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Following Virus-Induced Damage

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14. ABSTRACT The objective of these studies is to examine the impact of erbB-mediated signaling on myelin destruction and repair in a novel model of central nervous system (CNS) demyelination. In these studies, we utilize a model of demyelination triggered by direct injection of Theiler's murine encephalomyelitis virus (TMEV) into the spinal cord. This method of lesioning permits us to precisely identify the age and site of the lesion. We hypothesize that increased erbB-mediated signaling will protect animals from disease. Conversely, reduced signaling will worsen demyelination. Our studies have shown that animals that have been exogenously treated with glial growth factor (GGF; a member of the neuregulin family that binds to erbB receptors) have improved clinical function compared to animals receiving glial growth factor 2 or sensory motor derived factor (other members of the neuregulin family). Data to date suggest that the affect of GGF is likely indirect and does not directly affect myelination. Results to date support a role for microRNAs as mediators of this observation. In addition, EGFR levels are low and do not appear to modulate over the course of infection. Myelinating cells do not appear to undergo proliferation, but rather appear to migrate to the site of damage in mice with conditional erbB2 deletions. It is likely that these cells are Schwann cells.					
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Introduction

The immediate objective of the studies funded by this grant is to define the neuregulin-mediated interactions that enhance myelin preservation/repair in the spinal cords of mice following Theiler's murine encephalomyelitis virus (TMEV) injection by testing the hypothesis that neuregulins invoke erbB signaling and protects the central nervous system (CNS) by limiting Theiler's virus-induced pathology and triggering myelin repair processes, either directly or indirectly. These studies utilize two animal models. In the first model, we are directly injecting TMEV into the spinal cords of mice and characterizing the early events after infection. In these animals, there is rapid infiltration of the lesion with T cells, B cells, and macrophages. The area of virus localization coincides with demyelination, indicating a direct role for virus in this process. In the second model, we are utilizing animals that have genetic deletions of EGFR and/or erbB2 to determine the direct impact of these genes on myelination of the CNS. We have also performed some studies on the traditional intracerebral model of TMEV infection. These studies are the first to examine the role of neuregulin-mediated signaling processes on CNS damage and repair in a model of multiple sclerosis.

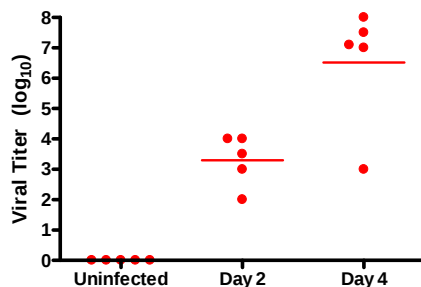
Body

Specific Aim 1. Identify the cellular mechanism(s) used for myelin repair/preservation following Theiler's virus-mediated damage to the spinal cord white matter.

Task 1: Directly inject spinal cords of FVB/nJ mice with Theiler's murine encephalomyelitis virus or virus diluent and collect spinal cord tissues at various time points post-infection (6, 12, 24, 48 and 72 hours post-infection). These tissues will be used in Tasks 1-6, 11, 12.

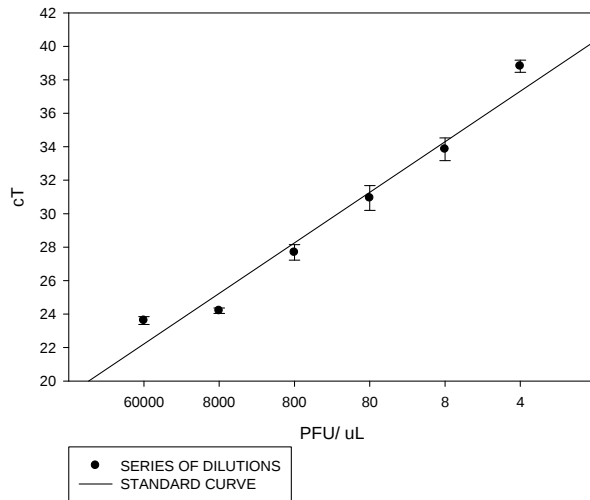
We have injected TMEV into the spinal cords of FVB/nJ mice. These tissues have been used primarily in Western Blotting experiments ([Task 4](#)), immunostaining, and real-time PCR experiments ([Task 5](#)). In some studies, we have also used intracerebrally injected FVB/nJ female mice to examine the impact of the most common experimental route of TMEV infection on erbB2 and EGFR expression.

TMEV replicates in the spinal cord after intraspinal cord injection. To test whether TMEV replicated at the lesion site, we injected FVB/nJ mice into the spinal cord, and sacrificed the animals at various timepoints post-injection to measure viral load by plaque assay. As shown in the figure below, TMEV replicates within the lesion site over several days, indicating that a production infection occurs.

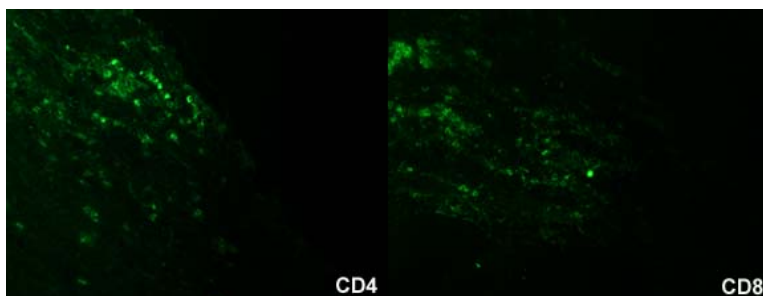


Real time PCR for TMEV has been optimized and can detect 4 to 8 plaque forming units (PFUs) of TMEV in a sample. Because we are using very small pieces of tissue in our assays, it became necessary to determine what the sensitivity of our assay was despite the fact that we are using relative quantification as our output. To test the sensitivity of our assay, we spiked spinal cord samples with a known numbers of PFUs of TMEV. Our dilution series ranged from 0.5 to 60,000 PFUs. Using this

assay, we can routinely detect between 4 and 8 PFUs of TMEV, and feel very comfortable stating that the limit of detection of this assay is <8 PFUs per sample. An example of the data obtained from these optimization experiments is shown below. This assay allows us to detect even very small amounts of virus in our samples, which we what we observe in some of the samples collected from areas distal from the spinal cord injection site. This assay is being used to substitute for our viral plaque assay that has a detection limit of 200 PFUs per mg tissue (~50X more sensitive).

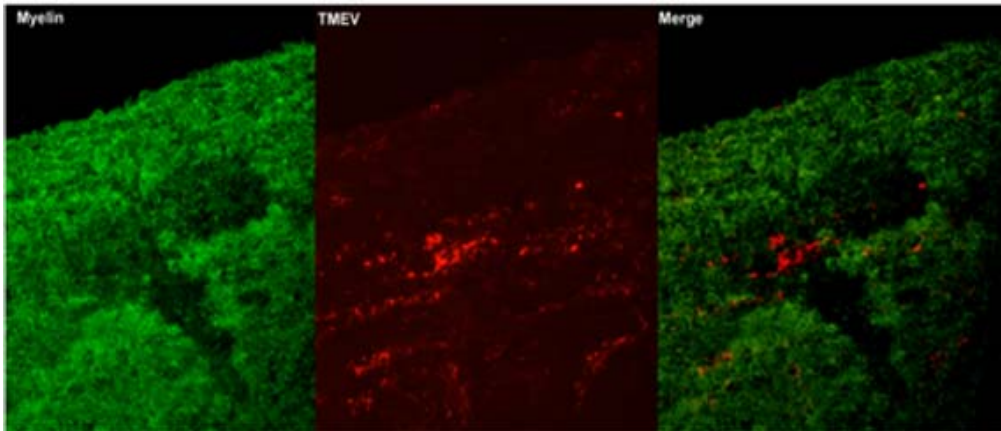


T cells infiltrate the spinal cords of mice that have been directly injected with TMEV. Immunostaining was used to determine whether cells of the adaptive immune system are attracted to the site of damage following direct injection of TMEV into the spinal cord. This is important, as one of the key characteristics of human multiple sclerosis is that T cells infiltrate the site of damage. In this experiment, we examined tissues for the presence of either CD4+ (helper) or CD8+ (cytolytic) T cells. As demonstrated below, within 5 days of injection, both CD4+ T cells and CD8+ T cells have infiltrated the lesion site.

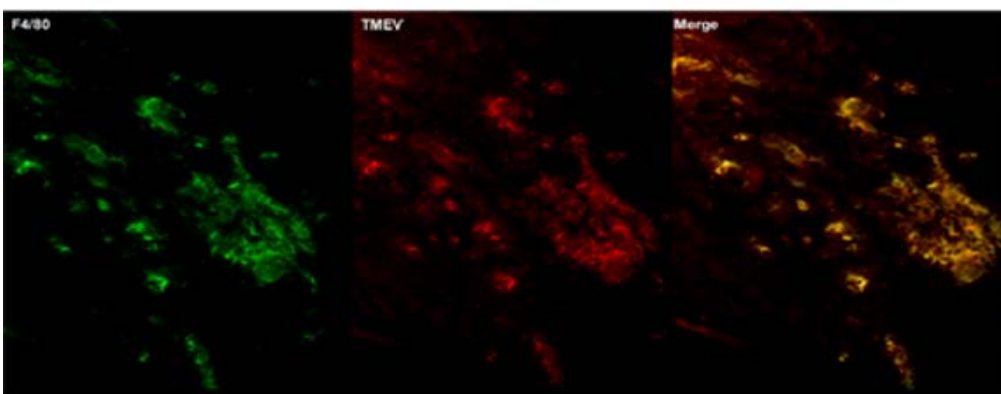


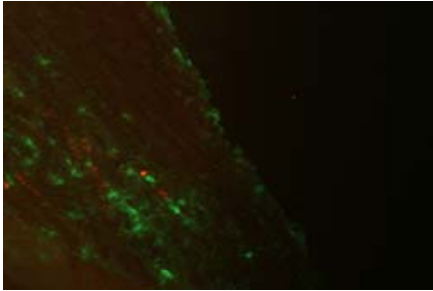
The primary virus burden is localized to demyelinated areas of the spinal cord. We examined the distribution of the demyelinating lesions relative to the location of virus early in infection (day 4 after injection of virus into the spinal cord). If virus localized to the areas of the spinal cord lacking in myelin, then it would be appropriate to postulate that early phases of demyelination could be due to direct damage of the myelin by the virus. If there was demyelination observed, but virus could not be localized to these areas, then we would postulate that the damage to the myelin was either the result of a) injection trauma or b) indirect. Double immunohistochemical staining was performed using a polyclonal antisera to TMEV and an antibody reactive to mouse myelin. Differentially-labeled detecting antibodies permitted us to stain sections simultaneously with both antibodies. The

secondary antibody used for the myelin antibody was Alexa-Fluor 568 labeled (green; far left panel, below) and the secondary antibody used for the virus was Alexa-Fluor 488 labeled (red, middle panel, below). As shown in the merge image (green and red, far right panel, below) TMEV is found in the spinal cord in an area devoid of myelin. Virus is localized to areas without intact myelin, supporting the hypothesis that virus infection, not trauma or an indirect effect, leads to demyelination in this model. Further supporting this conclusion is the finding that mice injected with UV-inactivated virus do not experience a loss of myelin as determined by immunohistochemistry.



Macrophages/microglia are the cells harboring the primary viral burden in the spinal cords of TMEV injected mice. The lesion sites were examined to determine what cell type harbored the majority of virus in the newly formed lesions in the spinal cords of mice injected with TMEV. As the primary reservoir of virus in the model of demyelination that develops following intracerebral infection are phagocytic cells (1), we performed double immunostaining using antibodies to TMEV and F4/80, a marker of macrophages and microglia, the primary phagocytic cells in the CNS. In these experiments, virus fluoresces red (Alexa-Fluor 488 labeled secondary antibody) while cells positive for F4/80 appear green (Alexa-Fluor 568 labeled secondary antibody). The far right panel shows the merged images of the green F4/80 staining and red TMEV staining. The yellow staining is indicative of cells positive for both virus and F4/80.





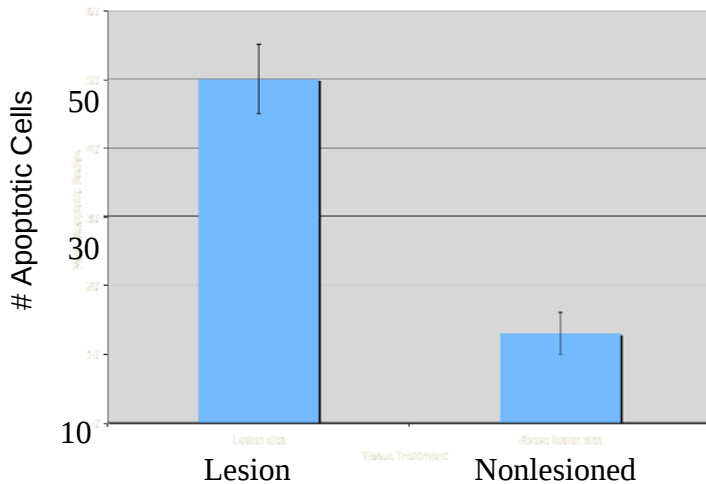
On the left, we utilized immunostaining for virus (red) and a marker of neuronal cells to assess whether neurons could be a reservoir of TMEV. Previous work has shown that in the early encephalitic stages of the disease, neurons are positive for virus. In our studies no overlap was observed between virus and neurons.

- Task 2: Determine if an increase in oligodendrocyte/Schwann cell proliferation occurs following injection of Theiler's murine encephalomyelitis virus into the spinal cord of FVB/nJ mice.
- Perform dual immunohistochemical staining on slides using antibodies for proliferating cells (Ki67 and BrDu) and a marker of myelinating cells (PLP). Quantitate the number of proliferating myelin-producing cells.

Myelinating cells in the spinal cords of female FVB/nJ mice do not undergo proliferation following TMEV injection of the spinal cord. To determine whether myelinating cells proliferated at the site of TMEV infection, we performed intraspinal cord inoculation of TMEV into the FVB/nJ mice and stained the spinal cords for Ki-67, a marker of actively proliferating cells at various time-points post injection. Using dual immunohistochemical staining techniques, the majority of cells undergoing proliferation in the spinal cords of mice within the first 72 hours following TMEV infection are not CNS resident cells, but rather infiltrating immune cells (T cells predominantly). These data indicate that myelinating cells are not increasing in number within the first 72 hours after infection. This is consistent with current viewpoints on mature oligodendrocyte stability.

- Task 3: Determine if oligodendrocyte/Schwann cell apoptosis occurs following injection of Theiler's murine encephalomyelitis virus into the spinal cord of FVB/nJ mice.
- Perform terminal deoxynucleotidyl transferase-mediated UTP nick end-labeling (TUNEL) on spinal cord sections. Slides will also be immunostained using an antibody to PLP to quantitate the number of apoptotic myelinating cells.

Apoptosis is increased in the lesion site of injection as compared to regions immediately adjacent to the lesion site. To assess the impact of intraspinal cord injection of TMEV on apoptosis in the spinal cord, we performed terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) assays on the tissue. Both the lesion area and the area immediately above the lesion were examined. The area of the spinal cord immediately above the lesion was used for comparison. As shown below, there is a high level of apoptotic cells within the lesion site (data are expressed as the mean number of apoptotic cells $\pm$ SEM). While the level of apoptosis in areas adjacent to the lesion are greatly reduced (by approximately 23%), the observation that there is significant apoptosis in these uninfected areas of the spinal cord suggests that damage is occurring prior to the development of demyelination. One possible mechanism of this damage is soluble mediator production. Other potential mechanisms of damage could be the result of axonal damage and/or influx of inflammatory cells. Rarely are apoptotic cells observed in the spinal cords of uninjected animals.

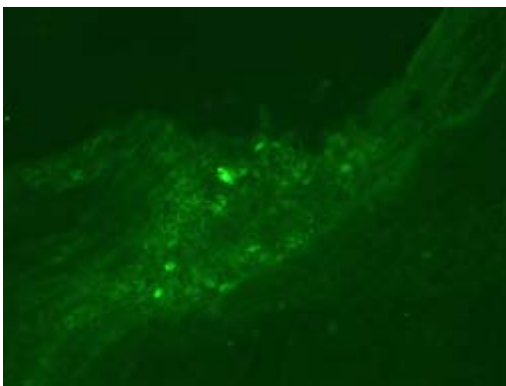


Myelinating cells at the lesion site of FVB/nJ mice undergo apoptosis following TMEV infections.

Using a combination of immunostaining and TUNEL assays, we have determined that the majority of cells undergoing apoptosis within the spinal cord white matter (>50%) are oligodendrocytes, indicating that the host is susceptible to a high degree myelin loss within the spinal cord. It is not surprising that the majority of cells experiencing apoptosis are oligodendrocytes, as this population comprises the majority of cell types in the spinal cord white matter.

IL-11 is localized into the lesion area of TMEV-infected FVB/nJ mice. Because we observed apoptosis of oligodendrocytes in the lesion and minimal proliferation, we were curious to know what other mechanisms could be invoked that would provide some degree of protection to the spinal cords of TMEV infected mice. One mechanism by which protection could be afforded is via the production of IL-11, a mediator known to be involved in oligodendrocyte survival and maturation (8).

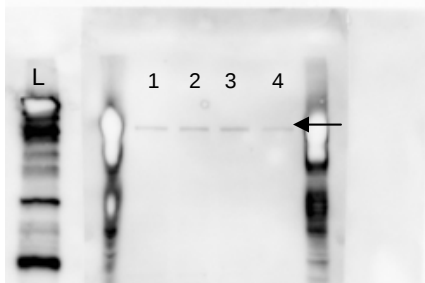
Immunohistochemical staining (below) revealed immunoreactivity (bright green) to IL-11 within the lesion, suggesting that one mechanism that could be involved in functional preservation (in the absence of oligodendrocyte proliferation) is via the maturation of oligodendrocyte precursor cells. We have performed staining for oligodendrocyte precursor cells and found no increase in oligodendrocyte precursor cells, supporting the hypothesis that CNS protection is due to enhanced maturation of myelinating cells, versus *de novo* generation of myelinating cells.



Task 4: Determine if an increase in phosphorylation of EGFR, erb-B2, or erb-B4 occurs in spinal cord of TMEV-infected mice.

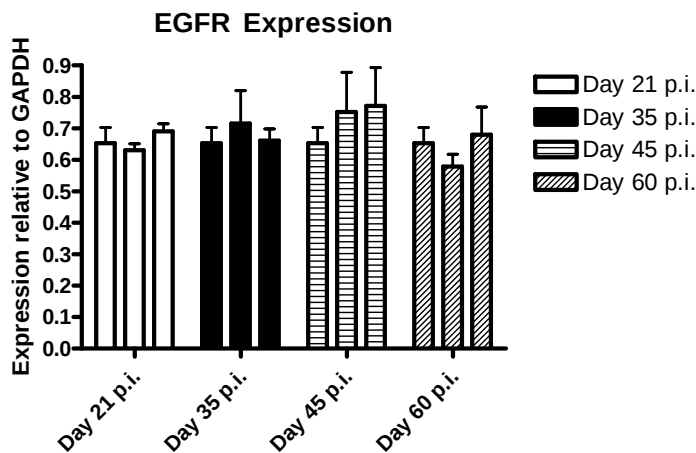
- Compare the level of EGFR, erb-B2, and erb-B4 in the spinal cords of virus- and HBSS-injected mice by Western blot.
- Compare the level of phosphorylation of EGFR, erb-B2, and erb-B4 in the spinal cords of virus- and HBSS-injected mice by Western blot.

For a period of time (~4 months) we were unable to obtain sufficient sensitivity in our assays to permit detection of these receptors. We have optimized this assay (using a different source of antibodies) and are able to detect these receptors. An example of these blots is shown below.

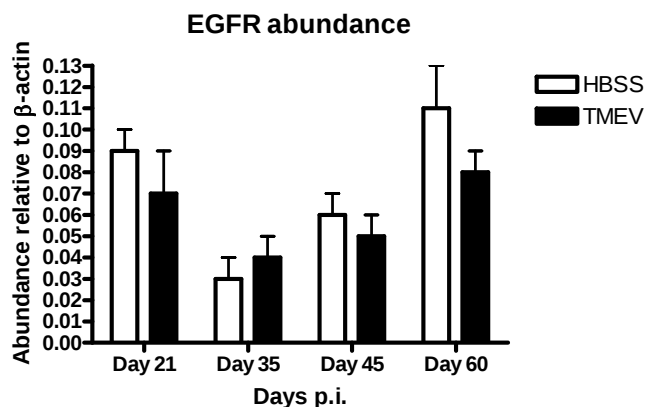


EGFR Western Blot. Lanes 1-4 represent varying amounts of protein (500, 400, 300, 100 ng/we11, respectively). Lanes on either side are the positive control sample. L = MW ladder. EGFR is indicated by the arrowhead.

