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## Neuroimmune interactions in a model of multiple sclerosis

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### Abstract

Psychological stress has been implicated in both the onset and exacerbation of multiple sclerosis. Our research has focused on the role of stress at the onset of MS using the mouse model: Theiler's murine encephalomyelitis virus-induced demyelination (TVID). Theiler's virus is a natural pathogen of mice that causes a persistent infection of the central nervous system and inflammatory immune-mediated demyelination which is very similar to MS. Our research has shown that restraint stress sufficiently increases corticosterone secretion to cause immunosuppression. Stressed mice develop decreased innate and adaptive immune responses including decreased chemokine and cytokine responses to virus which leads to increased viral replication within the CNS. Higher levels of virus then cause increased later demyelinating disease. These findings may have important implications in our understanding of the interactions between stress and the development of autoimmune diseases induced by infectious agents.

### Keywords

Theiler's virus; multiple sclerosis; demyelination; central nervous system; autoimmunity; viruses; stress; restraint stress; glucocorticoids; corticosterone; HPA axis; immune system; chemokines; cytokines; NK cells; interferon; macrophages; innate immunity; adaptive immunity; Th1; Th2; T cells; CD4+ T cells; CD8+ T cells; inflammation

## MULTIPLE SCLEROSIS

Multiple sclerosis is an autoimmune inflammatory demyelinating disease of the CNS occurring at a prevalence of 250,000-350,000 in the United States and an incidence approaching 1/1000.<sup>1,2</sup> Although the etiology of MS is unknown, epidemiological studies have implicated infectious agents as probable initiating factors.<sup>3</sup> For instance, there is an increased risk of developing MS in patients who developed mumps, measles or Epstein-Barr (EBV) virus infections at an older age.<sup>4</sup> Interestingly, elevated EBV antibody titers occurred 15-20 years prior to MS onset in a 2006 study.<sup>5</sup> In addition, several viruses have been isolated from MS brains, such as measles, mumps, parainfluenza type I and human herpes virus simplex.<sup>6,7</sup> Furthermore, exacerbations of MS are frequently preceded by viral

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infections such as those caused by Rhinovirus.<sup>8</sup> Interestingly, interferon-beta (IFN- $\beta$ ) an antiviral agent, is currently used to treat relapsing/remitting MS.<sup>9</sup>

In animals, viruses are also known to cause demyelination: measles virus in rats; visna in sheep; herpes simplex in rabbits; JHM mouse hepatitis virus, Semliki Forest virus and Theiler's virus in mice.<sup>10</sup> Theiler's virus is a natural pathogen of mice that induces a disease that is similar both in disease course and pathology, to chronic progressive MS in humans.<sup>11</sup> Theiler's virus infection in mice represents not only an excellent model for the study of the pathogenesis of MS but also a model system for studying disease susceptibility factors, mechanisms of viral persistence within the CNS and mechanisms of virus-induced autoimmune disease.

## STRESS AND MULTIPLE SCLEROSIS (MS)

Psychological stress has been implicated in the onset and exacerbations of several autoimmune diseases including MS.<sup>12-14</sup> Anecdotal accounts suggest that significant stressful life events frequently trigger the development of MS symptoms. Psychological stress has been shown to precede both the onset and recurrence of MS symptoms in 70-80% of cases, using standardized assessment of life stressors measures.<sup>15</sup> More recently, stressful life events have been shown to predict the development of new lesions and relapses in MS.<sup>16,17</sup> A meta-analysis of 14 studies concerning stress and MS concluded that there was "a significantly increased risk of exacerbation associated with stressful life events."<sup>18</sup>

