-remitting EAE. IL-16 is a promising candidate for a $CD4^+$ T cell targeted therapy of relapsing MS. Furthermore IL-16 emerges as a good candidate for treatment of other inflammatory and/or neurodegenerative neurological diseases.

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