ErbB2, but not EGFR, is expressed at high levels in the spinal cords of female TMEV-infected FVB/nJ mice. We assessed differences in expression of these mediators in uninfected and infected murine spinal cords (FVB/nJ mice). β -actin was used as a housekeeping gene to normalize samples for comparison. Based on real-time RT-PCR (top panel, below) and Western Blotting (bottom panel, below), we have noted no significant changes in expression of EGFR over the disease course. In addition, protein expression levels of EGFR were extremely low, even late into the disease course. Immunostaining studies detected minimal expression of EGFR in the spinal cords of TMEV-infected FVB/nJ mice.

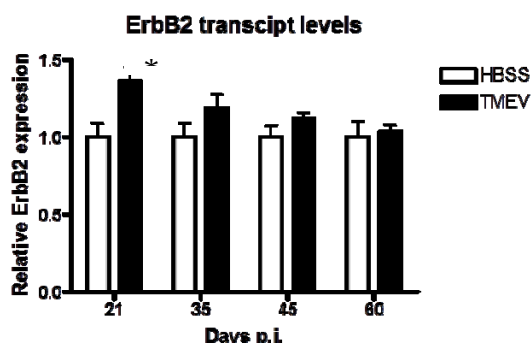


EGFR expression is stable in the spinal cords of female FVB/nJ TMEV infected mice over the course of infection. Each bar represents one animal assayed in triplicate. Transcript levels were analyzed by qRT-PCR and expressed as the mean fold change $\pm$ SEM relative to GAPDH levels. Statistical analysis was performed using ANOVA with HBSS treated animals as the control.



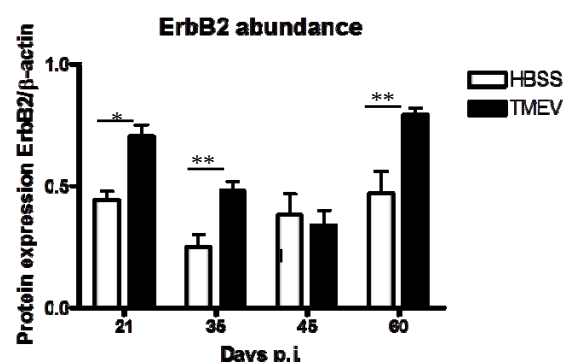
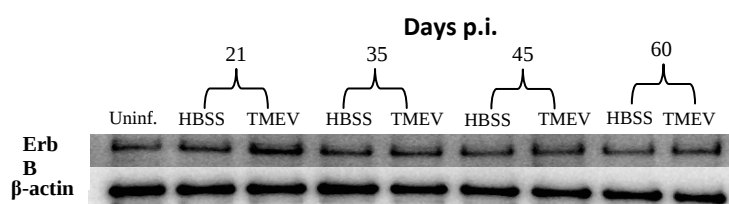
EGFR protein levels are low in the spinal cords of female FVB/nJ TMEV-injected mice. Protein levels were assessed by Western Blot using an antibody to EGFR. Samples were normalized to β -actin to ensure equal loading in each well. Each bar represents the mean $\pm$ SEM of 3 animals assayed in triplicate.

We then assessed the levels of ErbB2 (both transcript levels and protein levels) in the spinal cords of TMEV injected mice over the course of infection. As shown below, mRNA levels (as determined by qRT-PCR) were significantly higher in the spinal cords of the virus-injected animals as compared to the HBSS control injected animals. Protein levels were also altered as determined by Western Blot.



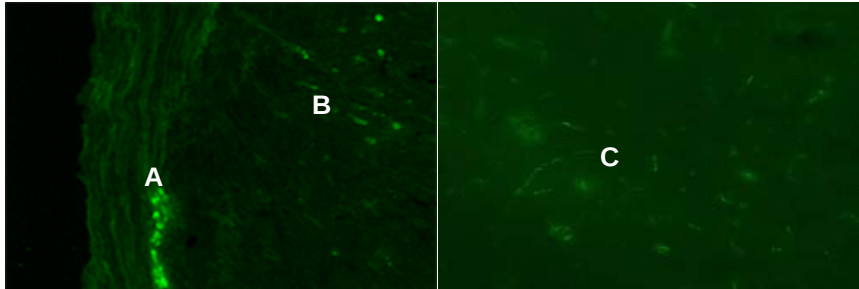
ErbB2 transcript levels are stable in the spinal cords of TMEV injected female FVB/nJ mice over the course of infection. Each bar represents three animals assayed in triplicate. Transcript levels were analyzed by qRT-PCR and expressed as the mean fold change $\pm$ SEM relative to GAPDH levels. Statistical analysis was performed using Student's t test with HBSS treated animals as the control. * $p \leq 0.05$

ErbB2 Levels in Spinal Cords of TMEV-Infected Mice



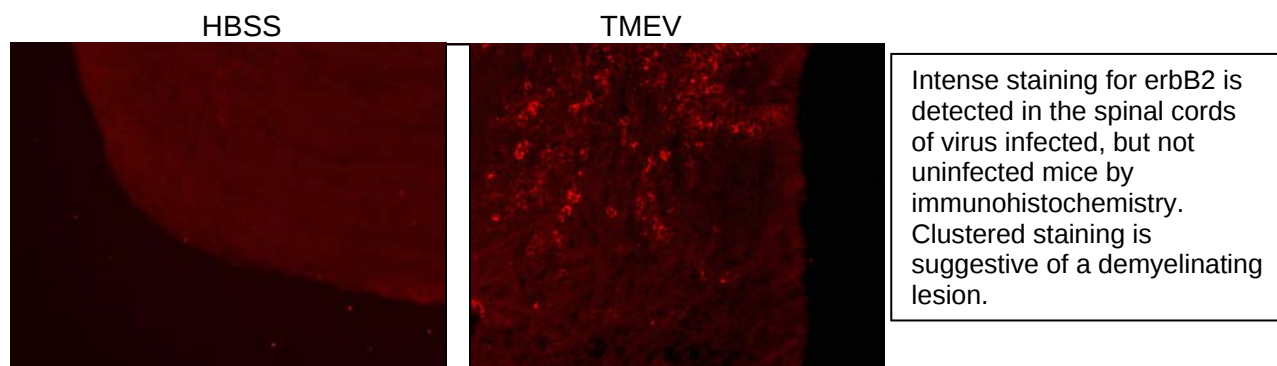
ErbB2 abundance in spinal cords isolated from TMEV-infected female FVB/nJ mice. 25 μ g of spinal cords lysate was loaded per well. Values represent the mean abundance level $\pm$ SEM, relative to β -actin abundance levels. Statistical analysis was performed using a two-way ANOVA, with HBSS-injected female FVB/nJ mice as a control, and $n = 3$. The blots shown on the left are representative of three experiments. * $p < 0.05$; ** $p < 0.01$.

ErbB3 expressed constitutively at low levels in the spinal cords of control mice, but highly expressed on infiltrating cells in TMEV infected mice. We examined whether erbB3, a receptor for neuregulin, was expressed in the spinal cords of control (uninjected; right panel) and TMEV-injected mice (left panel below). TMEV infected mice express erbB3 at high levels on cells infiltrating the CNS (A). Based on our knowledge of the model, these cells are likely to be T cells. In addition, there are immunoreactive cells in the gray matter of the spinal cord (B) that are likely resident cells (neurons). These immunopositive cells are also observed in uninjected spinal cords (C).



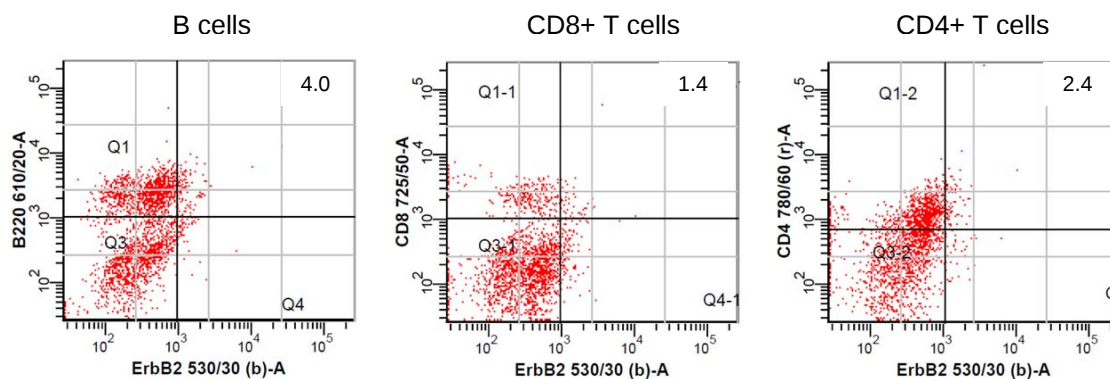
ErbB2 is predominantly expressed by immune cells infiltrating the demyelinating lesions. We performed immunostaining to determine the localization of ErbB2 positive cells in the spinal cords of TMEV infected mice. While we hypothesized that the main source of ErbB2 positive cells would be CNS resident cells, we found that this was not the case, and that immune cells were the primary cell types expressing ErbB2 protein.

An initial immunostaining experiment was performed using an antibody to ErbB2. As shown on the left, minimal immunoreactivity to the ErbB2 antibody is detected (positive staining is indicated by bright red) at day 21 post-infection in animals that have been sham infected (HBSS injected; left image). Following virus infection, large numbers of ErbB2 positive cells were localized in the spinal cord (right image). The location of these cells suggests is suggestive that they are within a lesion.

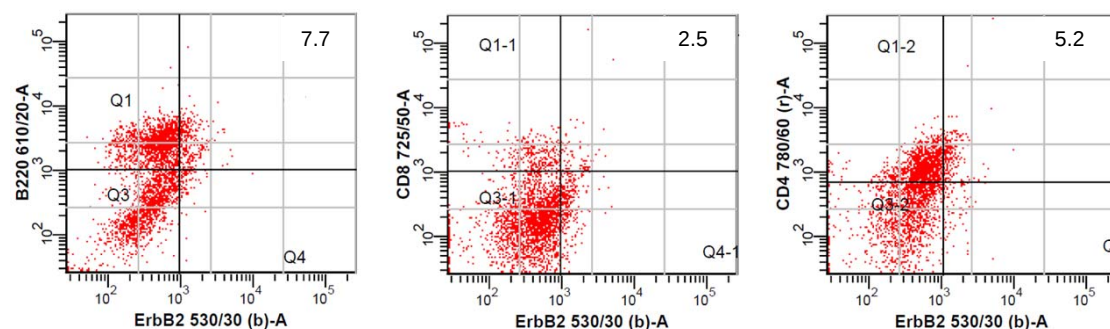


To further delineate the phenotype of the cells staining positively for erbB2, we then isolated splenocytes from mice and incubated the splenocytes with Concanavalin A, a known non-specific activator of T cells. We then performed flow cytometry to determine if 1) B cells (using B220 as a marker), CD4+ T cells or CD8+ T cells expressed erbB2; and 2) whether activating the cells increased erbB2 expression on the cells. As shown on the next page, activated T cells and B cells express higher levels of erbB2 than unstimulated cells. This is significant, as cells in the lesions represent T and B cells that have been activated. The number in the upper right hand quadrant indicates the percentage of cells that are positive for the particular cell-type specific marker and the erbB2 protein. Each graph represents one animal and is reflective of the data obtained in all animals assayed (n=5).

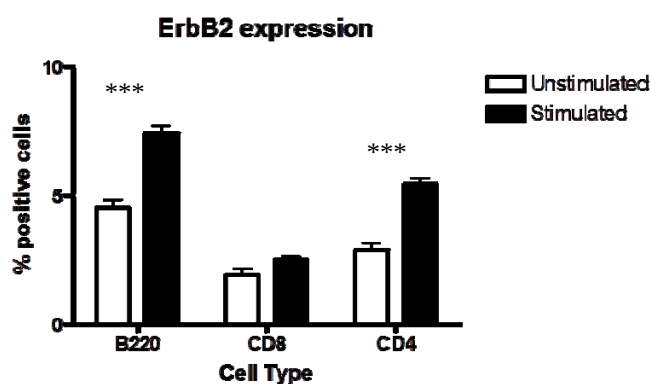
Unstimulated Splenocytes



Con A Stimulated Splenocytes

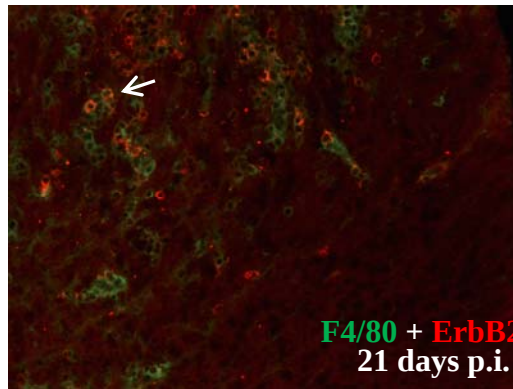
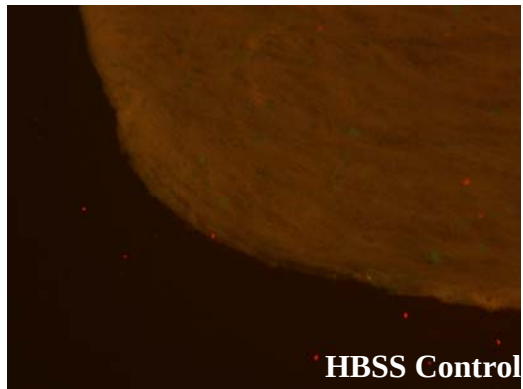


Summary data of the flow cytometry is shown below:

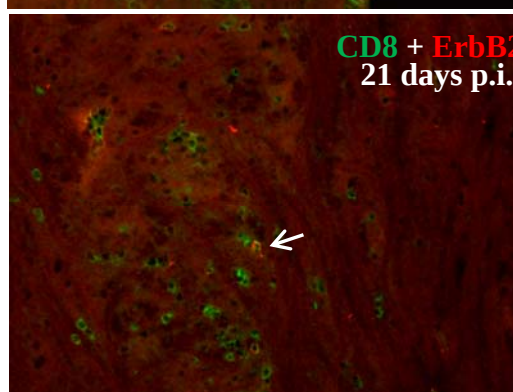
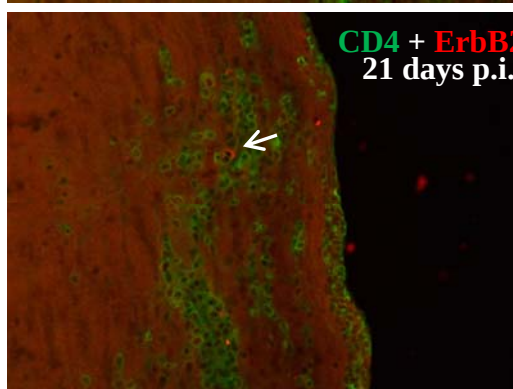
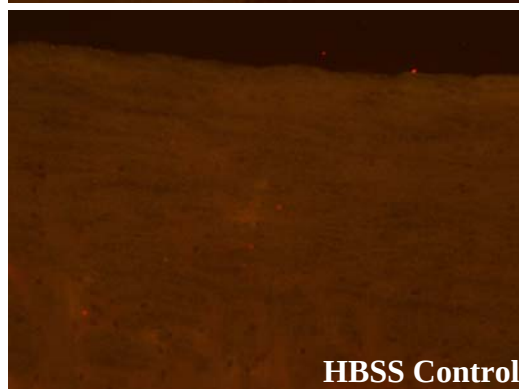
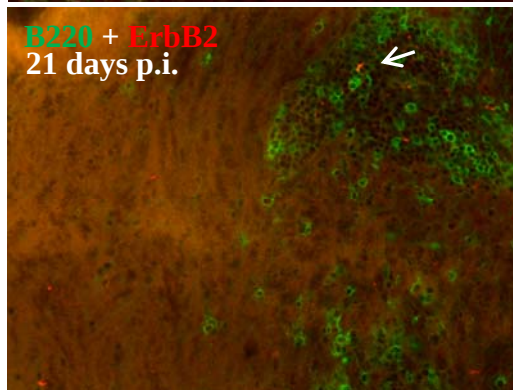


Splenocytes were treated with 2.5µg of Concanavalin A in RPMI media for 48 hours. Activated splenocytes were analyzed by flow cytometry (FacsAria). Values represent the mean % of positively stained cells $\pm$ SEM. Statistical analysis was performed using a two-tailed T-test, with unstimulated splenocytes as a control, and $n = 5$. *** < 0.001.

We performed double immunohistochemical staining using Alexa-Fluor labeled antibodies to identify cells that were positive for both erbB2 and a cell-type specific marker. Surface markers used in these studies were F4/80, a marker of macrophages; B220, a marker of B cells; CD4, a marker of T helper cells; and CD8, a marker of cytotoxic T cells. The results are shown on the following page.



ErbB2 staining (red) is seen on macrophages (F4/80), B cells B220), CD4⁺ T cells, and CD8⁺ T cells (red) within demyelinating lesions in spinal cords isolated from TMEV-infected female FVB/nJ mice.



- Task 5: Determine if there is increased kinase activation following erb-B receptor phosphorylation.
- Perform real-time PCR to determine the signaling pathways affected by virus insult.
 - Perform immunoprecipitation experiments on tissue lysates to identify phosphorylated kinases.

All tissues have been collected for this task. While this task is ongoing, there are issues with being able to get sufficient positive signal. This is due to the limited expression of ErbB2 relative to the regions where no ErbB2 is expressed.

- Task 6: Utilize pharmacologic agents to the kinases identified in (c) to determine if there are alterations in pathology/repair of the central nervous system following virus infection.
- Determine the impact of inhibition of the signaling pathways on proliferation of Schwann cells/oligodendrocytes (as in Tasks 2, 3).
 - Determine the impact of inhibition of signaling pathways on pathology.

There is minimal oligodendrocyte/Schwann cell proliferation, thus this task has not been focused on.

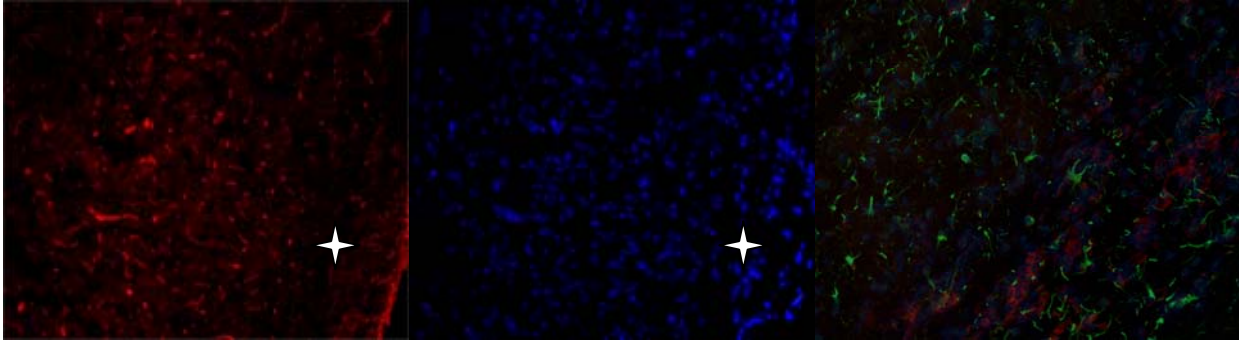
Specific Aim 2. Identify the contribution of erb-B family members to myelin repair following injection of Theiler's virus into the spinal cord.

- Task 7: Directly inject spinal cords of conditional knockout mice with Theiler's murine encephalomyelitis virus or virus diluent and collect spinal cord tissues at various time points post-infection (6, 12, 24, 48 and 72 hours post-infection). These mice will be used in Tasks 8-10. These Tasks will be discussed together as they vary only in the strain of mice used.

Injections have been performed and a large amount of the tissue collected for use in the following tasks.

- Task 8: Examine the effects of a conditional deletion of erb-B2 on myelination following virus insult.

ErbB2 is expressed throughout the spinal cord of infected mice. We performed immunohistochemical staining on the spinal cords of FVB/nJ mice that have had TMEV injected into their spinal cords. At 3 days post-injection with TMEV, we utilized an antibody to erbB2 to examine the distribution of this protein in the spinal cord. As shown on the next page (left), positive signal is found in the area of the spinal cord adjacent to the initial lesion site (star indicates lesion site). Minimal staining is observed in the lesion site, suggesting that one cell type expressing erbB2 is the myelin-producing oligodendrocyte. The DAPI-stained image is shown in the middle. Note the altered cellularity in the DAPI-stained image. The image on the far right shows a lesion from an erbB2^{-/-}EGFR^{-/-} mouse that has been stained for neuregulin (green), macrophages (red) and counterstained with DAPI (blue). Like the erbB2 staining of FVB/nJ mice, this image demonstrates that neuregulins are widely expressed in spinal cord tissue of mice following injection with TMEV.