## THEILER'S VIRUS-INDUCED DEMYELINATION (TVID) AS A MODEL FOR MS

Theiler's murine encephalomyelitis virus is a member of the *Picornaviridae* in the cardiovirus genus. Theiler's virus causes an asymptomatic gastrointestinal infection and occasionally paralysis.<sup>19</sup> The persistent TO strains of Theiler's virus (BeAn, DA, WW, Yale) cause a primary demyelinating disease in susceptible strains of mice that is similar to MS.<sup>20</sup> Theiler's virus must establish a persistent infection in the CNS in order to cause demyelination.<sup>21</sup> TVID-resistant strains of mice are able to clear the infection effectively from the CNS. A number of studies have reported that viral persistence and demyelination in susceptible strains of mice are under multigenic control. Major histocompatibility complex (MHC) class I genes, the T cell receptor genes and a gene locus on chromosome 6 not linked to the T cell receptor locus have been implicated in susceptibility to demyelination.<sup>22,23</sup> Two additional loci, one close to *Ifn $\gamma$*  on chromosome 10 and one near *Mbp* on chromosome 18, have been associated with viral persistence in some strains of mice.<sup>24</sup> Immune recognition of Theiler's virus is clearly an important element in susceptibility to demyelination, as indicated by the genetic association with MHC class I and the T cell receptor, although other undefined factors are also involved.

## THE EFFECTS OF RESTRAINT STRESS ON THE NEUROPATHOGENESIS OF ACUTE THEILER'S VIRUS INFECTION

In order to investigate the psychological effects of stress on the neuropathogenesis of Theiler's virus, we have employed a restraint stress model originally described by Sheridan and colleagues.<sup>25</sup> Restraint stress is both a physiological and psychological stressor and involves placing mice in well-ventilated tubes overnight. Our studies systematically dissected the effects of restraint stress on the various components of the immune response to Theiler's virus. Moreover, we have used three strains of mice with varying degrees of susceptibility to Theiler's virus: 1) SJL mice - high susceptibility to TVID; 2) CBA mice - intermediate susceptibility to TVID; and, 3) C57Bl/6 mice - resistant to TVID.

Our first study, involved male CBA mice subjected to chronic stress that consisted of five nights (12h/night) of restraint stress per week for a total of four weeks. The experimental groups were: Infected/Restrained; Infected/Non-Restrained; Infected/Food and water deprived; Non-Infected/Restrained; Non-Infected/Non-Restrained and Non- Infected/Food and water deprived. The food and water deprived groups were deprived overnight to match the restrained groups and did not differ from the non-restrained groups and therefore were not included in subsequent studies. Stress had a profound effect on survival, 80% of the stressed-infected mice died during the first three weeks of infection. Restraint stress resulted in increases in: corticosterone levels, signs of sickness behavior, viral titers in the CNS, circulating neutrophils and adrenal hypertrophy, thymic atrophy, decreased numbers of circulating lymphocytes and decreased inflammatory cell infiltrates into the CNS.<sup>26,27,28</sup> Similar results were found in a subsequent study with male and female SJL mice. Chronic restraint stress administered in the first four weeks of Theiler's virus infection, decreased body weight, increased clinical signs of infection, and increased plasma corticosterone concentration during the acute viral infection. Although all the stressed mice developed significantly increased corticosterone levels, female SJL mice showed higher basal and stress-induced increases in corticosterone.<sup>29</sup>

Clearly stress has a profound impact on the neuropathogenesis of Theiler's virus infection. We proposed the following mechanisms for this phenomenon: stress activates the hypothalamic-pituitary-adrenal (HPA) axis resulting in adrenal hypertrophy and increased production of corticosterone. High levels of corticosterone, in turn, cause thymic atrophy and immunosuppression that reduces both the innate and adaptive immune response to Theiler's virus, thus reducing the effective clearance of virus from the CNS. Evidence for glucocorticoids being the main mediators of increased mortality in Theiler's virus-infected mice were obtained by the replication of the restraint findings by the addition of corticosterone in the drinking water of infected mice.<sup>30</sup>

## EFFECTS OF STRESS ON THE INNATE IMMUNE RESPONSE TO THEILER'S VIRUS

### Interferon and Natural Killer (NK) cells in Theiler's virus infection

The early host responses, interferon production and NK cell activation, are crucial for the effective clearance of Theiler's virus from the CNS. Failure to clear virus results in the establishment of persistent infection of the CNS and subsequent demyelination.<sup>31,32</sup> Type I interferons activate NK cells, upregulate MHC class I expression and induce the anti-viral state. Type I interferons are critical in the early clearance of Theiler's virus from the CNS as demonstrated by experimentation with IFN- $\alpha/\beta$  receptor knock-out mice which die within 10 days of infection with severe encephalomyelitis.<sup>33</sup>

