PATHOGENESIS OF MULTIPLE SCLEROSIS

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Multiple sclerosis (MS) is an inflammatory, demyelinating disorder in which the target antigen(s) of the immune response are located in CNS myelin (1). Animal models of MS such as experimental autoimmune encephalomyelitis (EAE) have demonstrated that autoreactive, myelin-specific T lymphocytes (CD4+ or CD8+) can result in CNS inflammatory demyelination (2-4). In MS patients, the frequency of myelin-reactive T cells appears to be quite similar to that of healthy individuals. However, significant qualitative differences have been observed in the responses that are mediated by these T cell populations. Myelin-reactive T cells have been shown to be of a memory or activated phenotype when obtained from MS patients, while these same T cells have a naïve phenotype when obtained from healthy individuals (5,6). This would suggest that myelin-reactive T cells in MS patients have already encountered their antigen, resulting in this primed or activated phenotype. In EAE, using a molecule called CTLA-4Ig was able to inhibit the priming of myelin-reactive T cells and prevent clinical expression of the disease (7). A clinical trial in patients with MS using CTLA-4Ig or abatacept has recently been completed and the results of that study did not demonstrate a significant therapeutic response (35).

Using a flow cytometric technique which allows characterization of T cells from the peripheral blood directly *ex vivo*, significant differences in cytokine production and chemokine receptor expression also suggest that myelin-reactive T cells from MS patients have a greater pro-inflammatory phenotype than those T cells obtained from healthy individuals (8). In addition, myelin-specific CD8+ T cells have also been obtained from patients with relapsing MS and their suppressive capability appears to change depending on the stage of disease activity (9). During an acute MS exacerbation, the regulatory capability of CD8+ T cells appears to be reduced, but returns as the patient recovers from the attack.

Additional evidence that myelin-reactive T cells can result in inflammatory demyelination came from a clinical trial studying altered peptide ligands in MS (10). In that study, several patients developed either clinical exacerbations, or an increase in disease activity as measured by gadolinium-enhancing lesions on MRI. With this increase in disease activity, a significant increase in T cells responding to a particular component of myelin basic protein (MBP85-99) was also demonstrated suggesting a link between the observations (10). These data provided additional evidence that T cell responses directed against myelin antigens played a role in the pathogenesis of MS.

Evidence for humoral immunity in MS has been accumulating for several decades. Many studies have shown increases in B cells, plasma cells, and antibodies in the CSF of patients with multiple sclerosis. For example, Cepok and colleagues observed that both plasmablasts and memory B cells were in the CSF of MS patients (11). Plasmablasts were present in high numbers throughout the course of disease and their presence correlated with disease activity as measured by MRI. In addition, these plasmablasts were also responsible for the elevated IgG synthesis observed in the CSF of these patients. Given that the presence of oligoclonal bands (OCBs) in MS indicate an exaggerated Ig synthesis in the brain and spinal cord, it has been assumed by many that these antibodies may initiate and perpetuate disease activity. The molecular and cellular targets of OCBs in MS have not (yet) been identified despite the fact that plausible candidates, including myelin oligodendroycte glycoprotein (MOG), were investigated extensively. Thus, it cannot be entirely ruled-out that they are an epiphenomenon.

Work from Hemmer's group used a proteomic approach focusing on membrane proteins, and they identified the ATP-sensitive inward rectifying potassium channel KIR4.1 as the target of serum IgG of MS patients, but not controls. They identified IgG from the serum of a subgroup of MS patients (n=186/397), which could bind to glial cells (12). This antibody was specific for KIR4.1 an inward rectifying potassium channel located on astrocytes and oligodendrocytes. This antibody was present in 47% of MS patients in the study and only in <1% of patients with other neurologic disorders (n=329). Neuromyelitis optica (NMO) is an inflammatory, demyelinating disorder with antibodies primarily directed against aquaporin 4 (AQP4), a channel also located on astrocytes. Since KIR 4.1 localizes with AQP4 on astrocytes, it may be possible that KIR4.1 is also a target in