Tamoxifen-treatment of *erbB2*^{-/-}*-EGFR*^{-/-} mice under the control of the PLP promoter experience alterations in their immune cell subsets compared to untreated mice. To determine whether tamoxifen treatment altered immune cell subset distribution, we performed flow cytometry on splenocytes isolated from tamoxifen or control treated PLP-*erbB2*^{-/-}*-EGFR*^{-/-} mice. We examined cells for the expression of CD4, CD8 and B220. As shown below, mice that were tamoxifen treated for 8 days (1 mg/day) had reduced levels of CD4⁺ T cells in the periphery 1 month after treatment, indicating that tamoxifen treatment, indicating a long-term effect on the immune system due to tamoxifen treatment.

	CD4 ⁺	CD8 ⁺	B220
Treated	11.33±3.636	8.133±2.111	35.13±3.818
Untreated	32.30±1.457	8.200±0.776	35.87±2.949
*Statistically Significant; p=0.0029			

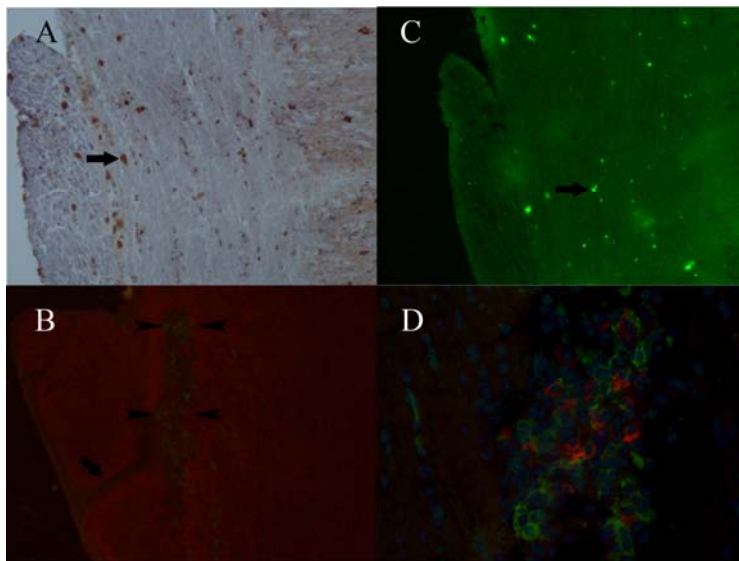
Additional data regarding myelin staining is included under Task 10.

Task 9: Examine the effects of a conditional deletion of EGFR on myelination following virus insult.

EGFR loss does not appear to impact myelination. EGFR is expressed at extremely low levels by both CNS resident cells as well as infiltrating cells. These studies were performed using immunohistochemical staining, Western Blotting, and qRT-PCR. Animals with a conditional knockout of EGFR do not experience worse pathology than mice that do not have EGFR knocked out in their oligodendrocytes.

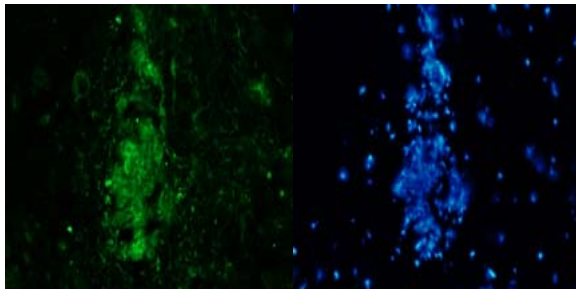
Task 10: Examine the effects of a conditional deletion of EGFR and *erbB2* on myelination following virus insult.

Lesion characterization of tamoxifen-induced PLP-*erbB2*^{-/-}*-EGFR*^{-/-} mice demonstrate that immune cells infiltrate the lesions. Immunohistochemical staining was performed to characterize the lesions that form in the tamoxifen-inducible knock out mice. Antibodies utilized are indicated in the legend. All staining was performed at 7 days post injection with TMEV.



Characterization of a lesion site in a tamoxifen-induced PLP-erbB2-/-EGFR-/- mouse at 7 days post-injection with TMEV. Despite intra-spinal cord injection, the animal had a normal gait at sacrifice. **A:** CD8+ cells (arrow) are present and concentrated in the lesion. 20x magnification. **B:** Foci of CD4+ cells are associated with demyelination in the lesion (arrowheads). The site of needle insertion is indicated by the arrow. **C:** Scattered apoptotic cells in the lesion and in tissue surrounding the lesion (arrow). **D:** F4/80+ phagocytic cells (green) are concentrated in an area of myelin producing cells (red). Nuclei are stained with DAPI (blue).

Mice that have a conditional deletion of erbB2 appear to have altered migration of myelinating cells to lesion site. We performed immunohistochemical staining using an antibody to myelin on tamoxifen-treated TMEV-injected erbB2-/-EGFR-/- or erbB2-/- mice to examine the lesion site. As shown below, mice appeared to have 'clusters' of myelin-positive sites in the areas around the lesion. Because we did not detect increased levels of proliferation in the spinal cords of mice injected with TMEV, we postulate that these clusters of myelin-positive cells are the result of increased migration of myelin-producing cells to the area of the lesion. We postulate that these cells are Schwann cells, not oligodendrocytes, as Schwann cells have migratory capacities and produce myelin.



Immunohistochemical staining of a spinal cord from a mouse after an intraspinal cord injection of TMEV. The mouse was treated with tamoxifen to induce the excision of EGFR and erbB2 in PLP expressing cells. The time-point is 5 days post-injection. On the far left, green indicates cells that are positive for myelin. Nuclei are stained with DAPI (blue). Note the hypercellularity of myelin positive cells. It is likely that these cells are Schwann cells that have migrated to the trauma site.

Task 11: Examine the effects of pharmacological blocking of erbB signaling on spinal cord repair/myelin preservation.

The following subtasks will be performed on Tasks 8-11.

- Quantitate the level of demyelination in spinal cords of mice.
- Localize the distribution of TMEV antigens by immunostaining
- Localize and identify the inflammatory cells infiltrating the spinal cord
- Perform real-time PCR to determine mRNA levels of transcripts involved in myelination
- Perform Western blotting to determine protein levels of MBP, PLP, and MAG.
- Determine the level of proliferation and apoptosis of myelinating cells.

Optimization of real-time RT-PCR to detect alterations in myelin gene transcripts.

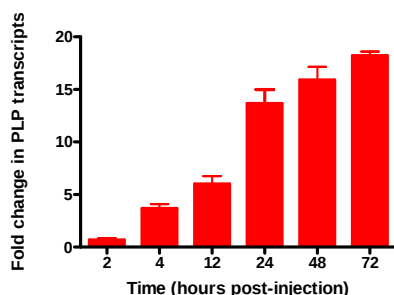
We designed a battery of real-time PCR primers specific for the transcripts of various myelin genes. The genes of interest are: peripheral myelin protein-22 (PMP), myelin protein zero (MPZ), proteolipid

protein (PLP), myelin associated glycoprotein (MAG), Krox-20 (KROX) and myelin basic protein (MBP). Using spinal cord and brain from mice that were infected with TMEV (intracerebral infection, not localized spinal cord injection), we isolated RNA and examined the difference in the level of mRNA transcripts in each of the above-described genes, using GAPDH as a housekeeping gene. Because we used the entire spinal cord (not just the lesioned area) the results are diluted relative to what is occurring at the lesion site. Transcript levels in infected control mice were reduced relative to the uninfected control samples. All data are expressed as the fold-change over a calibrator sample $\pm$ the standard error of the mean. In addition, transcript levels of MAG were significantly reduced in the infected spinal cord compared to the control (t-test; $*p \leq 0.05$).

Transcript	Uninfected Brain	Infected Brain	Uninfected Spinal Cord	Infected Spinal Cord
PMP	0.99 ± 0.17	0.76 ± 0.80	1.78 ± 0.46	1.05 ± 0.06
MPZ	0.50 ± 0.26	0.16 ± 0.05	2.01 ± 0.93	1.36 ± 0.15
PLP	1.03 ± 0.15	0.62 ± 0.06	1.28 ± 0.38	0.47 ± 0.03
MAG	0.99 ± 0.26	0.38 ± 0.06	1.13 ± 0.24	$0.26 \pm 0.17^*$
KROX	0.82 ± 0.14	1.28 ± 0.38	2.39 ± 1.05	2.00 ± 0.15
MBP	0.84 ± 0.12	0.87 ± 0.10	1.38 ± 0.35	0.90 ± 0.08

3 samples/group

TMEV was injected directly into the spinal cord of FVB/nJ mice and animals were sacrificed at various timepoints post-injection. The time-frame examined was from 2-72 hours after injection. We dissected out the injection site, isolated RNA and performed real-time PCR. The results of the PLP real-time are shown as follows:

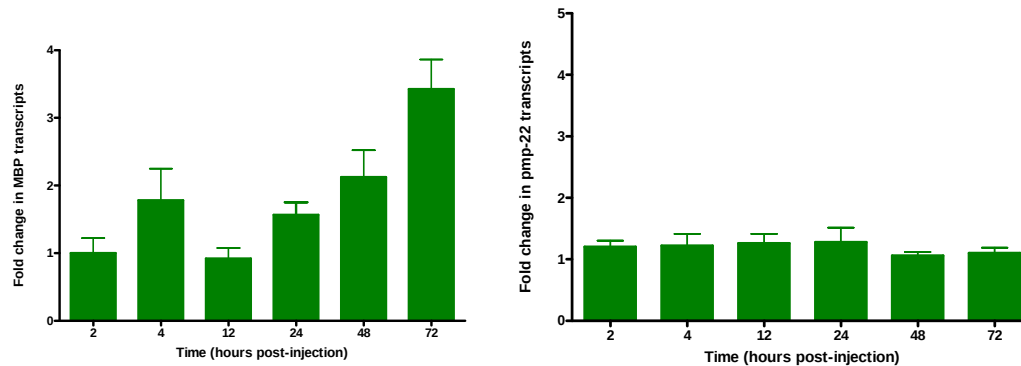


We observed a large increase in the level of PLP transcripts within 24 hours of injection of virus into the spinal cord. Given the results using the whole spinal cord homogenates (see table, above) this was surprising. There are several possibilities for the 'disconnect' between these data and the whole spinal cord results. 1) There could be a short period of time immediately after insult when the host attempts to invoke repair mechanisms in the CNS by upregulating the production of a various myelin transcripts. 2) The use of the long-term infected mice could reflect a time-point past which these mechanisms could be harnessed. 3) There could be areas of the spinal cord where transcripts are down-regulated during the pathogenesis process and these areas are 'diluting' the effects observed at the lesions site. Currently, we favor the first option, although further studies will be required to confirm this.

We have also tested the MPZ primers in these samples. MPZ is not expected to be altered in the CNS as it is primarily expressed in the peripheral nervous system. As expected, no significant changes in MPZ transcript levels were detected over the timecourse.

Changes in the levels of MBP but not pmp-22 transcription occur within the lesioned area of the spinal cord. Previously, we reported that we developed a series of real-time RT-PCR primers to detect various myelin transcripts including myelin basic protein (MBP) and peripheral myelin protein-22 (pmp-

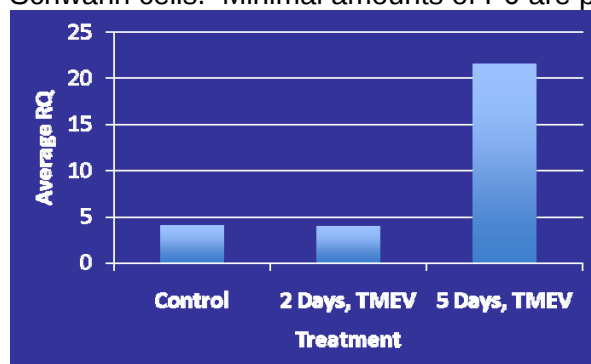
22) mRNA. We have performed real time RT-PCR assays on the spinal cord lesions from mice infected via the spinal cord to determine whether there were alterations in the level of MBP and/or pmp-22 following injection with TMEV. As shown below, there were increased levels of MBP transcripts detected following virus injection, but no change in pmp-22 levels. This is consistent with our expectations, as pmp-22 is largely localized to the peripheral nervous system. Furthermore, MBP is known to be upregulated very quickly after CNS insult. Data shown below are expressed as the level of myelin gene transcript relative the level observed in control injected mice. All data were normalized using a housekeeping gene (GAPDH).



We have localized TMEV within the demyelinated lesions, identified the infiltrating cells, and examined the lesions for proliferation and apoptosis. Because we are using lesioned areas (as opposed to the entire spinal cord) we need to combine spinal cord blocks (<1 cm of tissue) to get sufficient RNA to perform assays, while ensuring the quality of that sample. We are detecting changes in some myelin transcript levels relatively early after direct injection of TMEV into the spinal cord (within 24 hours) as compared with HBSS injected animals.

Real time PCR for P-zero transcripts indicate an increase in mRNA at 5 days post-injection with TMEV.

Real time PCR was performed using primers specific for P0, a myelin transcript made in abundance by Schwann cells. Minimal amounts of P0 are produced by oligodendrocytes. At 2 days post-injection,



minimal changes in P0 transcripts were observed at the lesion site. By 5 days post-injection with TMEV, there is a large increase in the levels of P0 levels, indicating that there is an increase in myelin products. Of note however, it is unlikely that this increase in P0 can be attributed to oligodendrocyte-mediated repair. It is our hypothesis that there is an influx in Schwann cells from the peripheral nervous system that are attempting to repair the demyelinated central nervous system. This hypothesis is supported by several other pieces of data 1) the lack of actively proliferating cells in the spinal cord following infection;

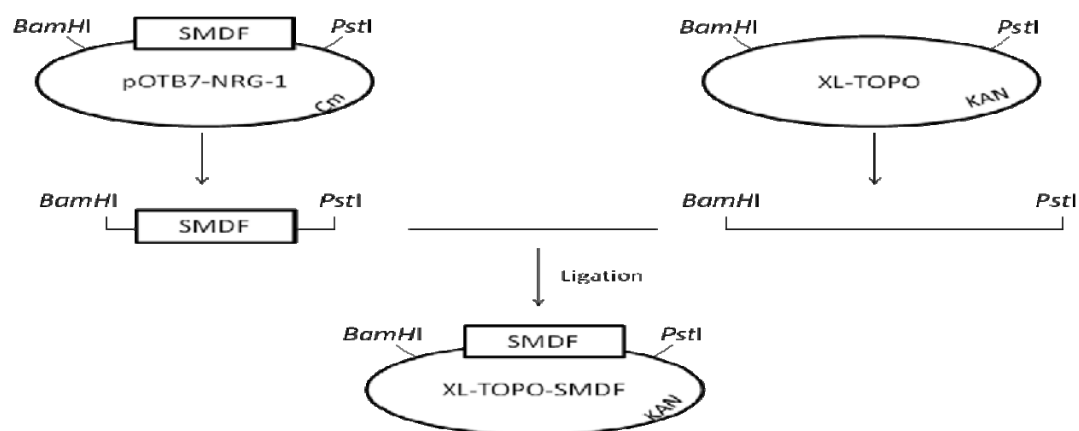
and 2) the increased hypercellularity of myelin-positive cells observed at the lesion site (shown under Task 10).

Specific Aim 3. Determine if myelin repair processes can be enhanced by administration of exogenous neuregulin following injection of Theiler's virus into the spinal cord.

- Task 12: Determine if exogenous administration of neuregulin-1 enhances spinal cord repair/myelin preservation.
- Generate viral vector expressing neuregulin-1.
 - Systemically inject viral vector or recombinant neuregulin-1 into FVB/nJ mice immediately prior to intraspinal cord injection with TMEV.
 - Determine if there are alterations in proliferation and apoptosis of myelinating cells (per Tasks 2, 3) as a result of treatment.
 - Determine if there are alterations in erb-B mediated signaling in TMEV-infected animals as a result of exogenous neuregulin treatment (per Tasks 4, 5).

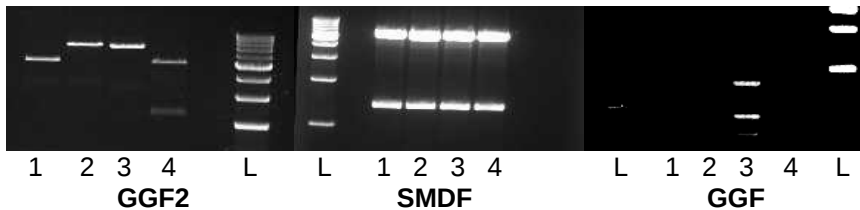
Generation of recombinant adenovirus vectors expressing glial growth factor (GGF), glial growth factor-2 (GGF-2) and sensory motor derived factor (SMDF). We generated recombinant adenovirus vectors that express a variety of neuregulin isoforms (required for Aim 3 of the grant). GGF, GGF2, and SMDF clones were obtained and subcloned into a shuttle vector (Dual CCM-CMVeGFP). The cloning strategy is outlined below:

Cloning of SMDF into pXL-TOPO Vector

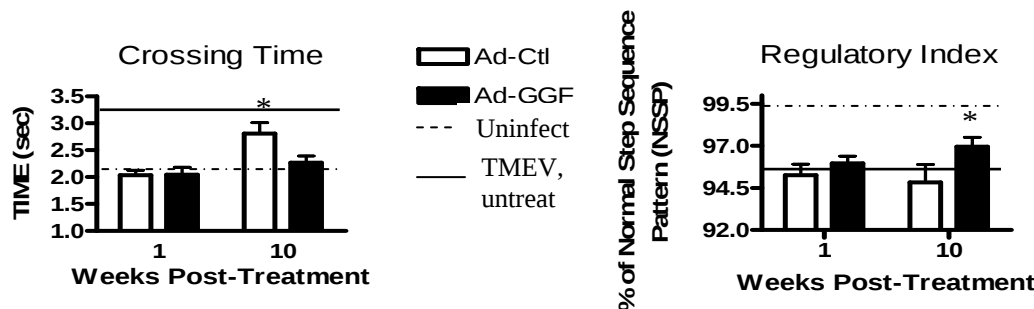


SMDF: Sensory and motor neuron derived factor
Cm: Chloramphenicol resistance
KAN: Kanamycin resistance

A similar strategy was used to generate GGF and GGF2 vectors. The cloned inserts are shown on the next page. Lane 4 is positive for the GGF2 vector, all lanes positive for SMDF and lane 3 positive for GGF. This was then inserted into the adenoviral genome. Following sequencing and verifying that the sequence was 100% homologous to the sequence in GenBank, the XL-TOPO-SMDF vector was cut with restriction enzymes and inserted into an adenovirus backbone. Resultant viruses were sequenced and titered prior to use. We generated high titer stocks ($2 \times 10^{11-12}$ pfus/ml; minimum of 5 mls) of each of the adenoviral constructs.

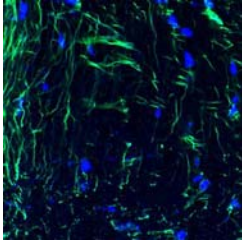


TMEV-infected FVB/n mice treated with Ad-GGF had fewer clinical deficits than TMEV-infected mice treated with Ad-Ctl. Administration of an adenovirus vector expressing the neuregulin family member glial growth factor (Ad-GGF) showed increased clinical function compared to control treated animals (Ad-Ctl) following infection with TMEV as determined using the CatWalk Gait Analysis System. TMEV-infected mice tolerated the adenovirus injections well. No differences in weight or survival were observed between the two treatment groups and untreated TMEV-infected control mice. Studies determined that crossing time (speed over a specific distance) and the regulatory index (the coordination of the mouse) are key measures of clinical function in this model (Salerno, Leland and Drescher, in preparation). Gait parameters were tested at 1 and 10 weeks after adenovirus injection (n=13-15/group). Ad-GGF mice experienced less severe clinical disease than Ad-Ctl mice ($p<0.05$; Student's t-test). One week after adenovirus treatment, Ad-Ctl- and Ad-GGF-treated, TMEV-infected mice performed similarly. By 10 weeks post-treatment, Ad-GGF-treated, TMEV-infected mice walked



faster across the CatWalk than the Ad-Ctl-treated infected mice ($p<0.05$). The mean regulatory index (RI) of the treatment groups at 1 and 10 weeks post-treatment with either Ad-Ctl or Ad-GGF. RIs were similar 1 week post-treatment, but the TMEV-infected mice receiving Ad-GGF had significantly improved coordination at 10 weeks post-treatment compared to the Ad-Ctl-treated mice ($p<0.05$). Swing speed, the rate at which an animal moves a particular paw during the step sequence is also significantly different between the 2 treatment groups (data not shown; $p<0.05$). The crossing time and RI of TMEV-infected, untreated animals was similar to that observed in Ad-Ctl treated mice (solid line on graphs). Uninfected mice perform at a level higher than that observed in all other groups of animals (dashed line on graphs).