Natural killer (NK) cells are activated early in viral infections and play an important role in infection with Theiler's virus. TVID-susceptible SJL mice have a 50% lower NK cell activity when compared to resistant C57BL/6 mice.<sup>34</sup> The low activity of NK cells in the SJL mice is due to a differentiation defect in the thymus that impairs the responsiveness of NK cells to stimulation by IFN- $\beta$ .<sup>35</sup> Resistant mice depleted of NK cells by monoclonal antibody to NK 1.1 or anti-asialo-GM1, and then infected with Theiler's virus developed severe signs of gray matter disease.<sup>34</sup> Thus, NK cells are critical in the early clearance of Theiler's virus from the CNS either directly via cytotoxic activity and/or antiviral effects of IFN- $\gamma$  or indirectly through IFN- $\gamma$  mediated activation of STAT1 and polarization of T cells towards a Th1 phenotype.

We examined the effects of stress on the NK cell response to Theiler's virus infection in male CBA mice using four experimental groups of mice: Infected/Restrained; Infected/Non-

Restrained; Infected/ Non-Infected/Restrained; Non-Infected/Non-Restrained. Restraint stress applied one day prior to infection with Theiler's virus resulted in 50% reduction in splenic NK cell activity 24 hours post infection.<sup>27</sup> Similar results have been obtained with TVID-resistant strains of mice C57Bl/6 and BALB/c.<sup>36</sup> Restraint stress did not alter the NK cell response in SJL/J mice infected with Theiler's virus because this strain has a deficiency in NK cell response.<sup>35</sup> However, restraint stress did impact the neuropathogenesis of Theiler's virus infection in SJL/J mice and therefore stress must mediate its immunosuppressive effects on the other components of the immune response. Stress-induced NK cell suppression may contribute to but is not sufficient to observe the stress induced exacerbation of acute and chronic Theiler's virus infection.

### **Stress reduces virus chemokine/cytokine expression**

CBA mice were subjected to the restraint stress protocol for seven nights, and infected after the first stress session. Then they were euthanized and RNA isolated from the brains and spleens. Ribonuclease protection analysis indicated that infection with Theiler's virus increased the following chemokine mRNA expression: lymphotactin (Ltn), interferon-induced protein (IP-10), macrophage inflammatory protein-1 (MIP-1), monocyte chemoattractant protein-1 (MCP-1) and TCA-3, in the spleen but not the brain at day two post infection (p.i.). Chemokine expression was increased first in the spleen which provides evidence that the immune response to Theiler's virus is initiated in the periphery. Ltn, normal T-cell expressed and secreted (RANTES) and IP-10 were elevated in both the spleen and the brain at day seven p.i. and were significantly decreased by stress in the brain. These chemokines are responsible for the recruitment of macrophages, NK cells, CD4+ and CD8+ T cells and thus may account for the diminished inflammatory cell infiltrate in the CNS of stressed mice and subsequently the reduced viral clearance and increased mortality in virus-infected stressed mice.<sup>26,28</sup>

The effects of stress on cytokine production in both the spleen and brain were measured by RPA in CBA mice following seven stress sessions. Theiler's virus infection elevated IFN- $\gamma$ , LT- $\beta$ , IL-12p40, IL-6, and IFN- $\beta$  in the brain at day two and seven p.i. Importantly, restraint attenuated the increases in IFN- $\gamma$ , LT- $\beta$ , IL-12p40, and IL-6, but elevated IFN- $\beta$ . The increased mRNA IFN- $\beta$  levels maybe as a result of the increased viral titers stimulating the production of this interferon.<sup>37</sup> In further experiments examining the effect of stress on cytokine expression, mice were subjected to the restraint stress paradigm and, at sacrifice, half the brain taken for measurement of virus titers and the other half for RPA analysis of cytokine mRNA levels. mRNA levels of IFN- $\gamma$ , LT- $\beta$ , and TNF- $\alpha$  negatively correlated with viral titers in the CNS such that mice with higher cytokine levels had lower virus levels. TNF- $\alpha$  protein levels, as measured by Western blots, gave similar results to the RPA data for this cytokine.<sup>37</sup> These cytokines have multiple immunomodulatory effects including anti-viral activity. Therefore, stress-induced decreased levels of LT- $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  may contribute to increased viral titers in the CNS.