NMO patients who are NMOIgG negative (13). Interestingly, the subgroup of MS patients with antibodies to KIR 4.1 and in NMO patients seropositive for NMO IgG, the presumed pathologic antibodies are rarely detected within CSF, but can occur with high titer within the serum. The vast majority of patients with multiple sclerosis eventually have detectable OCBs in the CSF. This indicates that the target of these clonally expanded antibodies in MS remains unknown in patients who have antibodies to KIR4.1 in the periphery (13). Interestingly, while another study found anti-KIR4.1 antibodies increased during MS exacerbations (14), another study found no evidence of these antibodies in the serum (15).

Because of the evidence of the role of B cells in MS pathogenesis, investigators believed that targeting B cells might be beneficial in patients with MS. Rituximab is a anti-CD20 monoclonal antibody that depletes CD20+ B cells by binding to CD20 and initiating complement-dependent cytolysis. Cross and colleagues initiated early studies with rituximab in patients with relapsing-remitting MS patients who did not respond to first line immunomodulatory agents (16) Examination of the CSF in these patients showed that 90% of CSF CD19+ B cells were deleted. CSF T cells were also reduced, suggesting that B cells played a role in the subsequent recruitment of T cells into the CSF. This decrease was attributed to decreases in the chemokines CXCL13 and CCL19. The decreased number of gadolinium-enhancing lesions, along with a lack of newly formed lesions, combined with no significant change in the IgG levels found in the CSF of rituximab treated patients suggests that the APC function of B cells, along with their creation and maintenance of specific cytokine and chemokine networks is more important than their antibody secretion in the pathogenesis of MS. Recently, trials utilizing a humanized anti-CD20 antibody called ocrelizumab showed benefit in both relapsing remitting and primary progressive multiple sclerosis (36, 37).

The cytokine-producing phenotype of myelin-specific T cells is an important factor in determining their pathogenic potential or encephalitogenicity. Organ-specific autoimmune diseases such as MS are thought to be mediated by interferon-□ (IFN□)-producing T helper 1 type cells (Th1) (17). Myelin-reactive T cells from MS patients produce cytokines more consistent with a Th1 response, while myelin-reactive T cells from healthy individuals are more likely to produce Th2 cytokines (8). In addition to Th1 cells, it is now clear that there are a subset of T cells called Th17 cells that may also contribute to inflammatory demyelinating disease such as multiple sclerosis (18). IL-12p40 associates not only with IL-12p35 to form IL-12, but also with a p19 chain to form IL-23 (19). IL-23 results in T cells producing IL-17, which some investigators believe is an important determinant of encephalitogenicity. Microarray studies on MS lesions in humans have shown an increased expression of IL-17, suggesting it can contribute to the development of an inflammatory, demyelinating lesion (20). Recent studies have also suggested that transcription factors such as T-bet and Stat-4, necessary for Th1 differentiation, are important in the differentiation of encephalitogenic T cells, both of the Th1 and Th17 phenotype (21-24).

To define the minimum signals required for development of encephalitogenic T cells which cause CNS autoimmunity, myelin-specific T cells were differentiated with various cytokine cocktails and pathogenicity was determined by transfer into mice. IL-6+IL-23 or IL-12+IL-23 generated encephalitogenic T cells and recapitulated the essential cytokine signals provided by antigen presenting cells, and both IL-6 and IL-12 induced IL-23 receptor expression on both mouse and human naïve T cells (38). IL-23 signaled through both STAT3 and STAT4, and disruption in STAT4 signaling impaired CNS autoimmunity independent of IL-12. These data explain why IL-12-deficient mice develop CNS autoimmunity, while STAT4-deficient mice are resistant. CD4+ memory T cells from multiple sclerosis patients had significantly higher levels of p-STAT3/p-STAT4, and p-STAT3/p-STAT4 heterodimers were observed upon IL-23 signaling, suggesting that p-STAT3/p-STAT4 induced by IL-23 signaling orchestrate the generation of pathogenic T cells in CNS autoimmunity regardless of Th1 or Th17 phenotype (REF). We are now poised to determine whether studies in humans will corroborate these animal observations and demonstrate that similar transcriptional programs are important in determining the pathogenic potential of myelin-reactive T cells in MS.