Adenovirus vectors are not detected in the CNS of TMEV infected mice 15 days after i.p. injection of adenovirus expressing glial growth factor (AD-GGF). To examine whether Ad-GGF was directly responsible for altered clinical function between the two treatments, we performed immunohistochemical staining for the coxsackie adenovirus receptor (CAR). CAR has been well described as being highly expressed in the CNS of young mice and children, with expression levels that drop over time. In uninfected mice, CAR was not detected (data not shown). Following TMEV infection, CAR was upregulated, particularly in lesions (see next page).



Spinal cord from an FVB/NJ/n female mouse with TMEV stained with antibody to CAR (green). The morphology of the positively-stained cells is consistent with that of microglia. Nuclei are stained blue (DAPI). Image was captured using a Nikon two photon confocal microscope (Creighton University Biomedical Imaging Core Facility).

We performed RT-PCR on samples from TMEV infected mice untreated and Ad-GGF treated using adenovirus-specific primers. No product was amplified from samples from untreated spinal cords. At 7 days after injection of adenovirus vectors, adenovirus was detected in all samples (n=5). At day 14 p.i., low levels of adenovirus were identified in one sample (1 of 5). By day 19 p.i. no adenovirus was detected (n=5). The confirmation that CAR is not expressed in the spinal cords of uninfected mice may provide insight into the reasons as to why treatment with Ad-GGF does not improve clinical function in mice infected with adenovirus at the same time as TMEV infection. It could be postulated that lesion formation is required for Ad-GGF to trigger the repair events as there is no CNS CAR expression in the absence of lesion formation.

Adenovirus vectors expressing various neuregulin isoforms induce alterations in lymphocyte subset phenotype compared to untreated mice. Flow cytometry was performed on peripheral blood mononuclear lymphocytes (PBLs) to determine if treatment with adenovirus vectors expressing neuregulin isoforms altered the levels of T and B-cell subsets in animals receiving these injections. FVB/nJ mice were injected with adenovirus vectors expressing glial growth factor (GGF), glial growth factor 2 (GGF2), sensory motor neuron derived factor (SMDF) or a control insert (enhanced green fluorescent protein). The peripheral blood lymphocyte (PBL) populations from control (uninjected mice) and adenovirus-injected mice were analyzed at 9 days p.i.

Animals receiving adenovirus vectors, regardless of the nature of the insert, had increased levels of CD8+ T cells as compared to uninjected control animals. As CD8+ T cells are increased following virus infection, it is not surprising that there is an increase in these cell types following injection with the adenovirus constructs. It is likely that the specificity of these cells is directed against the adenovirus. Furthermore, there were increased levels of CD4+ T cells in GGF and SMDF-treated animals, as well as decreased levels of B220+ B cells in the neuregulin treated animals as compared to controls. While it is likely that at least a portion of the CD4+ T cells are specific for adenovirus due to the requirement for CD4+ T cell help, it is unclear if all of the cells are adenovirus-specific. Furthermore, this explanation does not take into account the observation that mice receiving control adenovirus or GGF2-expressing construct do not have significant alterations in their CD4+ T cell compartment. Together, these data demonstrate that results generated using the adenovirus virus expression system need to carefully consider the impact of the vectors on the immune system. Our choice of using 3 different neuregulin isoforms as test vectors will further permit us to assess changes as related to either exposure to adenovirus, or insert-specific changes. All data analysis was performed using a one-way analysis of variance (ANOVA).

Treatment	CD4+ % $\pm$ SEM	CD8+ % $\pm$ SEM	B220 % $\pm$ SEM
GGF-adenovirus	60.350 $\pm$ 0.750*	16.600 $\pm$ 1.700*	11.400 $\pm$ 2.400*
GGF2-adenovirus	54.200 $\pm$ 1.609	17.867 $\pm$ 0.837*	11.967 $\pm$ 1.440*
SMDF-adenovirus	59.600 $\pm$ 2.498*	19.230 $\pm$ 0.882*	8.667 $\pm$ 0.593*
Control-adenovirus	48.750 $\pm$ 3.050	16.300 $\pm$ 0.000*	15.200 $\pm$ 5.800
No treatment	34.300 $\pm$ 6.300	9.350 $\pm$ 2.450	35.550 $\pm$ 5.350

* $p \leq 0.05$

Minimal alterations in chemokine and cytokine levels are observed in animals injected with neuregulin-expressing adenovirus constructs. To further explore the impact of the neuregulin-expressing adenoviruses on immune function (a critical experiment as the immune system is known to impact the development of demyelinating lesions), we utilized the Luminex bead array system to determine if there was a difference in the levels of 21 cytokines in serum samples 7-days post-injection and in spleen samples 9-days after injection with adenovirus vectors expressing three different neuregulin isoforms. Control vectors contained an irrelevant insert. The cytokines tested were: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, GM-CSF, IFN γ , KC, MCP-1, and MIP-1 β , RANTES, TNF α , and VEGF. Analysis of sera at 7 days post-infection revealed that RANTES levels were significantly increased in adenovirus/GGF2-treated mice compared to control-treated mice. No other differences were observed in any of the cytokine levels between the control group and the neuregulin-expressing adenovirus groups. In the spleen (9 days post-treatment) levels of all cytokines were similar with no observed differences between treatment groups. Together, these data suggest that if there are differences observed in demyelination and remyelination in animals treated with neuregulin-expressing adenovirus vectors, the alterations are not likely to be due to alterations in immune function.

MicroRNAs(miRNAs) are differentially expressed in TMEV-infected versus uninfected FVB/nJ mice.

The half-life of most miRNAs is unknown but some studies suggest that miRNA half-lives are in the order of weeks(7). Real-time PCR-based array analysis for microRNAs (RT² miRNA PCR Array, SABiosciences) was performed on spinal cords from uninfected and TMEV-infected mice. We found 32 miRNAs with at least a 10-fold change in expression between the two groups of mice. *In silico* analysis of predicted targets of the 2 miRNA sequences with the greatest change between the groups revealed an interesting finding. miR-684, upregulated 115.7-fold in spinal cords from TMEV-infected mice compared to control mice, and miR-466g, increased in the infected mice by 78-fold, are both predicted to target neurexophilin-1 (NXPH-1; Sanger miRNA Database). The neurexophilins form complexes with α -neurexin (2) and are expressed in some neurons. There are four known neuroexophilins – NXPH-1-4 (3;4;6). NXPH-1, -3, and -4 and are expressed in the human and mouse brains. NXPH-2 has is largely localized to the peripheral organs of humans and rats (1;4). Neurexophilins are secreted glycopeptides that undergo post-translational processing and contain a domain structure with homology to that observed in neuropeptides (5); it is postulated that these proteins are involved in neuronal signaling. miR-684 is also postulated to target neurexin, the receptor for neurexophilin (www.TargetScan.org). Neurexophilin-neurexin interactions promote adhesion between dendrites and axons. Others have shown that animals deficient in NXPH3 have decreased motor coordination compared to control littermates (1) as assessed using a rotarod. This finding supports our hypothesis that regulation of neurexophilins impacts clinical function in our mouse model of MS.

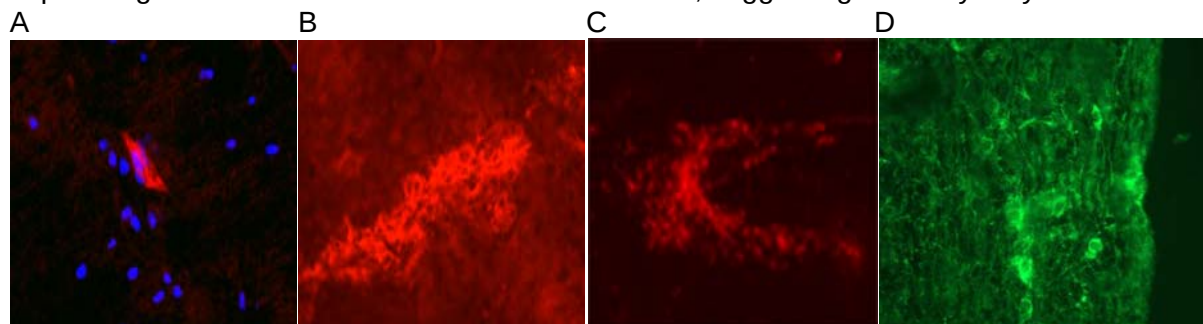
miR-684 and miR-466g are differentially expressed in the spinal cords of TMEV-infected mice treated with Ad-Ctl or Ad-GGF. We postulated that if miR-684 or mi-466g impacted clinical function in TMEV-

infected mice then the expression of these miRNAs would be altered in animals with CNS damage, but differing levels of clinical function. We found a 2.63-fold decrease in mi-466g and a 2.51-fold decrease in miR-684 expression in Ad-GGF- versus Ad-Ctl-treated mice using qRT-PCR. While these changes are not as large as those observed in the initial profiling experiment, it is important to note that the baselines for comparison of these 2 results are significantly different. In the first experiment, we compared uninfected and infected miRNA levels, while in this experiment we are comparing levels of miRNAs in groups of mice with significant CNS damage. Calibrating these data so as to permit comparison between the 2 experiments, the data are expressed as follows (data expressed as fold change relative to uninfected control):

miRNA	Infected	Infected, Ad-Ctl	Infected, Ad-GGF
miR684	-115.7	-98.3	-39.1
mi566-g	-78.1	-81.4	-30.6

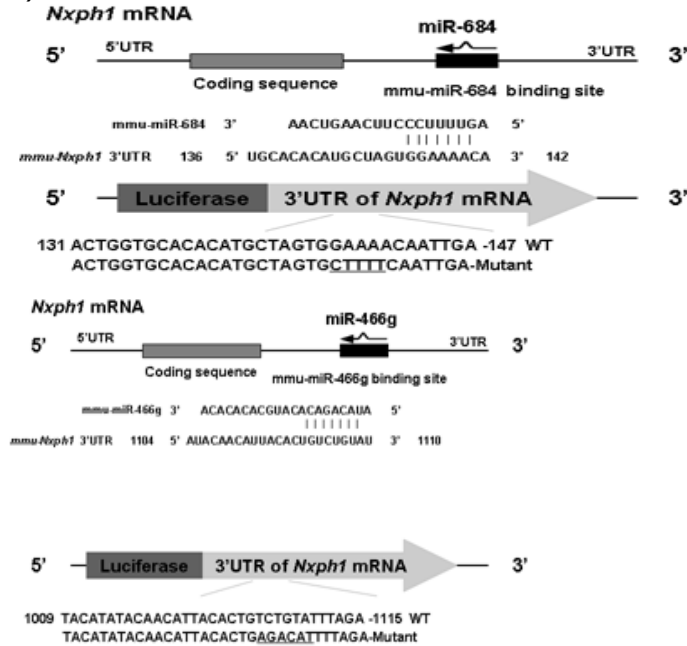
Together, these data suggest that increased levels of miR-684 and/or miR-466g are associated with decreased clinical function the TMEV model of MS.

Neurexophilin-1 is expressed in the spinal cords of mice infected with TMEV. Immunofluorescent staining was performed to localize NXPH-1 and neurexin in the spinal cords of TMEV infected mice. NXPH-1 was localized both within the spinal cord white matter (A), central canal (B). NXPH1 (C) and neurexin (D) were found within demyelinating lesions. Flow cytometry suggests one cell type expressing NXPH-1 and neurexin is are B220+CD5+, suggesting that they may be B-1 B cells.



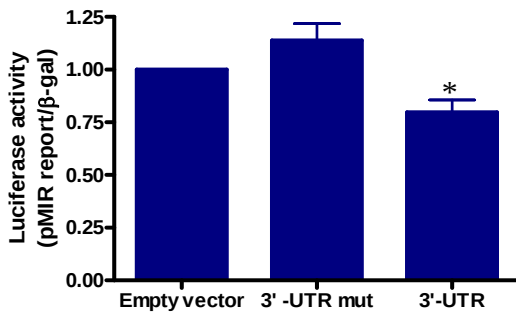
Reporter gene assays support the role of miR-684 and miR-466g in regulating neurexophilin-1 expression. Luciferase-based constructs containing predicted binding sites of the miRNAs in the 3' UTR of NXPH-1 were transfected into cell lines that constitutively expressed either miR-684 or miR-466g. The results demonstrate that with constructs expressing the wild-type miR-684 binding site in the 3'-UTR of NXPH-1 suppresses NXPH1 translational activity as measured by luciferase activity ($p < 0.05$ compared to mutated miR-684 binding site in the 3'UTR). Luciferase activity was restored using constructs containing the mutated miR-684 binding site in the 3'-UTR of NXPH1 (next page, left). Similar results were obtained using the constructs expressing wild type and mutated miR-488g binding sites (next page, right). These data support the hypothesis that miR-684 and miR-466g are involved in the translational regulation of neurexophilin-1.

A)

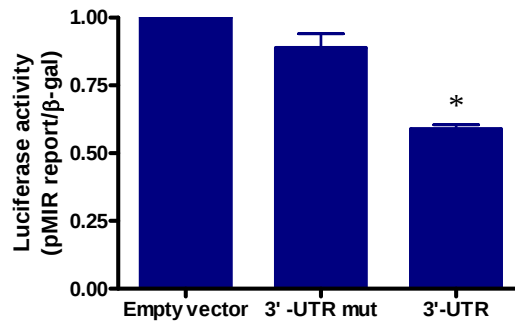


Luciferase reporter gene constructs expressing the neurexophilin-1 3'UTR containing the wild-type miR-684 (A) and miR-466g (B) binding sites were constructed. Additional constructs were made which contained mutated miR-684 (A) and miR-466g (B) binding sites.

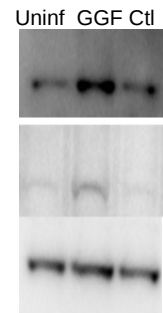
miR-684



miR-466g



Western blot shows changes in Nxph1 (top) and neurexin (middle) levels in the spinal cords of mice infected with TMEV. Bottom panel shows blot of actin used for normalization. GGF treated mice (middle lane) had the highest levels of Nxph1 and neurexin



Western blots demonstrate that there is differential regulation of both neurexin and neurexophilin-1 in the spinal cords of mice infected with TMEV following treatment with Ad-GGF compared to Ad-Ctl treated mice. There is an approximately 3.6 ± 0.3 fold increase in Nxph1 in GGF treated mice relative

to controls, and a 2.5 ± 0.02 fold increase in neurexin levels relative to controls ($p \leq 0.05$ for both neurexophilin and neurexin).

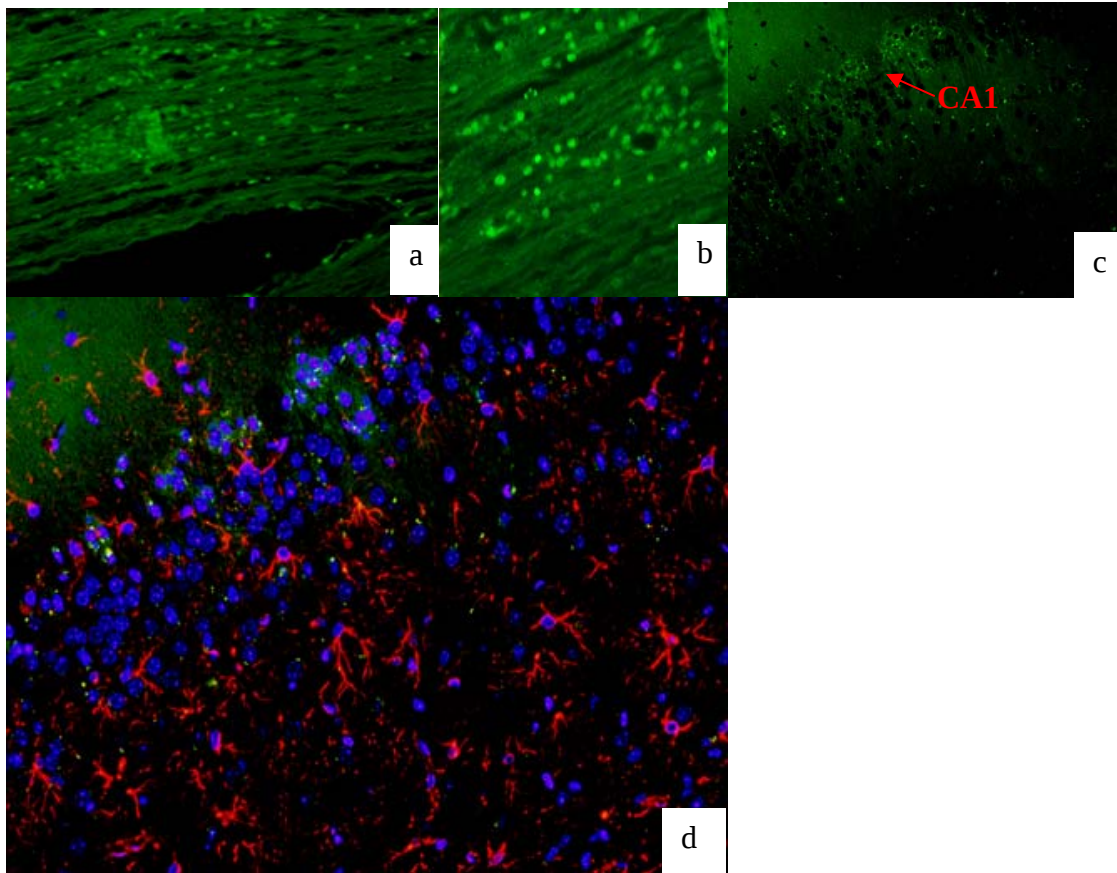
Neurexophilin-1 and neurexin are expressed in the spinal cords of mice infected with TMEV and B1 B cells are positive for both proteins. Based on the western blot data shown above, we examined the phenotypes of the cells expressing neurexophilin-1 and neurexin in the CNS. We performed both immunohistochemical staining and flow cytometry to identify the phenotype of the Nxph1 and α -neurexin positive cells. In uninfected mice, Nxph-1 expression was limited to the central canal, and the cells expressing Nxph1 were likely to be neurons based on their localization. Minimal staining for α -neurexin was found in uninfected mice. In infected mice, staining for α -neurexin was in areas of inflammation as well as the central canal. We performed flow cytometry on CNS (spinal cord) infiltrating cells and one cell type positive for both Nxph1 and α -neurexin type are B220+CD5+, suggesting that B-1 B cells may be a source of these proteins. B-1 B cells have been implicated in some autoimmune diseases.

miR-466-g and miR-684 are expressed in both resident CNS cells and immune cells. MicroRNA was isolated from CNS tissue of uninfected mice as well as splenocytes to assay for miR-466g and miR-684. Using real time RT-PCR, miR-466g and miR-684 were detected in the CNS (spinal cord) tissue (C_T values of 28.4 and 30.6, respectively) and splenocytes (C_T s of 29.1 and 29.4, respectively). As microRNA was isolated from whole spleen, we postulated that the sample could be contaminated with organ enervating tissue, and the miR-466g and miR-684 were from PNS tissue. We then isolated microRNA from CNS (brain and spinal cord) infiltrating lymphocytes. Similar results were obtained. These preliminary studies suggest that both resident and infiltrating cells express miR-466g and miR-684.

To further confirm the phenotype of the immune cells expressing the microRNAs, we isolated splenocytes and used flow cytometry to isolate cells of the following phenotypes: CD4+CD8-, CD8+CD4-, B220+CD5-, and B220+CD5+. MicroRNA was then isolated from each of these discrete cell populations and realtime RT-PCR performed. We also dissected brains from mice and performed realtime RT-PCR on cortex, hippocampus, cerebellum and brainstem. Ct values for each immune cell type and brain region were as follows:

	CD4+	CD8+	B220+CD5-	B220+CD5+	Cortex	Brainstem	Cerebellum	Hippocampus
miR466g	31.5	33.3	29.7	30.4	32.0	29.3	30.7	35.8
miR684	35.0	33.8	34.4	34.3	35.6	34.1	34.8	36.2

Collectively, these data support the hypothesis that miR-466g and miR-684 are expressed by both resident CNS and infiltrating cells. To further explore the cell types expressing these miRNAs, we performed in situ hybridization using biotin labeled probes specific for each of the miRNAs. An avidin labeled Alexa Fluor 488 conjugate was used for visualization of the hybridization. Both infiltrating cells and CNS resident cells are positive for the miRNAs (green signal; below and data not shown). We also used antibodies to GFAP and F4/80 to determine if astrocytes and microglia/macrophages were the sources of the positive signal. No co-localization was identified. Based on our collective data to date, neurons appear to be the primary cell type of the CNS positive for both of these microRNAs. We are continuing with the co-localization studies to confirm this observation. Both T and B cells are also positive for miR-466g and miR-684.