### **Stress reduces the inflammatory cell infiltrate in the CNS**

CBA mice were restraint stressed and infected with Theiler's virus and their brains examined histologically at either day 7 and 24 p.i.<sup>26</sup> Restraint stress profoundly reduced the inflammatory cell infiltrate into the CNS at day seven p.i. with Theiler's virus.<sup>26,28</sup> In addition, stress reduced microglial activation. The reduced inflammation maybe a result of reduced chemokine expression in the brain, in particular, decreased Ltn, RANTES and IP-10 levels. In contrast, by day 24 p.i., the stressed mice had increased levels of inflammation in the CNS compared to the unstressed infected mice. This phenomenon maybe due to recovery of the immune system and increased activation due to the increased viral titers in the CNS.<sup>26</sup>

## RESTRAINT STRESS FACILITATES DISSEMINATION OF THEILER'S VIRUS DURING EARLY DISEASE

The restraint stress model was used to investigate the effect of stress on the systemic dissemination of Theiler's virus during the early stage of disease in CBA mice.<sup>38</sup> Restraint stress increased viral replication in the CNS and also systemic organs: the spleen, lymph nodes, thymus, lungs and heart. Restraint stress also resulted higher titers of virus in the heart which caused granular degeneration of the myocardium which was not evident in infected unstressed mice. These profound effects may explain the exacerbated clinical symptoms and higher mortality in stressed and infected animals. The fact that stress results in a higher level of viral replication, may allow for the increased likelihood of emergence of mutant viruses with altered tissue tropism. Stress may render a virus pathogenic for diverse organs and result in the development of novel diseases.

## RESTRAINT STRESS REDUCES ADAPTIVE IMMUNE RESPONSES TO THEILER'S VIRUS

### CD8+ and CD4+ T cells in Theiler's virus infection

In early infection with Theiler's virus, both CD8+ and CD4+ T cells have been shown to play an important role in viral clearance.<sup>39-42</sup> However, in later disease, these T cell subsets become pathogenic and mediate the demyelinating process.<sup>39,42-44</sup> In early disease, CD4+ T cells activate CD8+ T cells and also assist B cells in the production of antibodies, which are important mediators of Picornavirus clearance.<sup>39,45</sup> CD4+ T cells also produce IFN- $\gamma$ , a potent inhibitor of Theiler's virus infection *in vitro* and *in vivo*.<sup>46-48</sup> CD8+ T cells mediate viral clearance as demonstrated by *in vivo* depletion experiments and studies with gene knock-out mice.<sup>40,49-50</sup> CD8+ T cell-depleted mice fail to clear virus from the CNS and developed more severe demyelinating disease than the immunocompetent infected controls.<sup>40</sup> Similarly, demyelination developed in Theiler's virus-infected  $\beta_2$ -microglobulin knock-out mice (constructed on a TVID-resistant background). In addition, introduction of resistant H-2D<sup>b</sup> or H-2D<sup>d</sup> transgene into susceptible strains of mice render these animals resistant to TVID.<sup>51,52</sup> Taken together, these investigations clearly implicate CD8+ T cells in viral clearance and resistance to demyelination. The CTLs may function protectively, by recognizing viral determinants or by inhibiting delayed type hypersensitivity (DTH) responses.<sup>40</sup>