As noted above, a conundrum in MS research is that the myelin-specific T cells that potentially participate in MS pathogenesis exist in all individuals (5, 6, 8). The factors that predispose MS naïve T cells to become activated and cause inflammation in MS remain obscure. While Genome-Wide Association Studies have demonstrated genetic associations with MS (25-28), a large portion of hereditary susceptibility remains unaccounted for (28). MicroRNAs (miRNAs) have recently emerged as important regulators of gene expression (29, 30) and their expression can be influenced by both genetic and environmental factors, making them attractive candidates in influencing T cell differentiation in MS.

miRNAs are small RNAs, 19-24 nucleotide-long, that bind the 3'UTR of target mRNAs, thereby inhibiting translation or inducing mRNA degradation (29, 30). The role of miRNAs in suspected autoimmune disorders such as MS is just beginning to be elucidated. Studies in peripheral blood mononuclear cells (PBMC) from MS patients have identified miRNAs dysregulation in MS (31, 32). However, it is unclear whether these miRNA differences underlie the etiology of the disease.

We have investigated miRNA expression in highly purified naïve CD4+ T cells from MS patients (33). The naïve $CD4^+$ T cell population represents cells that have not been activated, allowing us to examine whether miRNA influence T cells from MS patients to differentiate into proinflammatory phenotypes. We identified miR-128, miR-27a/b and miR-340 as being increased in MS patients' naïve T cells. These miRNAs acted to suppress Th2 differentiation by targeting Bmi-1 (a molecule that helps stabilize GATA-3, the transcription factor thought to be the master regulator of Th2 differentiation) and IL-4 expression and set the stage for pro-inflammatory Th1 autoimmune responses, illustrating the biological significance of miRNAs in the susceptibility to MS.

We have also examined the expression of T-bet in CD4+ T cells in MS patients and healthy controls. T-bet levels are elevated in MS patients, which would be in agreement with our observations of the important role of this transcription factor in T cell encephalitogenicity (34). We also found that a miRNA (miR29b) that targeted both T-bet and IFN-□ was overexpressed in memory CD4+ T cells in MS patients. We would have expected that the increased levels of miR29b would result in reduced levels of T-bet and IFN-□. But, as noted above, T-bet levels were actually increased in MS patients, which is not what one would have expected. To address this issue, we activated memory CD4+ T cells from MS patients and healthy controls and found that miR29b levels increased in healthy controls, but decreased in MS patients. Thus, while resting memory CD4+ T cells appear to be primed to regulate a Th1 response in MS patients by controlling T-bet and IFN-□ levels, this regulatory mechanisms fails upon T cell activation in patients with MS. This failure allows for the high T-bet expression observed in MS CD4+ T cells and promotes effector functions that are associated with the CNS pathology observed in MS patients.

Tregs in patients with multiple sclerosis, while normal in number, demonstrate diminished suppressive effect on myelin-specific autoreactive T cells, low FOXP3 expression, and a less diverse T cell receptor (TCR) repertoire. 24 of the 85 differentially expressed miRNAs in naïve CD4 T cells were predicted to target the TGFβ signaling pathway (39). TGFβR1 and SMAD4 were significantly decreased in MS patients. The differential expression of miRNAs predicted to target TGFβR1 and SMAD4 in the miRNA profiling. These data suggested that overexpression of miRNAs in MS patients may limit TGFβ signaling and may be the mechanism responsible for the Treg defect in MS patients.

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