Localization of miR-466g (a,b) and 684 (c,d) in spinal cord (a,b) and brain (c,d). Low power view of infiltrate in spinal cord white matter of a TMEV infected mouse demonstrating presence of miR466g by the infiltrating cells (a). Higher power view of lesion (b). No signal was observed in the tissue (a,b) using the red channel, indicating the signal was not autofluorescence, which would be expected if the cells were red blood cells. Brain of TMEV infected mouse localizing miR684 (green; c). Arrow indicates the CA1 region of the hippocampus. Higher power view of the brain in (c), which is also stained with an antibody to GFAP (red) and DAPI (blue). The localization of the positive cells and lack of co-localization of them with GFAP or F4/80 (not shown) suggests that the miR684 positive cells are neurons.

Key Research Accomplishments

- We have identified macrophages as the primary immune cell type infected with virus in a model of intraspinal cord injection of TMEV.
- Replicating virus has been isolated from the spinal cords of injected mice.
- Determined that myelinating cells undergo minimal proliferation, but significant apoptosis following TMEV injection into the spinal cord.
- The identification of IL-11 within the TMEV-induced lesion may represent a key mechanism for preservation of myelin in this model, and may account for the increased levels of MBP that are observed within the spinal cords of infected animals.
- Determined that glial growth factor administration can improve clinical function in a mouse model of multiple sclerosis.
- We reported results from gait analysis experiments of animals infected with TMEV and treated with adenovirus constructs expressing glial growth factor, glial growth factor 2, sensory and motor neuron derived factor or a control construct and determined that the most stable clinical function is found in mice that have been treated systemically with adenovirus expressing GGF. Correlative data suggest that these changes in function may be the result of changes in microRNA expression that may ultimately impact protein levels of mediators of myelin-axon interactions. Luciferase reporter assays support the regulation of neurexophilin-1 by these microRNAs.
- We performed in situ hybridization studies to localize microRNAs in the CNS and in infiltrating cells. Both neurons and immune cells appear to express miR-466g and 684.
- We developed and optimized an assay to detect low levels of TMEV RNA in CNS samples. This assay was used in the Salerno et al. manuscript (see Appendix; Salerno et al.). This paper also establishes the methodology for the changes in clinical function we have observed following treatment with neuregulin family members.
- The effects on the immune response of the recombinant adenoviral vectors expressing three isoforms of neuregulins have been characterized both in FVB/nJ mice as well as the tamoxifen-treated erbB2^{-/-}-EGFR^{-/-} mice.
- TMEV lesion sites have been characterized in the tamoxifen-treated erbB2^{-/-}-EGFR^{-/-} mice.
- Real-time RT-PCR experiments suggest that there is Schwann cell-mediated repair of the central nervous system occurring.
- Apoptosis assays demonstrate that damage to the spinal cord occurs prior to the spread of demyelination and virus. These data indicate that there are other mechanisms (that is non-viral mediated) of damage occurring to the spinal cord.
- Expression of erbB2 and neuregulins have both been shown to be widespread throughout the spinal cord, indicating that the components required for signaling are intact in mice with spinal cord lesions.
- EGFR levels are low and unaltered over the course of TMEV infection.
- Myelin expressing cells are clustered in the lesions of TMEV-infected mice with conditional deletions of erbB2. These cells are likely Schwann cells.
- ErbB2 is expressed on immune cells – B cells, macrophages, CD4⁺ T cells, and CD8⁺ T cells.
- ErbB2 is upregulated on activated CD4⁺ T cells, CD8⁺ T cells, and B cells.
- ErbB2 levels increase in the spinal cord following TMEV infection.

Reportable Outcomes:

Grant Applications:

Neurexophilin-1:alpha-neurexin interactions impact function in a model of MS, LB 692, April 2010 (not funded).

Impact of miR-684 on alpha-neurexin expression and clinical function in a mouse model of multiple sclerosis, Department of Defense, June 2010 (not funded)

Impact of miR-684 and 466g on α -neurexin expression and gait in a model of MS, National Institutes of Health, October 2010 (not funded).

Impact of miR-684 and 466g on α -neurexin expression and gait in a model of MS, National Multiple Sclerosis Society, February 2011

Presentations:

Drescher KM and Tracy SM (2008) Development of a model allowing study of the early stages of demyelination. International Society of Neuroimmunology, Fort Worth, TX October 2008. Oral Presentation.

Rasband-Lindquist, A and Drescher KM (2009) Role of neuregulins in myelin preservation and repair in the central nervous system. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Lindquist JD and Drescher KM (2009) The Roles of ErbB2 and EGFR in the development of TMEV-induced lesions. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Poster Presentation. Military Health Research Forum. Kansas City, Missouri. September 1, 2009

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Oral Presentation. Military Health Research Forum. Kansas City, Missouri. September 2, 2009

Drescher, KM (2010) Impact of erbB ligands on function in a mouse model of multiple sclerosis. Molecular Biology Research Group, Creighton University School of Medicine.

Drescher, KM. Glial growth factor treatment improves clinical function in a mouse model of multiple sclerosis. March 29, 2010. Medical Microbiology and Immunology seminar series, Creighton University.

Hendricks, T and Drescher, KM. (2011). Expression of miR684 in a mouse model of multiple sclerosis. Midwest Medical Student Biomedical Research Forum, Omaha, NE. February 2011. Poster Presentation.

Schenk WB, Drescher KM, Aggarwal A, and Chen XM. (2012) miRNA-684 regulation of neurexophilin-1 and α -neurexin expression. Midwest Medical Student Biomedical Research Forum, Omaha, NE. February 2012. Poster Presentation.

Iarocci AL, Drescher KM. (2012). ErbB2 levels increase in spinal cords of Theiler's murine encephalomyelitis virus-infected mice. St. Albert's Day Student Research Forum, Omaha, NE. March 2012. Poster Presentation.

Other:

Served on Abstract Selection Committee for the Military Health Research Forum held in Kansas City, Missouri (August 31 – September 3, 2009).

Medical Student Fellowships:

Allison Lindquist (M1), recipient of a research fellowship from the Dean's Research Fund for a proposal entitled "Role of Neuregulins in Myelin Preservation and Repair in the Central Nervous System" 2008

Jonathan Lindquist (M1), recipient of a research fellowship from the Nebraska Medical Foundation for a proposal entitled "The Roles of ErbB2 and EGFR in the Development of TMEV-Induced Lesions" 2008

Thomas Hendricks was awarded a fellowship from the Nebraska Medical Foundation for his proposal entitled "Expression of miR684 in a mouse model of multiple sclerosis."

W. Brad Schenk (M1) was awarded a Medical Dean's Fellowship to perform summer research entitled "Neurexophilin 1 expression in a mouse model of multiple sclerosis." 2011

Publications/Manuscripts:

Drescher KM and Sosnowska D (2008) Being a mouse in a man's world: what TMEV has taught us about human disease. *Frontiers in Bioscience* 13: 3775-3785.

Hendricks TJ, Iarocci AL, and Drescher, KM. Mast cells, complement, and TLRs: influence of the innate immune system on demyelination (under revision).

Salerno DM, Leland KM, and Drescher KM. Development of gait alterations in immunocompetent mice resistant to TMEV-induced demyelination (under revision).

Iarocci AL and Drescher KM. ErbB2 expression increases in the spinal cords of Theiler's murine encephalomyelitis virus-infected FVB/nJ mice (in preparation).

Salerno DM, Leland KM, and Drescher KM. Altered clinical function in TMEV-infected mice following administration of neuregulin-1 encoding adenovirus vectors (in preparation).

Hendricks TJ, Schenk WB, Leland KM, Chen XM, and Drescher KM. MicroRNA-684 and -466g in the spinal cords of TMEV-infected mice (in preparation).

Awards:

Drescher KM and Tracy SM. A Model of Demyelination that Permits the Study of Early Phases of Lesion Development. October 2007. Creighton University Faculty Club: Innovation in Basic Science and Clinical Research. Third Place.

Conclusions

Because myelin basic protein transcripts increased despite the increase in apoptotic cell death, we postulated that myelinating cells were undergoing proliferation. This is unlikely based on the data that we have obtained. Rather it currently appears that precursors to myelinating cells are undergoing a maturation process due to the increase in IL-11 levels. Further studies will be performed to confirm this. The sensitivity of the real-time RT-PCR assay that we developed will greatly enhance our analysis of the spinal cord tissues. This assay will better be able to detect whether some of the apoptosis that we observe in areas distal to the injection site are in fact, negative for virus or have virus burdens below the level of detection of the plaque assay.

The data acquired support the role of erbB/EGFR interactions with their ligands, in particular glial growth factor, as mediators of CNS health. In particular, these interactions are important in the preservation of clinical function. While we are not observing CNS repair, we are observing stabilization of disease, as measured by clinical function (as opposed to pathological parameters of CNS health such as axonal numbers or oligodendrocyte cell death). Two participants in this process appear to be miR466g and miR684, microRNAs that affect the regulation of neurexophilin-1.

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Appendix

Abstracts:

Drescher KM and Tracy SM (2008) Development of a model allowing study of the early stages of demyelination. International Society of Neuroimmunology, Fort Worth, TX October 2008. Oral Presentation.

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Military Health Research Forum. Kansas City, Missouri

Rasband-Lindquist, A and Drescher KM (2009) Role of neuregulins in myelin preservation and repair in the central nervous system. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Lindquist JD and Drescher KM (2009) The roles of ErbB2 and EGRF in the development of TMEV-induced lesions. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Hendricks TJ, Leland KM, and Drescher KM (2011). Expression of NXPH-1 in a mouse model of multiple sclerosis. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2011, Poster Presentation.

Schenk WB, Drescher KM, Aggarwal A, and Chen XM. (2012) miRNA-684 regulation of neurexophilin-1 and α -neurexin expression. Midwest Medical Student Biomedical Research Forum, Omaha, NE. February 2012. Poster Presentation.

Iarocci AL, Drescher KM. (2012). ErbB2 levels increase in spinal cords of Theiler's murine encephalomyelitis virus-infected mice. St. Albert's Day Student Research Forum, Omaha, NE. March 2012. Poster Presentation.

Publications/Manuscripts

Drescher KM and Sosnowska D (2008) Being a mouse in a man's world: what TMEV has taught us about human disease. *Frontiers in Bioscience* 13: 3775-3785.

Hendricks TJ, Iarocci AL, and Drescher, KM. Mast cells, complement, and TLRs: influence of the innate immune system on demyelination (under revision).

Salerno DM, Leland KM, and Drescher KM. Development of gait alterations in immunocompetent mice resistant to TMEV-induced demyelination (under revision).

Iarocci AL and Drescher KM. ErbB2 expression increases in the spinal cords of Theiler's murine encephalomyelitis virus-infected FVB/nJ mice (in preparation).

Other

Drescher, KM. Curriculum Vitae

DEVELOPMENT OF A MODEL ALLOWING STUDY OF THE EARLY STAGES OF DEMYELINATION

Kristen M. Drescher¹ and Steven M. Tracy²; ¹ Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, NE and ² Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE

Studying the earliest phases of the development of demyelinating lesions can be difficult given the current animal models available. We have modified the currently existing Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis to permit us to study the lesion site hours to days after insult. In this model, we directly inject virus into the spinal cord of mice, thereby allowing for precise localization and aging of the lesion site

Following surgical exposure of the spinal cord, female SJL/J mice were injected with 2×10^4 pfus of TMEV (Daniel's strain) directly into the spinal cord white matter. Using antibodies directed against myelin proteins, immunohistochemistry revealed large areas of demyelination within three days of virus injection. Staining with a polyclonal antiserum to TMEV revealed that the area of demyelination overlapped with the region that was positive for TMEV antigens. Animals injected with DMEM (virus vehicle) did not experience demyelination. Tissue was isolated from the injection site, and plaque assay revealed the presence of replicating virus at levels approximately 3 logs greater than the initial inoculum. Immune cells were recruited to the site of the demyelination within 5 days post-injection. CD4+ and CD8+ T cells were all found at the lesion site, as well as F4/80 reactive macrophages. Real-Time PCR was used to assay the levels of various myelin gene transcripts from lesion sites dissected from the spinal cords over time. Alterations in gene transcript levels over time were unique for myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG).

Together, these data represent a model of TMEV-induced demyelination which permits the study of the earliest phases of lesion development. The events that occur in the hours and days after insult are largely undefined, in particular with regards to the exact sequence of mediators and events that are triggered. Studies are ongoing to determine the earliest immune system participants in the demyelination process in this model.

Presented as an oral presentation at the International Society of Neuroimmunology Meeting, October 2008, Fort Worth, Texas

Development of a Model of MS Permitting Study of Early Stages of Lesion Development

Kristen M. Drescher and Helene Thal-Jantzen

Background and Objectives: The cause of multiple sclerosis (MS), the most common central nervous system (CNS) demyelinating disease, is unknown although it is likely that both host genetics and environmental influences are involved in disease progression. The cellular immune response however, is partially responsible for the ongoing demyelination of the CNS. Because diagnosis occurs well after lesion formation, the earliest stages of lesion formation in humans have not been well-characterized. We have modified the existing Theiler's murine encephalomyelitis virus (TMEV) model of MS to permit the study of the lesion site within hours to days after the initial insult. In this model, TMEV is directly injected into the spinal cord of mice, thereby allowing for precise localization and aging of the lesion site

Methodologies: The DA strain of TMEV was utilized in all studies. Viral load was assayed by plaque assay. Immunohistochemistry and real time RT-PCR were used to characterize protein and gene expression within the lesion, as well as in areas distant from the lesion. TUNEL assays were also performed to determine the extent of apoptosis in the CNS relative to the lesion site.

Results: After surgical exposure of the spinal cord, female SJL/J mice were injected with 2×10^4 pfus of TMEV directly into the spinal cord white matter. Immunohistochemistry was performed using an antibody raised against myelin proteins and large areas of demyelination were observed within 3 days of injection. This demyelination was not observed in animals injected with vehicle (Hanks Balanced Salt Solution). Staining for viral proteins with a polyclonal antisera to TMEV revealed that demyelinated regions overlapped in areas where TMEV antigens were localized. The injection site was dissected out of the spinal cord and infectious virus was assayed. Replicating virus levels were nearly 3 logs greater than the initial inoculum. Immune cells were recruited to the site of the demyelination within 5 days post-injection: CD4+ and CD8+ T cells were all found within the lesion, as well as F4/80 reactive cells. Morphology of these cells was consistent with macrophages. Real-time RTPCR was used to assay levels of various myelin gene transcripts from lesion sites dissected from the spinal cords as a function of time post-injection. Alterations in myelin gene transcript levels over time were unique for myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG), indicating a differential impact of infection on each of these genes.

Conclusions: Together, these data describe a new model designed to examine the earliest phases of demyelinating lesion development. This will permit definition of events that occur within minutes of initial CNS damage, thereby permitting insight into the earliest pathogenic (and possibly protective) responses invoked by the host. Studies are ongoing to determine the earliest immune system participants in the demyelination process in this model.

Impact: These studies permit, for the first time, insight into the spatiotemporal development of CNS demyelinating lesions. Knowledge of these events may assist in the development or refinement of therapies for MS.

Presented at the Military Health Research Forum, Kansas City, MO in both poster and oral presentations.

ROLE OF NEUREGULINS IN MYELIN PRESERVATION AND REPAIR IN THE CENTRAL NERVOUS SYSTEM

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The precise role of neuregulins in myelin preservation and repair in the central nervous system is not well defined, particularly in disease processes such as multiple sclerosis. The role of neuregulins, specifically glial growth factor (GGF), glial growth factor 2 (GGF2), and sensory and motor derived factor (SMDF), as well as their primary receptor (erbB2) was evaluated in the Theiler's murine encephalomyelitis virus (TMEV) infection. The role of neuregulins in TMEV was assessed through measurement of cytokines, flow cytometry, RT-PCR, immunostaining and apoptotic cell detection. The concentration of 21-cytokines did not statistically differ between TMEV-infected and uninfected control mice 7 days and 10 days after infection. However, after 18 hours, animals that received adenovirus vectors expressing GGF, GGF2, or SMDF had increased numbers of T-cells, both helper and cytotoxic, and decreased numbers of B-cells. The level of myelin basic protein (MBP) transcripts was statistically decreased in spinal cord tissue from areas near TMEV-injection sites 3-days post-injection compared to uninfected spinal cord tissue. The expression of proteolipid protein (PLP) transcripts was statistically decreased in the spinal cords of mice following infection of TMEV and treatment with SMDF-expressing adenovirus when compared to uninfected spinal cord tissue. Fluorescent immunostaining was performed for macrophages, myelin basic protein, erbB2, and coxsackie and adenovirus receptor (CAR). Immunostaining revealed that NRG3 and CAR were localized to the demyelinated lesion. Taken together, these results show that growth factors impact the environment of demyelinated lesions. Additional experimentation done on neuregulins and growth factors will help to clarify the role of all of these relationships in demyelination/remyelination.

Acknowledgements: This research was supported by the Department of Defense (W81XWH-07-1-0223)

Presented as a poster at the Midwest Medical Student Biomedical Research Forum, Feb 2009

THE ROLES OF ERBB2 AND EGFR IN THE DEVELOPMENT OF TMEV-INDUCED LESIONS Jonathan D. Lindquist and Kristen M. Drescher, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, NE 68178

The roles of ErbB2 and EGFR in remyelination of the adult spinal cord are poorly defined. ErbB2 and EGFR inducible knockout mice in the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis were used to address the role of these receptors in spinal cord repair. Following induction of the knockout, CD4+ T cell populations were altered in B6/129 mice. Immunostaining showed infiltration of spinal cord lesions with CD4+, CD8+, and F4/80+ cells in both treated (ErbB2, EGFR knockout) and control (B6/129) mice 7 days post-infection. Staining also revealed foci of preserved myelin-producing cells associated with F4/80+ macrophages. Real-time PCR demonstrated alterations in P₀ transcripts, but not PLP and MBP transcripts in ErbB2, EGFR knockout mice. Together, these findings suggest that the increase in P₀ is due to Schwann cell infiltration of the CNS lesion.

Acknowledgements: This research was supported by the Department of Defense (W81XWH-07-1-0223)

Presented as a poster at the Midwest Medical Student Biomedical Research Forum, Feb 2009

EXPRESSION OF NXPH-1 IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

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Creighton University School of Medicine, Omaha, NE

Abstract

PURPOSE: Demyelinating disease induced by Theiler's murine encephalomyelitis virus (TMEV) is a well-established model for multiple sclerosis (MS). Previous data from the lab has shown that treatment of TMEV-infected mice with glial growth factor (GGF) improves clinical disease. These studies examined the potential role of miR684 and its targets in disease pathogenesis.

MATERIALS AND METHODS: These studies made use of real time RT-PCR, indirect immunostaining, flow cytometry, and luciferase reporter gene assays.

RESULTS: CatWalk Gait Analysis of TMEV-infected mice treated with an adenovirus expressing a glial growth factor insert (Ad-GGF) or a control insert (Ad-Ctl) demonstrated that mice receiving the Ad-GGF insert had improved clinical function relative to controls. However, this effect was not evident until 70 and 96 days post-infection, suggesting the improved function was not due to direct effects of the Ad-GGF treatment but rather downstream mechanisms. These changes correlated with changes in the level of expression of miR684, which is predicted to translationally suppress Neurexophilin-1 (Nxph-1). miR684 was increased in mice experiencing clinical dysfunction, and decreased in mice with improved function. The temporal expression of Nxph-1 and its receptor, Neurexin II α (NrxnII α), by real time RT-PCR demonstrated that the changes in clinical function correlated with changes in Nxph-1 and NrxnII α expression. Immunostaining revealed these proteins to be expressed on infiltrating cells in spinal cord white matter. Follow-up flow cytometry was performed to identify the phenotype of these cells, and the findings indicate that NrxnII α and Nxph-1 are expressed by CD5+CD19+(B220) cells, a phenotype consistent with the B-1 B cell subpopulation. The luciferase reporter gene assay confirmed that miR684 is responsible for the translational suppression of the Nxph-1 protein.

CONCLUSIONS: Taken together, these results suggest that mice experiencing TMEV-induced demyelinating disease experience an upregulation in miR684, resulting in decreased NrxnII α and Nxph-1 expression by B-1 B cells. That these proteins were increased following administration of GGF and that this was associated with improved clinical function suggests that these proteins are key component of a healthy CNS. This is also suggests a therapeutic role for GGF in the management of demyelinating disease.

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Presented at the Midwest Medical Student Biomedical Research Forum, Feb 2010

miRNA-684 Regulation of Neurexophilin-1 and α -Neurexin Expression.

WB Schenk, KM Drescher, A Aggarwal, and XM Chen.

While microRNA expression patterns of many physiologic processes have been described, description of the role of miRNAs in the progression of multiple sclerosis (MS) is lacking. It is therefore the effort of this research effort to both define the expression and mechanism by which these molecules contribute to the disease.

MS is characterized by a decrease in expression of several proteins vital to neuron health. These studies focus on two such proteins, neurexophilin-1 and α -neurexin. Using the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis, candidate miRNAs that are involved in demyelinating processes were identified. The studies described herein focus on miR-684, hypothesized to have binding sites in the 3' untranslated region (UTR) of neurexophilin-1 and α -neurexin. In these studies, mutations were made in the miR-684 putative binding sites of the 3' UTR of neurexophilin-1 and α -neurexin. The effect of these mutations on expression was ascertained via a luciferase assay.