Our next series of experiments focused on the effects of stress on the T cell response to Theiler's virus in SJL mice, since the immunodominant Theiler's virus specific T cell epitopes have been identified in this strain of mice.<sup>53-56</sup> SJL mice were assigned to the experimental groups shown in Table 1 with the stressed mice being subjected to eight nights of restraint stress. At termination, splenic and CNS T cell responses to Theiler's virus were measured using an ELISPOT assay. Theiler's virus infection increased the number of IFN- $\gamma$  producing cells in the periphery in response to either CD8 epitope (FNFTAPFI corresponding to VP3<sub>159-166</sub>) or the CD4 T cell epitope (QEAFSHIRIPLPH corresponding to VP2<sub>74-86</sub>). Restraint stress significantly decreased both the splenic virus-induced CD4+ and CD8+ T cell response. Furthermore, restraint stress dramatically decreased (50%) the infection related increase in CD8+ T cell responses within the CNS.<sup>57</sup>

There are reports that stress inhibits Th1 responses and increases Th2 responses. Therefore, we examined the expression of both Th1 and Th2 cytokines in the serum of the experimental animals. Restraint significantly decreased both Type 1 (IL-12(p40), IL-12(p70), IFN- $\gamma$ ) and Type 2 (IL-4 and IL-5) serum protein concentrations as measured by Bioplex. The transcription factors T-bet and GATA-3 are the drivers of Th1 and Th2 polarization,

respectively.<sup>58,59</sup> Therefore, we also measured splenic mRNA expression levels of these factors in the experimental mice and found significant decreases in both when normalized to non-infected/non-restrained control mice.<sup>57</sup> Thus our findings do not support the hypothesis that stress selectively inhibits the Th1 response and promotes the Th2 response, rather that stress has a global immunosuppressive effect.

Restraint stress also decreased serum concentrations of RANTES and MCP-1, but increased IL-6, KC, and G-CSF protein concentrations. The chemokines RANTES and MCP-1 are involved in the chemoattraction of both memory T cells and monocytes to the site of infection to mediate early viral clearance from the CNS.<sup>57</sup> These findings confirm the previously reported RPA data from the spleen.<sup>28</sup> Interestingly, the chemokine KC (CXCL1) and growth factor G-CSF increased in the serum of stressed mice. KC plays a major role in the trafficking of neutrophils and the hematopoietic factor G-CSF is, in part, responsible for the maturation of neutrophils from the bone marrow. The stress-induced increases in KC and G-CSF may explain the increase in neutrophilia that we previously reported.<sup>26,27</sup>

## RESTRAINT STRESS DURING ACUTE INFECTION, EXACERBATES THE LATER DEMYELINATING DISEASE INDUCED BY THEILER'S VIRUS

Demyelination induced by Theiler's virus is partly mediated by viral lysis of oligodendrocytes<sup>60</sup>; the continued attempts of the immune system to clear virus which include bystander demyelination mediated by virus-specific DTH T cells<sup>61</sup>; and also cytotoxic T cell reactivity directed against virus-infected oligodendrocytes<sup>44</sup> autoimmunity<sup>39,42,62,63</sup> and epitope spreading.<sup>64</sup> The autoimmune reactivity seen in TVID may result from viral damage to oligodendrocytes, myelin uptake by macrophage/microglial cells and subsequent presentation to and activation of autoreactive T cells. These autoimmune T cells have been shown to be pathogenic and are able to demyelinate *in vitro*.<sup>65</sup>

Life stressors have been implicated in the onset of MS and we have shown that chronic stress during acute infection with Theiler's virus leads to decreased viral clearance from the CNS. Other studies have shown that increased viral load during acute disease leads to increased later demyelinating disease.<sup>40</sup> Thus, we speculated that stress applied during the acute viral infection would result in higher viral load in the CNS which would allow the establishment of persistent CNS infection and subsequently lead to increased demyelination. To test this hypothesis we subjected SJL/J mice to restraint stress for four weeks (eight hours per night for five nights per week) and then monitored the course of disease and assessed the spinal cord lesions 14 weeks post infection. During early infection, both male and female stressed mice displayed decreased body weights and locomotor activity, with increased behavioral signs of illness and plasma corticosterone levels. During the subsequent demyelinating phase of disease, previously stressed mice had greater behavioral signs of demyelination, worsened rotarod performance, and increased inflammatory demyelinating lesions of the spinal cord.<sup>29</sup>

Interestingly, correlational analysis of the dependent variables, revealed that plasma corticosterone levels during stress in the acute phase correlated with disease severity in the chronic disease and thus may be a good predictor of disease course in the chronic phase.<sup>29</sup> This study suggests that restraint stressed mice develop high levels of corticosterone which in turn induces immunosuppression and therefore higher viral titers and consequently more severe demyelinating disease.