The results demonstrated that expression was decreased in cells transfected with the wild type 3'UTR binding sites. Luciferase activity was restored by transfecting cells with a construct containing a mutated miR-684 binding site. Together, these data demonstrate that miR-684 binding to the 3'UTR of either neurexophilin-1 or α -neurexin decreases expression of these proteins, thereby functioning as a post-transcriptional level of control.

Midwest Medical Student Biomedical Research Forum, Omaha, NE. February 2012. Poster Presentation.

Title: ERBB2 LEVELS INCREASE IN SPINAL CORDS OF THEILER'S MURINE ENCEPHALOMYELITIS VIRUS-INFECTED MICE

Andrew L. Iarocci, Kristen M. Drescher

Purpose: Previous studies, using the *Catwalk Automated Gait Analysis System*, demonstrated improved motor function in Theiler's murine encephalomyelitis virus (TMEV)-infected female FVB/nJ mice treated with an adenovirus vector expressing a cDNA clone of human glial growth factor (GGF). GGF is a splice variant of the neuregulin-1 (NRG1) gene. Neuregulins (NRGs) are a family of epidermal growth factors that interact with epidermal growth factor receptors ErbB2, ErbB3, and ErbB4. ErbB2 is implicated to be the primary receptor with which ErbB3 and ErbB4 heterodimerize. Our goal was to determine the level and distribution of ErbB2 in female FVB/nJ mouse spinal cords throughout a time course of TMEV-infection.

Materials and Methods: Female FVB/nJ mice were injected intra-cerebrally with TMEV (experimental) or HBSS (control). Mice were sacrificed on days 21, 35, 45, and 60 post-infection. Spinal cords were isolated and homogenized for protein and RNA isolation. Protein and RNA were used for western blotting and RT qPCR analysis. Whole spinal cords were placed in OCT medium for tissue sectioning. Sections were used for immunofluorescence and immunohistochemistry studies.

Results: Western blots indicate that ErbB2 is increased throughout a time course of TMEV infection. Immunohistochemical staining studies suggest that one cell type expressing ErbB2 is infiltrating immune cells. Double staining studies demonstrate that the cells expressing ErbB2 include CD4+ T-cells, CD8+ T-cells, B-cells, and macrophages.

Conclusion: Positive immunoreactivity for ErbB2 is found in demyelinating lesions in TMEV-infected mice. A subset of T-cells express ErbB2, indicating that GGF's effects are not limited to resident central nervous system cells. Together, these data suggest that GGF may alter the phenotype of infiltrating cells, which are participants in the pathology observed in TMEV-induced demyelination.

Being a mouse in a man's world: what TMEV has taught us about human disease

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1. ABSTRACT

Choosing an appropriate animal model to study a disease is guided by a variety of factors including but not limited to the questions being asked, availability of reagents, knowledge of the animal species, personal biases of the researcher, and in some cases, cost and availability of facilities to effectively investigate the model. The validity of an animal model can be further complicated when the etiology of the disease is incompletely defined. Examples of these diseases include multiple sclerosis (MS) and type 1 diabetes (T1D). In addition to host genetics, epidemiological studies have implicated infectious agents, in particular viruses as triggers of these diseases. Thus many studies of these diseases have focused on modeling the interactions of viruses and the host immune response *in vivo* in small animals. Theiler's murine encephalomyelitis virus (TMEV) infection of mice has been used for over 30 years as a model of virus-induced demyelination. TMEV induces a MS-like disease in susceptible strains of mice but does not cause pathology in humans. While some researchers may question the rationale for using a non-human pathogen to model human disease, the TMEV model of central nervous system (CNS) demyelination has permitted study of some aspects of human MS which would have been difficult to address in other models of the disease. Despite being 'merely a disease of mice,' many of the findings in the Theiler's virus model are directly applicable to the human condition, and studies from the model are responsible for our current understanding of mechanisms of pathology and clinical disability in human MS. In this review we will present some of the key findings from the TMEV model in the context of human disease.

2. AN OVERVIEW OF THE THEILER'S MODEL OF DEMYELINATING DISEASE

Theiler's murine encephalomyelitis virus (TMEV) is a positive-stranded RNA virus belonging to the family Picornaviridae (genus *Cardiovirus*). There are two subgroups of TMEV – GDVII and TO. The GDVII subgroup viruses (FA and GDVII) are highly neurovirulent and cause necrotizing encephalitis and death in the murine host within 7 days of intracerebral (i.c.) infection even at very low doses (1). Intracerebral infection of mice with TO subgroup viruses (DA and BeAn) induces acute encephalitis between days 5 and 10 post-injection (p.i.) in all strains of mice. During this phase of infection virus replicates primarily in neurons and viral titers escalate rapidly (2). Within two weeks of infection, virus is cleared from the gray matter of the brain. In mouse strains resistant to persistent infection (H-2^d, ^b) (3-5) TMEV is effectively cleared from the central nervous system (CNS) by a strong cytolytic T lymphocyte (CTL) response directed against the VP2 viral capsid protein (6,7). These animals recover and experience no obvious functional deficits as a result of the infection. Some strains of mice are unable to efficiently clear virus from the CNS and a persistent infection is established in the white matter of the brain and spinal cord, where demyelination and chronic inflammation develops. The infiltrate is comprised of CD4⁺ T cells, CD8⁺ T cells, B cells, and activated microglia/macrophages (8). Low-levels of virus can be detected in oligodendrocytes (9,10), microglia/macrophages (2,11,12), and astrocytes (10). In the BeAn strain the macrophages are the main reservoir of virus in chronically infected mice (11). Despite the high degree of sequence homology between DA and BeAn (13), the disease observed in DA-infected mice is distinct from

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BeAn-induced disease. DA-infected mice have higher levels of spinal cord demyelination, increased functional deficits, higher levels of virus-specific RNA and protein, and lower levels of TMEV-specific antibody compared to BeAn-infected animals (14).

The initial demyelination in the TMEV model is due to direct viral damage to the myelin-producing oligodendrocyte. Ongoing chronic demyelination is attributed to the development of autoimmune responses to previously sequestered self-epitopes, a phenomenon known as epitope spreading (15-17). Clinical signs of chronic TMEV infection are similar to those observed in patients with chronic progressive MS. These include spasticity, incontinence, weakness of the extremities, and eventually paralysis (2,18). Akin to the oligoclonal bands found in the cerebrospinal fluid (CSF) of MS patients, i.e. infection of susceptible strains of mice with TMEV results in intrathecal antibody production (19).

The wide availability of mutant and knockout mice has permitted the study of the contribution of various immune system components on susceptibility or resistance to demyelination (20-31), neuronal damage (20,21,32-34), and in some cases, death of the host (24,32,35). Mice lacking adaptive immune systems die within two weeks of i.c. infection even when on a genetic background that is resistant to infection (32,35,36). In addition, some immune component deficiencies may result in altered patterns of brain pathology in the acute phases of disease (20,21,32). While Theiler's virus infection of mice has been used primarily as a model of demyelination, TMEV infection of mice has been used to explore many more scientific questions beyond modeling MS. TMEV infection of mice has also been successfully used to model T cell priming and antigen presentation in the CNS (17,37-41), mechanisms of virus transport from the PNS to the CNS (42-44), and understanding mechanisms of myelin damage (45) and repair (46-48).

3. MULTIPLE SCLEROSIS – A SHORT COURSE

Multiple sclerosis is the most common demyelinating disease of the CNS in humans, with more females affected than males (49-51). The disease is focal in nature and the lesions contain inflammatory infiltrates consisting of T cells, B cells, and macrophages (52). The disease course in individuals diagnosed with MS is unpredictable although most individuals experience increasing loss of function as time progresses. Autoreactive T cells have been identified against several myelin components including myelin basic protein (MBP) (53-55) and proteolipid protein (PLP) (53, 56, 57). As most studies have focused on individuals after diagnosis, the contribution of the autoreactive T cells to disease onset is murky.

The etiology of MS is unknown, but both host genetics and environmental factors are likely involved in disease development (58-64). A variety of genetic associations have been made between various HLA alleles and an increased relative risk rate of MS

development (58, 59, 65-68). The first genes implicated in the development of MS were HLA class I alleles – key participants in the immune response to viruses (61), suggesting that the response to intracellular pathogens may be important in disease development. In addition to particular HLA alleles, polymorphisms in other genes that affect immune function have been made (69-76). Despite extensive study, no consensus has been reached with regard to which genes are most important in MS susceptibility. The results of the genetic studies do support a role for multiple genes in determining MS risk.

While the risk of disease development increases when a first degree relative is affected, genetics alone do not adequately explain disease development (77-83). Long-term studies in the Faroe Islands in the decades following World War II and migration studies have supported a role for infectious agents in disease onset (60, 62-64, 84, 85). Since these original epidemiological studies, much work has focused on identification of pathogens that may trigger disease. The most commonly implicated class of pathogens in the development of MS is viruses (86-94). Viruses have been implicated or eliminated as causes of the disease based on their presence or absence in a demyelinating lesion, or the level of virus-specific antibody in the patient at or near diagnosis. Some of the pathogens proposed as triggers of MS include rabies (86,87), human herpes virus 6 (95, 96), measles (96-100), adenovirus (88-89), parainfluenza type 1 (90-93, 101), rhinovirus (102), and Epstein Barr virus (94, 103, 104).

Based on studies in the TMEV model of MS, mechanisms of demyelination that are triggered by virus may or may not require the presence of viral antigen at the lesion site at the time of clinical disease onset. Studies from the laboratory of Stephen Miller have demonstrated that while virus is required to initiate demyelination, it is ultimately the ongoing immune response to newly exposed self-epitopes that is responsible for the chronic, increased levels of demyelination that are observed (15-17). The specificity of the autoreactive cells that develop changes over time in a predictable manner (15-17). Extending these findings to the human disease, one could postulate that while a virus (or viruses) may trigger the disease in humans, it is long-gone by the time of diagnosis.

Diagnosis of MS is difficult, as similar symptoms related to other disease conditions must be considered. Diseases that must be excluded include Lyme Disease, sarcoidosis, vascular disease, syphilis, genetic diseases, or structural conditions such as herniated disks or tumors that may impair nervous system function. Other diagnostic criteria include the presence of oligoclonal bands in the CSF and gadolinium-enhanced plaques in the spinal cord and brain upon magnetic resonance imaging (MRI) (105-107). Currently lesion activity, as defined by MRI visualization, is the key pathological feature of MS both in diagnosis and in monitoring disease progression.

4. AXONAL INJURY IN DEMYELINATING DISEASE. DOES DEMYELINATION REALLY MATTER?

4.1. Axonal injury in multiple sclerosis

Human multiple sclerosis has long been characterized as a primary demyelinating disease. The hallmark pathology of the disease is a loss of myelin, with relative sparing of the axon (108). Despite this categorization, the clinical course of the disease is perplexing because no firm association between the extent of demyelination lesions and patient disability has been described. This lack of correlation between lesion load and clinical disability is referred to as the clinico-radiological paradox (109,110). Until relatively recently, axonal damage has been thought to be a long-term sequelae that results from the assault on the denuded axon by the immune response (108). Increasing evidence however currently suggests that axonal damage occurs significantly earlier than previously thought, and demyelination alone is not the cause of patient disability (108,111-116). Axonal injury results in axonal transection, a condition for which there is no treatment. In the past decade numerous studies have demonstrated that at least in some instances, axonal damage occurs in areas of normal appearing white matter of MS patients (111-114).

To examine whether there were alterations in axonal density in normal appearing white matter in patients with MS, Bjartmer *et al.* examined autopsy tissue from a patient with acute MS. Using immunohistochemical staining with an antibody to neurofilament protein as a marker for axons, these studies demonstrated that there was a decrease in axonal density of approximately 22% in normal appearing white matter in the MS patient as compared to the axonal density in the CNS tissue from individuals with no known neurological pathology (111). In the course of these studies, the authors also demonstrated that despite the significant level of axonal dropout, myelin sheaths devoid of axons were also apparent. These myelin sheaths were either intact or collapsed upon themselves. In addition, macrophages containing myelin debris were also detected, indicating that myelin was also disrupted (111). Similar immunohistochemical studies have confirmed the observation that axonal dropout occurred in normal appearing white matter in autopsy tissue from MS patients. Decreases in axonal density of up to 65% were observed (112-114). The axons at greatest risk were the small axons (113,114). Despite the sex bias in MS patients, no difference in the level of axonal dropout has been reported between the sexes (114).

While these findings were of interest, these studies did not address whether the axonal pathology was old or relatively recent. To address this question, immunohistochemical staining of biopsy tissue from MS patients was performed using amyloid precursor protein (APP) as a marker of acute axonal injury (117). Acute axonal injury was defined as damage that occurred within the last month. The amount of acute axonal injury varied with the subtype of MS in the patient. Patients with primary progressive MS had reduced levels of APP

staining; patients with secondary progressive MS had much higher APP-positive staining than other forms of MS (117). APP was found in areas of demyelination, remyelination, and normal appearing white matter (117) indicating that axons in areas undergoing damage, invoking repair mechanisms, and normal appearing tissue were all vulnerable to axonal damage.

The main focus of treatments used in MS patients is targeted toward reducing lesion activity and relapses as a measure of success (118-122). Given that significant numbers of axons are damaged early on in some forms of MS, the view that clinical sequelae can be prevented if myelin sheaths can be repaired relatively quickly after the onset of demyelination may be naïve and outdated. This is not to say that reducing or repairing demyelination in the treatment of MS is not warranted. As myelination impacts conduction velocities along the axon, reduced myelination most certainly negatively impacts clinical symptoms in the patient. However, it is imperative that alternative measures be used to assess treatment efficacy.

4.2. What has Theiler's virus taught us about demyelination and axonal injury?

The study of chronic infection of mice with Theiler's virus has been used to demonstrate the disparity between the extent of demyelination in the host and the level of clinical disability. Studies from this model first provided an experimental model demonstrating that clinical deficits and demyelination were independent of each other and ultimately providing a potential mechanism explaining the clinico-radiological paradox (123-126). Using beta2 microglobulin-deficient mice on a background resistant to demyelination (C57Bl/6J x 129), Rivera-Quinones *et al.* demonstrated that demyelinating lesions developed following intracerebral infection with Theiler's virus (123). These beta2 microglobulin-deficient mice, devoid of both MHC class I expression and CD8+ T cells, were unable to mount CTL responses (127). Despite the presence of these large areas of demyelination, spontaneous clinical activity and hindlimb evoked potentials in the virus-infected mice were similar to those observed in mice that were infected and efficiently cleared TMEV from the CNS (123). The retention of normal function in the TMEV-infected beta2 microglobulin-deficient mice parallels human cases of asymptomatic MS (128). TMEV-infected beta2 microglobulin-deficient mice also had increased sodium channel levels in the CNS, as well as relatively well-preserved axons, findings that would provide the basis for the normal evoked potentials measured in these animals (123). The development of this model permitted further studies on the role of CD8+ T cell/MHC class I interactions in the development of functional deficits in demyelinating diseases.

The beta2 microglobulin knockout mice have been the focus of intense study since their initial characterization (23, 124-126). One possibility for the observed differences in clinical function between beta2 microglobulin-deficient mice and immunocompetent control mice susceptible to the development of large areas of demyelination and functional deficits was that the two

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strains of mice developed lesions in different areas of the spinal cord. Simply put, the lesions in the beta2 microglobulin-deficient mice, while large, were in areas of the spinal cord that were less critical to motor function. To address this potential mechanism of clinical function preservation in the beta2 microglobulin-deficient mice, geographic distribution of the lesions and the extent of remyelination were examined. Beta2 microglobulin knockout mice and SJL/J mice, a strain of mice that experience significant loss of function following TMEV infection were used in these studies (125). The hypothesis being examined was that the location of the demyelinated lesions was the key determinant as to whether clinical deficits developed. Morphometric studies determined that both the lesion size, geographic distribution of the lesions, and the degree of remyelination in these two strains of mice were similar. Using retrograde neuronal labeling to measure the level of axonal injury, it was determined that compared to beta2 microglobulin-deficient mice, SJL/J mice had reduced retrograde labeling of neurons in the major motor tracts compared to the beta2 microglobulin-deficient mice (125). Together, these studies provide further evidence for the hypothesis that demyelination and axonal damage are independent of each other. These studies also implicate a role for CD8⁺ T cells in impacting axonal health.

4.3. Mechanism of axonal injury in the TMEV model

Key to induction of CTL response are that MHC class I molecules displaying a peptide in the binding cleft that corresponds to an appropriate T cell receptor on a CD8⁺ T cell. Under normal conditions, MHC molecules are not expressed in the brain and spinal cord. However, damage (infection, trauma, physiological) can result in the induction of MHC molecules in the affected area (129, 130). Thus, following damage or infection, CNS resident cells acquire the ability to present antigen to T cells. Increased expression of class I on the demyelinated axons in patients with MS has been reported, thereby demonstrating that one of the requirements for CTL-mediated damage to the axons is fulfilled (131). In studies exploring the interactions between CD8⁺ T cells and neurons in an *in vitro* setting, the hypothesis that cytotoxic T cells were directly responsible for damage to neuritis was tested (132). Murine neurons were pulsed with lymphocytic choriomeningitis virus (LCMV)-derived peptides and then co-cultured with LCMV-specific CD8⁺ T cells. The CD8⁺ T cells attached to the neuritis and within 3 hours, changes to the neurite cytoskeleton consistent with translocation of the neurite were observed (132). No structural abnormalities were observed in neuritis when control peptides or neurons devoid of class I expression were used, indicating that the structural changes were the result of antigen-specific class-I mediated responses (132).

While studies in the beta2 microglobulin-deficient mice demonstrated that demyelination and axonal damage were not interdependent, they did not address the observation in human tissue that there are damaged axons in the normal appearing white matter (111-114). The concept that axonal injury is a sequela to demyelination has also been examined in the TMEV model. Using

nonphosphorylated neurofilament protein as a marker of axonal damage, studies using SJL/J mice demonstrated that axonal injury was detected by one week p.i. with the DA strain of TMEV (133). At this time-point in infection the majority of virus was localized to the neurons. As the infection progressed the number of nonphosphorylated NPP immunoreactive axons increased in the spinal cord. Histologically there was an increase in the amount of axonal swelling in normal appearing white matter over time. These studies were significant as they provided evidence that axonal injury did not occur solely as a secondary event following demyelination (133). TMEV antigens rarely co-localized with axons indicating that direct virus-induced axonal damage was likely. Furthermore, similar to the pathology described in MS, empty myelin sheaths were observed indicative of axonal degeneration (111,133). Using the highly neurovirulent GDVII strain of TMEV, similar studies determined that this strain of TMEV also induced high levels of axonal swelling and degeneration in normal appearing white matter. As GDVII-infected animals do not demyelinate, these data demonstrate the independence of these distinct pathologies.

Two proteins are involved in CTL-mediated killing. Perforin is responsible for the generation of channels on the target cells, and granzymes enter the cell and cause damage to the target cell's DNA. To test the contribution of perforin to axonal degeneration and clinical deficits, perforin-deficient animals on a C57BL/6J background (that is, animals that can mount a vigorous CTL response and clear virus) were infected with TMEV and examined six months later. TMEV-infected perforin-deficient mice developed demyelinating lesions throughout the spinal cord but motor function and large diameter axons remained preserved (126). The levels of function and axonal preservation were similar to those observed in immunocompetent wild-type mice. In contrast, TMEV-infected mice with CD4⁺ T cell deficits experienced similar levels of demyelination as the perforin deficient mice but also experienced a loss of function. In addition, CD4-deficient mice infected with TMEV experienced a loss of large diameter axons (126). In these studies, demyelination alone was not a predictor of clinical function although the extent of loss of large diameter axons could be correlated to clinical disease. Similar to the *in vitro* studies demonstrating the antigen-specific nature of the CTL-mediated damage to the neuritis *in vivo* studies demonstrated that depletion of the VP2 121-130-specific T cells significantly reduces the damage in the TMEV model (124).

Because MS is typically categorized as an autoimmune disease the main focus of study in MS patients, as well as animal models, had been the host immune response. Despite the obvious interest in the immune response, the role of non-immune factors in the establishment of disease has also been of great interest. Recently, the role of myelin in the establishment of TMEV persistence has been explored. In these studies two mouse strains with myelin defects were studied and have presented us with new paradigms to understand the mechanism of viral persistence in this model. *Shiverer* mice have a large

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deletion in the myelin basic protein gene resulting in extremely low levels of myelin production (134-137). Rumpshaker mice have an X-linked mutation in proteolipid protein gene which results in dysmyelination and increased numbers of oligodendrocytes (138). Even when infected with high doses of TMEV, it is not possible to induce a persistent infection in these mouse strains (139). In contrast, infection of wild type control mice with much lower doses of TMEV results in virus persistence and subsequent demyelination. These data cannot be explained solely by the immune response in the context of epitope spreading as the immune response to myelin basic protein is not one of the early identified self-reactivities (16).