## SUMMARY

Our research into the role of the restraint stress in Theiler's virus infection has clearly demonstrated the importance of the psychological status on the host's immune response to infection and how these interactions contribute to the development of autoimmune disease. To summarize our findings: restraint stress affects immune cell development by inducing high levels of corticosterone that reduce circulating lymphocyte numbers and cause thymic atrophy (Table 1). Stress increases KC and G-CSF that leads to increased neutrophils in the circulation. Stress affects the innate immune response to Theiler's virus by reducing NK cell activity and macrophage IL-12 production (Figure 1). In addition, stress reduces the expression of chemokines in the CNS: Ltn, RANTES and IP-10 that are responsible for the recruitment of CD4+, CD8+ T cells, macrophages and NK cells to the site of infection and this may explain the reduced inflammation observed during acute infection. Reductions in innate immunity are probably partially responsible for the observed stress-mediated reduction in the acquired immune response to Theiler's virus. Stress-induced decreases in the acquired immune response to Theiler's virus are evidenced by reduced virus-specific CD4+ and CD8+ T cell responses, reduced T-bet and GATA-3 mRNA levels and decreased virus-induced pro-inflammatory cytokines: TNF- $\alpha$ , IFN- $\beta$  and LT- $\beta$ . As a result of this global immunosuppression, the ability to clear virus from the CNS is diminished and stressed mice subsequently develop more severe demyelinating disease.

Extrapolating these findings to the development of autoimmune diseases in humans, stressful events that occur prior to or during infection, may result in immunosuppression and failure to eliminate the pathogen. Persistent infection then may lead to the development of autoimmune disease such as multiple sclerosis. Stress-induced immunosuppression may also facilitate the emergence of mutant pathogens with enhanced and/or altered pathogenicity giving rise to novel disease processes.

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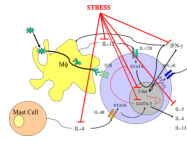
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**Figure 1. The effects of restraint stress on the immune response to Theiler's virus**

Restraint stress causes global immunosuppression of the response to Theiler's virus.

Theiler's virus is taken up by macrophages (mo) or dendritic cells and presented to T cells via their T cell receptor (TCR) in the context of MHC. Natural killer cell activity is reduced by stress and also the production of IFN- $\gamma$  which in turn reduces the activation of macrophages and T cells through STAT1 signaling. Th1 cells are driven by the transcription factor T-bet and activated by IL-12 through STAT4 signaling pathways. Th2 T cell responses are driven by GATA-3 and activation by IL-4 secretion and STAT6 signaling. Stress reduces the amount of IL-12 secreted by the antigen presenting cells then reduces the activation state of the Th1 cells. Stress also reduces mRNA expression of both T-bet and GATA-3 and thereby reduces both Th1 and Th2 responses.

**Table 1**

The effects of stress on the immune response to Theiler's virus

Response	Increases	Decreases
Hormones	Glucocorticoids	
Chemokines	IL-6, KC, and G-CSF	Ltn, normal T-cell expressed and secreted (RANTES) and IP-10
Cytokines	IFN- $\beta$	IFN- $\gamma$ , LT- $\beta$ , IL-12p40, IL-12(p40), IL-12(p70), IL-4, IL-5
Transcription factors		Tbet mRNA GATA-3 mRNA
Cellular response	Neutrophils in blood	Lymphocytes in blood NK cell activity
		Virus-specific CD4+ T cells
		Virus-specific CD8+ T cells
Virus	Titers in brain, heart, spleen, thymus, cervical and mesenteric lymph nodes	
CNS pathology	Inflammatory demyelinating disease in late disease	Microgliosis and inflammation in early disease