To explore the basis of this protection from persistent TMEV infection, the optic nerve was used to model axon, myelin, and virus interactions (45). These studies demonstrated that the axons of infected neurons are a key component in permitting infection of the cytoplasmic channels of myelin. It was postulated that the virus attempts to gain a survival advantage in the host by establishing itself in an environment distal from the demyelinating lesion, which is the main target of the immune response (45). What is the relevance of these findings in the context of human multiple sclerosis? The data, while relatively new, provide a mechanism by which viruses could induce MS in humans, and provide an explanation as to the lack of viruses that have been identified at the lesion site.

5. LESSONS FROM THE ACUTE PHASE OF TMEV INFECTION

All strains of mice, regardless of their genetic background, experience the acute phase of TMEV infection characterized by high levels of virus replication in the neurons (2, 18). This virus-induced encephalitis has permitted the study of the role of immune system components in protection of discrete areas of the brain from TMEV-mediated disease (20, 21, 32-34). It has been observed in viral encephalitis in humans that certain viruses induce distinctive patterns of pathology in the brain. For example, rabies localizes primarily to the pons and medulla, while herpes simplex virus-1 induces disease that is localized to the frontal and temporal lobes (140). While one possibility is that specific patterns of brain disease are related to virus receptor distribution, the host immune response also appears to significantly impact where pathology will occur.

To examine the role of specific immune system components on brain pathology, a series of mice with various immune system participants knocked-out were i.c. infected with TMEV and sacrificed at day 16 p.i. This time-point was chosen as by this time virus has been cleared from the brains of immunocompetent mice that are capable of generating CTL responses sufficient to clear virus from the host. Using mice deficient in MHC class I or II, alpha/beta TCR or antibody, it was demonstrated that class I-mediated immune responses are critical in clearing virus from areas of the brain rich in white matter, while areas abundant in neurons (i.e., gray matter) are protected

primarily by antibody (32). Given that white matter areas profoundly upregulate MHC class I levels following virus insult (141), it is logical that protective responses are induced that exploit this arm of the immune system. As neurons less efficiently upregulate MHC after virus infection, the dependence on antibody-mediated protective responses would be expected. Further studies using mice deficient in other immune systems components (ICAM-1, CD40, IL-6) have supported these initial observations (20, 21, 33, 34).

The continuous stream of knockout mice available to investigators will permit further dissection of immune system components to protection from virus-induced damage. Furthermore, utilization of this approach with different viruses will allow us to determine whether the patterns of brain pathology are unique for TMEV or reflect general patterns for particular classes of viruses.

6. INFECTION OF THE PERIPHERAL NERVOUS SYSTEM WITH TMEV

Peripheral nervous system infection with TMEV is an area of research that has been examined in a minimal number of studies. The natural route of CNS infection with TMEV in the wild is unknown. Because TMEV is transmitted via an oral-fecal route in the wild, it is likely that the CNS infection occurs via the peripheral nervous system or possibly, the blood. A small number of studies have examined the dynamics of virus spread from the peripheral to the central nervous system (42-44). To examine whether TMEV could enter the CNS from the PNS via axonal transport, mice were injected into the footpad with the highly neurovirulent GDVII strain of TMEV (44). Within one week, virus was detected in the spinal cord. Initially paralysis was observed in the injected limb, and subsequently in the contralateral hindlimb. Cholchicine, an inhibitor of fast axonal transport, was used and prevented transport of the virus into the CNS, demonstrating a microtubule-dependent mechanism of transport of TMEV from the periphery to the CNS (44).

More recently, studies were performed that propose a route for infection of the CNS with TMEV in the wild. Injection of either the tongue or the hypoglossal nerve with TMEV resulted in spread of the virus to the CNS as measured by the induction of paralysis (43). The results of the intratongue injections are significant, in that one could envision a scenario in the wild whereby a natural infection could travel to the CNS via a breach in the surface of the tongue, similar to one of the proposed mechanisms of transmission of prion diseases (142).

Our laboratory recently developed a model of direct injection of virus into the sciatic nerve with a goal of using this model to study myelin repair of the peripheral nervous system (42). While it has been well-described that the PNS is more efficient at repair than the CNS, few opportunities exist to directly examine the differences in the processes, as the lesioning methods used in the PNS and CNS vary. Further development of this sciatic nerve model, as well as our model of direct CNS lesioning (143),

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will permit study of these processes without the complication of an additional variable (that is, the method by which the lesion was made).

7. SUMMARY AND PERSPECTIVES

The study of non-human pathogens that are not of agricultural interest is sometimes denigrated by those working with human pathogens (aka, 'my virus is better than your virus'). The concept that one cannot advance the understanding of human disease by studying a mouse virus is, in our view, short-sighted. Certainly, our understanding of axonal damage in multiple sclerosis would not be as advanced as it is without the TMEV model. The ability to utilize a small animal model in concert with human histopathological studies provides investigators with an excellent opportunity to test and understand mechanisms of pathology, and to gain confirmatory data from human samples. Furthermore other aspects of the model, such as the acute phase of disease or infection of the peripheral nervous system provide ample opportunity for further study of human diseases other than multiple sclerosis.

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Abbreviations: CNS: central nervous system; CSF: cerebrospinal fluid; CTL: cytotoxic T lymphocyte; i.c.: intracerebral; MBP: LCMV: lymphocytic choriomeningitis virus; MRI: magnetic resonance imaging; MS: multiple sclerosis; NFP: neurofilament protein; p.i.: post-infection; PLP: proteolipid protein; PNS: peripheral nervous system; TCR: T cell receptor; TMEV: Theiler's murine encephalomyelitis virus

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Mast cells, TLRs, and the complement system: Influence of the innate immune system on demyelination

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Abstract:

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Individuals diagnosed with MS mount strong CD4⁺ T cell responses against central nervous system (CNS) autoantigens. These responses trigger immunological events that result in demyelination and axonal loss. Although the innate immune system is considered to play a less pathogenic role than the adaptive immune system, the innate immune system is imperative for the activation of adaptive immunity. In this review, we discuss how mast cells, toll-like receptors (TLRs), and the complement cascade influence the development of demyelination in the context of disease progression and CNS protection.

Introduction:

Those affected by MS are classified by clinical course: either relapsing-remitting (RR), primary progressive (PP), or secondary progressive (SP)¹⁻⁴. Approximately 85% of MS cases are RR-MS which is characterized by acute attack (relapse) followed by partial recovery (remission)^{2,3}. The majority of RR-MS patients transition to SP-MS and no longer exhibit remission^{2,3}. About 10% of MS cases are PP-MS, which experience no remission at onset. A hallmark of MS is the formation of demyelinating lesions in the white matter of the CNS. These demyelinating lesions are characterized by inflammatory cell infiltrates including: T cells, B cells, and macrophages/microglia⁵⁻⁷. These cells secrete proinflammatory mediators that contribute to the demyelinating response which can eventually lead to substantial disabilities in sensation, motor, autonomic, and neurocognitive function⁸⁻¹⁸.

The etiology of MS remains unclear, but it is hypothesized that the disease develops in genetically susceptible individuals and may require additional environmental triggers^{2, 3, 19}. Several studies have addressed genetic contributions associated with MS. Specifically, certain inherited HLA alleles predispose individuals to the neurodegenerative disease²⁰⁻³⁹. Also, polymorphisms within immune function genes have been implicated to play a role in development of MS⁴⁰⁻⁴⁵.

Many studies have attempted to elucidate the etiology of MS, with a major focus on the adaptive immune response. Autoreactive CD4+ T cell mediated immune responses against myelin components such as myelin basic protein (MBP) and proteolipid protein (PLP) are commonly identified in patients with MS⁴⁶⁻⁵⁷. However, the events prior to activation of adaptive immunity remain poorly understood. The innate immune system has become a topic of great interest within the field of MS research. Understanding events prior to ubiquitous demyelination and axonal loss may permit the development of targeted therapeutics for individuals with genetic predispositions to the disease. In this review we will discuss how mast cells, TLRs, and the complement cascade influence MS pathogenesis.

Mast cells and MS pathology

Mast cells are key mediators of inflammation and are most often studied in the context of allergy and asthma⁵⁸. More recent work has focused on the role mast cells have in the initiation and maintenance of chronic pathological changes in a variety of diseases, including autoimmune diseases such as: type 1 diabetes⁵⁹, pemphigus vulgaris⁶⁰, Graves' disease⁶¹, Guillain-Barre⁶², and MS^{7, 63-65}. In the brain, mast cells reside within the leptomeninges, the choroid plexus, thalamus, and hypothalamus⁶⁶⁻⁶⁹. They are activated by cross-linking with FcεRI:IgE

complexes⁷⁰⁻⁷², by interacting with complement cascade components^{73, 74}, and by interacting with TLRs^{75, 76}. Upon activation, mast cells produce a variety of inflammatory mediators that may affect MS pathogenesis. The breakdown of the blood brain barrier (BBB) precedes any clinical signs or pathology of MS, as demonstrated by MRI-gadolinium studies and trans-BBB leakage of albumin⁷⁷⁻⁷⁹.

Activated mast cells release proinflammatory mediators such as histamine, which can permeabilize the BBB and allow non-resident cells to cross into the immune privileged CNS⁸⁰. Trafficking immune cells into an immune privileged site can cause aberrant processes to ensue, such as priming against CNS specific proteins. In addition to histamine, activated mast cells release a wide-array of soluble mediators such as TNF- α (Figure 1). TNF- α activates endothelial cells, which increases the expression of adhesion molecules and promotes the influx of inflammatory leukocytes and lymphocytes into the CNS⁸¹. Mast cell degranulation can be directly stimulated by cross-linking Fc ϵ RI:IgE with myelin antigen *in vitro*⁸²⁻⁸⁵.

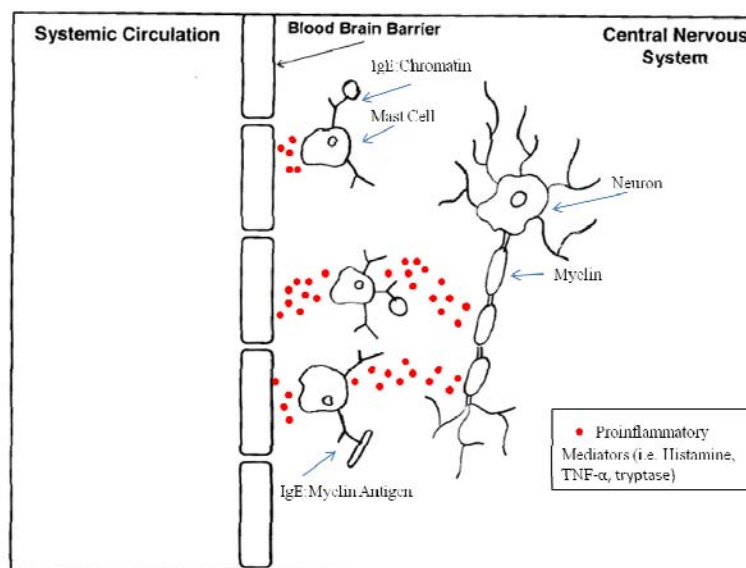


Figure 1: Mast cell degranulation and cytokine secretion can act directly on the blood-brain barrier (BBB) and on myelin. Studies implicate that both IgE:chromatin complexes and IgE:myelin complexes can activate mast cells which produces a proinflammatory response. Proinflammatory mediators can permeabilize the BBB and breakdown myelin, further contributing to MS pathogenesis.

Recent work has demonstrated that mast cells are found both within MS lesions and surrounding the lesions in the normal appearing white matter (NAWM)^{7, 63-65}. Tryptase, a serine protease released in mast cell degranulation, has also been identified in cerebrospinal fluid (CSF) of patients with MS at significantly higher levels compared to healthy patients⁸⁶. Tryptase can activate peripheral mononuclear cells to secrete TNF- α and IL-6 which further contribute to inflammation and BBB permeabilization⁸⁷.

A variety of factors have been examined to define processes that influence mast cell accumulation in demyelinating lesions. RT qPCR data demonstrated increased transcript levels of Fc ϵ RI, chymase, and tryptase in brain tissue taken from MS patients compared to healthy individuals and individuals with other neurological diseases such as amyotrophic sclerosis and Alzheimer's⁷. Mast cell numbers are highest in chronic active lesions compared to active and chronic inactive lesions⁷. This suggests that mast cells not only play a role in disease initiation, but also in disease persistence. Elevated levels of mast cell-associated transcripts were not confined to MS lesions. Tryptase and Fc ϵ RI β -chain transcripts were also elevated in NAWM in MS patients⁷. Studies have indicated that axonal dropout occurs within NAWM in MS patients⁸⁸⁻⁹¹, and the latter data possibly indicates that activated mast cell byproducts contribute to axonal loss.

Studies utilizing experimental autoimmune encephalomyelitis (EAE), demonstrated delayed onset of demyelination in mast cell-deficient mice (W/W^v)⁹²⁻⁹⁴. Clinical disease is less severe in W/W^v mice compared to controls⁹². Cytokine profiles showed similar levels between EAE and controls indicating that differences in severity of clinical disease is not attributed to cytokine production⁹⁵⁻¹⁰⁵. Disease incidence and severity reverts back to that observed in wild

type (WT) mice following reconstitution of the mast cell lineage⁹⁴. These data support a pathological role for mast cells in the demyelinating disease. One study adoptively transferred thymic T cells isolated from W/W^v mice into TCR β -deficient mice. EAE was induced and disease severity was identical to WT mice, indicating that W/W^v T cell populations were intact and functioning¹⁰⁶.

In a follow-up study, W/W^v mice were reconstituted with differentiated GFP-labeled bone marrow-derived mast cells. The GFP-labeled mast cells did not repopulate the CNS⁹⁴. Other studies demonstrated that intracerebral injection of bone marrow-derived mast cells is required to restore the meningeal mast cell population⁹³. These data indicate that CNS-specific mast cells may contribute more to the pathology observed in MS, than mast cells trafficking from the periphery into the CNS.

Sayed *et al.* examined the role meningeal mast cells play in the recruitment of immune cells into the CNS after EAE induction⁹³. Earlier studies determined that Th1 and Th17 T cell subsets existed at similar levels in both W/W^v mice and their WT controls⁹². Immune cell infiltration into the CNS was decreased in W/W^v mice following EAE induction^{92, 93}. No perturbations of the BBB were identified at any time prior to or following EAE induction⁹³. Further studies determined that W/W^v mice express decreased levels of cell adhesion molecules, LFA-1 and VLA-4⁹³. In contrast, WT controls experienced BBB permeability at day 10 post-EAE induction, with maximal permeability at day 15⁹³. While these data indicate that mast cells play a role in lymphocyte recruitment and BBB permeability, other data suggest that mast cell populations existing *outside* of the CNS are responsible for lymphocyte trafficking into the CNS⁷⁸.

Histidine decarboxylase-deficient mast cells were intracerebrally injected into W/W^v mice to test histamine's influence on EAE progression⁹³. EAE pathology was similar to that seen in WT mice⁹³, supporting the previously described data that increased BBB permeability may be due to mast cell degranulation *outside* of the CNS⁷⁸. When TNF- α ^{-/-} mast cells were intracerebrally injected into W/W^v mice following MOG-EAE induction, disease severity decreased⁹³. This data indicates that TNF- α may indirectly facilitate lymphocyte trafficking into the CNS post-EAE induction.

Mast cells could participate in MS pathogenesis in multiple ways: they could (1) release proinflammatory mediators that permeablize the BBB; (2) lead to demyelination via Fc ϵ RI:IgE myelin specific degranulation; (3) activate peripheral mononuclear cells and induce production of TNF- α and IL-6; (4) and lead to axonal degeneration. The exact mechanisms by which these cells are producing these pathological responses are poorly defined, but it is clear that mast cells are responsible for initial stages of disease and BBB permeability.

TLRs and MS pathology

TLRs are widely expressed throughout the CNS, particularly in areas of inflammation¹⁰⁷⁻¹¹³. Various CNS-resident cells express TLRs including microglia^{107, 114-116}, astrocytes^{107, 115, 117, 118}, oligodendrocytes^{107, 109, 119}, and neurons^{110, 120-125}. TLR 3 and TLR 4 are highly expressed within MS lesions, but not in the NAWM surrounding the lesions¹⁰⁷. TLR expression within the CNS targeted studies towards possible MS risk factors associated with polymorphisms within TLR genes¹²⁶⁻¹³¹.

TLR 4 recognizes bacterial lipopolysaccharide (LPS)¹³², an integral pathogen associated molecular pattern (PAMP) on gram negative bacteria. Some studies have shown that *Chlamydia pneumoniae* may contribute to MS pathogenesis through TLR 4 stimulation and immune system activation¹³³⁻¹³⁶. A study utilizing a cohort of Australian MS patients examined the frequency of Asp299Gly and Thr399Ile polymorphisms in the TLR 4 gene compared to healthy controls¹²⁷. No differences in frequency were observed. A different study examined single nucleotide polymorphisms (SNPs) in the TLR 4 gene within a population of Spanish MS patients¹³¹. No differences were seen in any of the 12 SNP haplotypes. A more recent study examined a total of 30 SNPs in TLRs 1-10 and found no association with disease severity or age of diagnosis¹³⁰.

The lack of reported associations between TLR gene polymorphisms and disease development does not negate a role for TLRs in MS pathogenesis. Primary microglia obtained from MS patients were found to express mRNA encoding a wide range of TLRs¹³⁷. Upregulating these TLRs could exacerbate disease through immune system activation and subsequent proinflammatory cytokine production¹³⁸. Such events can damage myelin directly or attract lymphocytes to the lesions. Persistent chronic inflammation in the CNS of MS patients results in release of various intracellular components, some of which stimulate TLR signaling pathways.

Recent studies have determined that high mobility group box chromosomal protein 1 (HMGB1), a nuclear DNA-binding protein, is found in active demyelinating lesions in MS and EAE¹³⁹. HMGB1 can act as a potent proinflammatory signal that interacts through TLR 2 and TLR 4¹³⁹. It has also been determined that proinflammatory cytokine IL-23p19 is expressed by

macrophages and microglia in demyelinating lesions of MS patients¹⁴⁰. IL-23p19 expression is induced through interactions with TLR 2 and TLR 4¹⁴⁰.

Several animal models have been utilized to study TLR contributions to MS pathogenesis^{107, 141-143}. MyD88^{-/-} mice are resistant to MOG₃₅₋₅₅-EAE induction¹⁴¹. Disease resistance is partially due to the inability to generate proinflammatory mediators through NF-κB activation¹⁴⁴. Deficiencies in IL-6 and IL-23 reduce differentiation of Th0 CD4⁺ T cells into Th17 CD4⁺ T cells¹⁴⁴. TLR 9^{-/-} mice demonstrated conflicting results between two studies utilizing EAE. One study showed decreased severity in disease while another study demonstrated increased severity^{141, 144}. These conflicting data may be a result of how EAE was induced.

Further studies generated bone marrow chimerae that limited MyD88 and TLR 9 deficiencies to radioresistant cells. Mice lacking MyD88 in the CNS were resistant to EAE development. Demyelination and axonal loss were decreased in mice lacking TLR 9¹⁴¹. While CpG island motifs are the major PAMPs recognized by TLR 9, one report suggested that chromatin:IgG complexes can trigger TLR 9-mediated signaling¹⁴⁵. Chromatin is released from apoptotic and necrotic cells within lesions, possibly causing aberrant pathological consequences in EAE.

Studies utilizing TLR 4^{-/-} mice detected increased levels of IL-17 in serum isolated post-EAE induction¹⁴⁴. EAE pathology increased in severity. Splenocytes isolated from the knockouts produced increased levels of IL-6 and IL-23, indicative of a more robust Th17 response¹⁴⁴. Excising the TLR 4 gene depleted Th1 T cell subsets¹⁴⁴. Considering cytokine profiles and Th0 differentiation, the absence of Th1 T cells may promote increased Th17 T cell differentiation. This phenotype could lead to increased disease severity.

One study utilized Theiler's murine encephalomyelitis virus (TMEV) which induces a MS-like disease in mice with susceptible genotypes in the H-2 locus. Turrin¹⁴³ detected an upregulation in TLRs 2, 3, 6, 7, 8, and 9 in the CNS of SJL/J mice post-intracerebral infection with TMEV. Considering the viral nature of TMEV, TLR upregulation was expected and inflammatory responses ensued. Studies have also shown that TLRs 3, 7, and 9 can be triggered by nucleotides released from apoptotic cells, causing further advancement in the inflammatory response¹³⁸.

In vitro studies utilizing an immortalized murine macrophage cell line (RAW264.7) demonstrated that TLR 3 and TLR 7 are upregulated following TMEV infection¹⁴⁶. Signaling through TLRs 3 and 7 induced cytokine production that ameliorates (IFN- β via TLR 3) and exacerbates (IL-23 via TLRs 3 and 7) *in vivo* disease pathology¹⁴⁶. Another study infected primary microglial cells with TMEV and they showed increased expression of TLRs 2, 3, 5, and 9¹¹⁶. This study reported an increase in both anti-viral (IFN- α and IFN- β) and proinflammatory (IL-1 β , IL-6, IL-12, TNF- α , and IL-18) cytokine transcript levels post-TMEV infection¹¹⁶. Chemokine production (RANTES, MIP-1 α , MCP-1) and antigen presentation molecules (B7-1, B7-2, ICAM-1, and MHC class I and II) were also upregulated post-TMEV infection¹¹⁶. These data support a role for TLRs expressed by both CNS-resident cells and infiltrating cells in TMEV pathology.

TLRs are expressed on various CNS and peripheral nervous system (PNS) cells and may participate in other mechanisms not associated with immune activation^{147, 148}. TLR 8 is expressed on both neurons and axons^{120, 149, 150}. During murine neurogenesis alterations in TLR 8 expression are seen and expression decreases once brain development is complete. When TLR

8 is stimulated with an agonist it activates apoptotic pathways in neurons and inhibits neurite outgrowth¹⁴⁹, suggesting a role for TLRs in CNS development. TLR 3 also inhibits neurite outgrowth. In these studies, TLR 8 and TLR 3 activated mechanisms independent of the NF- κ B pathways^{120, 149}. These data suggest that expression of particular TLRs may regulate development and/or repair damage in the CNS via non-immune mechanisms.

Further studies into the non-immune functions of TLRs were examined utilizing EAE induction. TLR 8 was detected in axons and often co-localized with SMI32, a marker of damaged axons¹⁴². TLR 8 was expressed relatively early in lesion development, supporting the hypothesis that TLR 8 is a mediator of axonal damage in EAE¹⁴². Several studies have shown that there is significant axonal dropout in the NAWM of MS patients^{2, 3, 46-53}. This finding possibly indicates that TLR 8 is partially responsible for axonal dropout in the NAWM of MS patients.

TLRs are proposed to contribute to MS pathogenesis in a variety of ways: (1) TLR 2 and TLR4 interact with HMGB1 which can induce expression of various proinflammatory cytokines such as IL-23p19; (2) TLR 9 recognizes products released from apoptotic cells such as chromatin:IgG complexes which contribute to proinflammatory processes; (3) TLRs 3, 7, and 9 can be activated by nucleotides released from apoptotic cells; and (4) TLR 8 is associated with axonal damage. TLRs are necessary for innate immune responses and are key linkers to the adaptive immune system. Future studies will further elucidate their function in MS induction and pathogenesis.

The complement system and MS pathology

The complement system is a key mediator of inflammation. It provides a link between the innate immune system (MBL and alternative pathways) and the adaptive immune system (classical pathway). Complement clears bacteria and viruses from the host by enhancing phagocytosis, generating anaphylaxis, and signaling pathogen lysis. The complement cascade is highly regulated; uncontrolled activation can result in serious tissue damage and can be fatal to the host¹⁵¹. Head trauma injuries and CNS infections can activate the complement cascade¹⁵¹⁻¹⁶². Complement components have also been found in various neurodegenerative diseases such as Alzheimer's^{152-154, 157, 163} and neuromyelitis optica^{157, 164}. Numerous studies have examined how complement contributes to MS pathogenesis^{159-162, 165-169}. Complement proteins are found in MS lesions, but the pathological role they play in demyelination is poorly understood.

Studies have characterized MS lesions based on plaque geography and infiltrating cell phenotypes. These studies identified four fundamentally different patterns of demyelination. Patterns I and II share similar features where active demyelination is associated with T cell and macrophage-dominated inflammation¹⁶⁵. The features distinguishing the two patterns are the prominent deposition of IgG and complement C9neo in active lesions found exclusively in Pattern II. Patterns III and IV are characterized by oligodendrocyte dystrophy, reminiscent of viral or toxin-induced demyelination^{159, 165}. The presence of complement components within active demyelinating lesions was initially indicative of complement-mediated lysis of CNS-specific proteins. More recent studies have discovered that complement deposition is a product of plaque evolution rather than plaque heterogeneity between patients^{160, 161, 166}. Complement activation in MS is dependent on lesion formation. C3d and C4d were present on myelin sheaths, while C3d, C1q, and C5b-9 were found on degraded myelin and within macrophages/microglia^{160, 168, 170}. Additionally, complement may be involved in remyelination.

Relapsing-Remitting MS (RR-MS) lesions generate distinct areas of remyelination which may be enhanced by complement-mediated debris removal¹⁶⁵.

One study suggested that the lack of remyelination in the other subtypes of MS is due to the loss of oligodendrocytes¹⁶⁵. Patients with RR-MS were examined ~24 hours prior to relapse. The study determined that oligodendrocyte death occurs before complement and immunoglobulin deposition in lesions¹⁶¹. Other studies found significant oligodendrocyte apoptosis in primary-progressive MS, but complement deposition was minimal¹⁶⁵. Some data suggests that assembly of the membrane attack complex (MAC) on oligodendrocytes actually inhibits oligodendrocyte apoptosis¹⁷¹⁻¹⁷³. Minimal oligodendrocyte death is seen in RR-MS, which is consistent with the previously described data¹⁶⁵. These data suggest that oligodendrocyte death is not complement-mediated in human MS pathology.

Two recent studies examined C4 levels in serum isolated from MS patients. One study found increased levels of C4a in plasma and in CSF compared to healthy controls. C4a was elevated in patients with RR-MS during relapse compared to remitting periods¹⁷⁴. In a similar study, total C4 levels were within normal ranges in MS patients. Analogous to the previous study, C4a levels were elevated in patients with RR-MS experiencing relapse¹⁷⁵. A key diagnostic feature of MS is the presence of oligoclonal bands (via immunoblotting) in the CSF, which are indicative of intrathecal antibody production^{176, 177}. The presence of both IgG and cleaved C4 in CSF of patients with RR-MS is likely due to classical activation.

Almost half of MS patients are unresponsive to corticosteroids, but these patients are responsive to plasma exchange therapy^{178, 179}. Based on the latter clinical results, new studies focused on why plasma exchange was successful. Following plasma exchange, patients with

pattern II lesions were more likely to experience neurological improvement compared to patients developing pattern I, III, or IV lesions¹⁷⁸. All patients in the study began with similar clinical symptoms. Following treatment, all patients with pattern II lesions experienced marked clinical improvement. Patients with pattern I or III lesions experienced no clinical improvement following treatment¹⁷⁸. Pattern II lesions are characterized by the presence of both immunoglobulin and complement within demyelinating lesions¹⁶⁵. After plasma exchange, these proteins are expected to be significantly decreased. It must once again be noted that the Patterns are representative of plaque evolution, not heterogeneity between patients.

Studies utilizing animal models of MS further support complement-mediated demyelination. Following EAE induction, C3^{-/-} mice experienced reduced disease severity compared to controls^{180, 181}. This data suggests that activation of one or more complement pathways is necessary to enhance disease in mice. Other studies have shown no effect on disease severity in C3^{-/-}^{123, 182}. These conflicting results are most likely due to differences in EAE-induction methodology and/or age and strain differences. Another study deleted C3 convertase regulator complement receptor 1-related protein y (Crry) from C57BL/6 mice¹⁸³. Crry regulates complement mediated priming of microglial cells. They showed that Crry^{-/-} experienced accelerated and exacerbated disease post-EAE induction¹⁸³. This data indicates that complement-mediated microglial priming confers susceptibility to other challenges in EAE.

Similar studies utilized animals that have been transiently depleted of complement¹⁸⁴. Administration of cobra venom factor (CVF) transiently depletes complement by stabilizing C3 convertase¹⁸⁴. C3 convertase catalyzes proteolytic cleavage of C3 which further activates other components of the complement cascade. In the presence of CVF, complement cascades cannot

be inhibited and complement is eventually depleted. EAE induction in SJL/J mice and in C57BL/6 mice 2 days post-CVF administration resulted in decreased disease severity¹⁸⁴. Further studies utilizing CVF-treated mice determined that CD4⁺ T cell infiltration into the CNS decreased, T cell responses against MOG diminished, and antibodies against MOG were reduced^{185, 186}. These data suggest that complement depletion may ameliorate disease severity in EAE via mechanisms that reduce inflammation.

The complement cascade is an important mediator of innate immunity. Its effects on MS pathogenesis are being defined, but to date researchers have determined that: (1) C9neo is found in Pattern II demyelinating lesions; (2) MAC formation on oligodendrocytes may prevent oligodendrocyte apoptosis; (3) C4a levels are elevated in RR-MS patients undergoing relapse; (4) C3^{-/-} mice experience reduced EAE severity; and (5) mice transiently depleted of complement experience decreased EAE severity. These data indicate that complement is associated with MS pathogenesis, but further studies must be performed to identify more specific functions.

Conclusion

The adaptive immune response is a critical component of MS pathogenesis. Studies have focused on the autoimmune aspects of disease, neglecting the innate immune properties that aid in disease initiation. The increasing availability of immune-specific genetically engineered animal models have provided insight into elements involved in disease initiation and progression. Studying and defining early immunological events of MS pathogenesis may provide a better prognosis for the patient. Further studies must continue to draw parallels between animal models of MS and human disease which will hopefully push development of novel therapeutics.

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Development of Gait Alterations in Immunocompetent Mice
Resistant to TMEV-Induced Demyelination

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Abstract

Intracerebral infection of immunocompetent mice with Theiler's murine encephalomyelitis virus, Daniel's strain (TMEV-DA) induces an acute encephalitis characterized by high levels of TMEV-DA replication in the gray matter of the brain and apoptosis of central nervous system (CNS) resident cells. Despite the occurrence of these disease processes, minimal work has been done to assess the long-term consequences of TMEV-DA induced encephalitis in mice resistant to TMEV-induced demyelination. We intracerebrally (i.c.) infected female C57BL/6J mice, a strain of mice resistant to chronic TMEV infection and demyelination, and tested the animals for gait abnormalities using the CatWalk Automated Gait Analysis System. We identified deficits in the gaits of TMEV-infected animals compared to mock-infected, age- and sex-matched control mice. The sum of the gait parameters affected by TMEV injection indicate that the observed changes in gait were not the result of impaired coordination, as no changes were detected in parameters associated with an unstable gait, but rather factors related to dynamic gait parameters including walking speed. These studies represent the first characterization of gait deficits in this model resulting solely from the acute encephalitogenic phase of TMEV infection.

Findings

Intracerebral (i.c.) inoculation of mice with Theiler's murine encephalomyelitis virus (TMEV) serves as a model of virus-induced encephalitis in the acute phase of infection (10;12;15;16;28;35), while the chronic, persistent infection with TMEV has been used as a well-characterized model of multiple sclerosis (MS) (5;6;10;13;28;33). In all strains of immunocompetent mice, i.c. inoculation with TMEV, Daniel's strain (TMEV-DA) induces an acute encephalitic disease due to the high levels of virus replication in the neurons for five to ten days after inoculation. In immunocompetent mice, virus is cleared from the central nervous system (CNS) gray matter within 16 days post-infection (p.i.) (28). Some strains of mice then experience demyelination of the CNS white matter. This demyelination is initially the result of viral insult to the CNS. The chronic ongoing demyelination is due to the development of an autoimmune response to newly exposed CNS epitopes (33;34). The resultant pathology is similar to that observed in humans with chronic MS. This susceptibility of a mouse strain to demyelination is primarily determined by the H-2D locus of the MHC (3;38;39;41), although other genes are known to influence susceptibility to demyelination (4-6;26;40).

Clinical deficits often occur as a sequeale to encephalitis in humans infected with a variety of viruses (2;37;43;44). Despite the existence of well-described complications resulting from virus infection of the brain parenchyma in humans, the clinical impact of the acute phase of TMEV infection has not been well characterized. Recent work by one group has described the development of seizure activity in C57BL/6 mice (a strain of mice resistant to demyelination but susceptible to the acute encephalitis) following TMEV infection (23;27;45;46), and described both short-term (27;45) and long-term (45;46) effects of TMEV infection on seizure activity. One study analyzed motor function

in TMEV-infected C57BL/6J mice using the rotarod to test motor function during the early phases of disease (days 5-13 post injection). No significant differences in the ability of TMEV- versus mock-injected mice to perform on a rotarod were detected. Of interest, however, was that the righting reflex was impaired in TMEV-infected compared to mock-infected mice, regardless of whether the mice experienced seizures (27).

A small number of studies have quantitated clinical disease in TMEV-infected mice using non-subjective measures of clinical defects including footprint (30;31;35;48) and rotarod (1;30;51) analysis. While both methodologies provide objective clinical data, each of these methodologies measures a unique aspect of an animal's gait. Footprint analysis primarily measures static gait parameters such as print and stride length, as well as the animal's base of support (9;24). Dynamic gait measures (for example, the speed of an animal over a given distance or the speed at which each individual limb moves) cannot be assessed using footprint analysis. Rotarods measure an animal's coordination and endurance using a negative feedback system (*i.e.*, mild electrical shock) but do not assess an animal's normal gait (7;25). The development of an automated gait-analysis system such as the CatWalk Gait Analysis System (Noldus; Leesburg, VA), permits simultaneous acquisition and assessment of a large number of both dynamic gait and static stance parameters, as well as other functional parameters including interlimb coordination (11;20;50).

We utilized the CatWalk Gait Analysis System to determine whether a wide array of gait parameters was altered in mice after clearance of TMEV from the CNS gray matter. We hypothesized that clinical deficits would result from acute virus replication in the neurons and these deficits might be detectable using a sensitive measure of clinical function. Female C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were

inoculated via the i.c. route with 2×10^5 plaque-forming units (pfu) of TMEV-DA (29) in a volume of 10 μ l as previously described (12;13). The C57BL/6J strain of mice are susceptible to the encephalitic, but not demyelinating, phase of TMEV-induced disease (42). All animal procedures were approved by the Creighton University Institutional Animal Care and Use Committee and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

To assess the extent of viral replication in the brain during the encephalitic phase of TMEV-induced disease, mice were killed at various time points during the first week after virus infection using an intraperitoneal (i.p.) injection of sodium pentobarbital (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). Using previously published methodology (14), immunofluorescent staining for virus was performed using a rabbit polyclonal antiserum to TMEV (Lampire Biological Laboratories, Pipersville, PA) and an Alexa-Fluor 568-labeled goat anti-rabbit IgG (Invitrogen; Carlsbad, CA) secondary antibody for detection. As shown previously (12;16;27;46), high levels of TMEV-positive cells were identified in the brains of TMEV-inoculated mice by 3 days p.i. High levels of immunoreactivity were found in the hippocampus (Fig. 1A), an area of the brain rich in neurons. By day 17 p.i., TMEV was no longer detected in the brains (Fig. 1B) using immunofluorescent staining. No immunoreactivity to the TMEV antiserum was observed in mock-infected control mice (data not shown). Consistent with the immunostaining data, apoptotic cells were identified in the brains of TMEV-infected mice early in infection using the TUNEL assay (ApopTag; Millipore; Billerica, MA) (Fig. 1C). By day 17 p.i., apoptotic cells were no longer detected in the brains of TMEV-infected mice (Fig 1D). Apoptosis was not detected in the brains of mock-infected control mice at any time point (data not shown).

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121 To quantitate the levels of TMEV in distinct areas of the brain early in infection,
 122 we performed real-time RT-PCR using the SYBR Green methodology (Qiagen, Valencia,
 123 CA) with primers specific for the TMEV genome. GAPDH was used as a housekeeping
 124 gene to standardize the reactions. We dissected out the hippocampus (predominantly
 125 gray matter) and brainstem (predominantly white matter) from TMEV-infected and
 126 control mice and analyzed these regions separately. We chose these areas of the brain,
 127 as the hippocampus is a known site of TMEV replication in the early phases of disease
 128 (12;16;27;46) in all strains of mice, and the brainstem represents a region of the brain
 129 known to be positive for TMEV during the later phase of disease in animals susceptible
 130 to demyelination (12;28). Total RNA was isolated (Trizol; Invitrogen/Life Technologies;
 131 Gaithersburg, MD) using standard methodology (14), and quantitative RT-PCR for was
 132 performed using the following primers: TMEV forward : 5'-GTCTAAGCCGCTCGG
 133 AATA (47); TMEV reverse: 5'-ATCTTATCCACGTGGCTTTTG (47); GAPDH forward :
 134 5'-GTGGCAAAGTGGAGATTGTTG; GAPDH reverse: 5'-CATTCTCGGCCTTGACTGTG.
 135 No PCR product ($C_T > 40$) was detected when GAPDH primers were used with samples
 136 that did not undergo reverse transcription. ABI 7000 SDS 7.1 software (Applied
 137 Biosystems, Foster City, CA) was used to analyze the data using the comparative $\Delta\Delta C_T$
 138 method (22;32). At day 3 p.i., the majority of viral RNA was localized in the
 139 hippocampus (Fig. 1E); viral RNA levels peaked at day 5 p.i. and then began to subside
 140 over time (Fig. 1E). The levels of TMEV RNA in the brainstem increased over the first
 141 seven days of infection, but did not reach the levels of virus observed in the
 142 hippocampus ($2.2 \pm 0.4 \times 10^3$ pfus/100 ng hippocampus versus 303 ± 58.9 pfus/100 ng
 143 brainstem) (Fig. 1F). Viral RNA was not detected in the hippocampus or brainstem of
 144 mock-infected control mice ($\Delta C_T > 40$; data not shown). Together, the immunostaining

and RT-PCR data demonstrate that TMEV-DA replicated to high levels in the hippocampus in the first week after i.c. injection with TMEV, with less robust replication in the brainstem (12). Furthermore, during the acute encephalitis (days 3-7 p.i.) induced by TMEV infection, apoptosis was evident within the hippocampus of the infected mice (Fig. 1C), but not in the brains of the mock-infected control mice (data not shown).

To determine whether TMEV-induced encephalitis affected clinical function in C57BL/6J mice, we utilized the CatWalk Gait Analysis System to test clinical function in infected mice on a weekly basis for the first five weeks after infection. This instrument has been used to assess gait alterations in a variety of disease models including Parkinson's disease (49), sciatic nerve injury (11), allodynia (17), and ataxia (8). The CatWalk Gait Analysis System consists of an illuminated glass walkway with a camera positioned at a fixed point underneath the glass walkway. Images are captured continuously as the animal walks across the walkway. Data were collected in a darkened room and analyzed using Noldus CatWalk software (version 7.0; Noldus). The CatWalk exploits the mice's normal exploratory behavior we did not find it necessary to fast the animals prior to performing gait analysis, nor were food rewards necessary to entice the animals to cross the walkway. Age- and sex-matched mock (HBSS)-infected mice were used as controls in these studies. At each time-point, animals performed 5 complete crossings of the CatWalk per day for 3 consecutive days (total of 15 runs per animal). A complete crossing was defined as the mouse walking across a predefined region of the walkway without pausing. A minimum of five steps were measured for each crossing. Mice were killed at each time-point by an i.p. overdosage of sodium pentobarbitol.

A previous study demonstrated that stride length was shorter in SJL/J mice (susceptible to demyelination) previously inoculated with TMEV compared to uninoculated control mice (30). We hypothesized that mice resistant to demyelination that had been infected with TMEV-DA would take longer to cross the walkway than uninfected control mice. By 3 weeks p.i., the walkway crossing time for TMEV-injected mice increased by 55 % compared to age-matched, mock-infected control mice (Fig. 2A). These changes in crossing times were maintained throughout the five-week study. As the crossing times were stably maintained and did not increase over the course of the study, this suggested that there was no ongoing (progressive and increasing) damage following the initial viral insult to the brain.

Increased crossing time can be attributed to a variety of locomoter parameters including a shortened stride length. The stride length of the infected mice was not altered as compared to the stride length of the mock-infected control mice (data not shown). Two additional components that contribute to the speed at which an animal moves its limbs during the step sequence are the duty cycle and the contact time. The duty cycle is a measure of the percentage of time during the step cycle that a particular foot is in contact with the walkway. This gait parameter has been shown to be affected in some models of CNS lesioning (19). The duty cycle in both of the hind limbs of TMEV-infected mice in the present study was increased ($p \leq 0.05$) compared to the duty cycle in control mice beginning at week 2 p.i. (data not shown). These changes were observed throughout the course of the study, indicating that the altered duty cycle was not solely a function of acute virus replication and that the effects of this burst of virus replication persist beyond the encephalitic phase of the disease. In keeping with the increased duty cycle time, the initial contact time of the paws with the walkway were also significantly ($p \leq 0.05$) increased by 2 weeks p.i. By the end of week 5, contact times

increased by $50.1 \pm 6.3\%$ and $47.4 \pm 5.1\%$ in the right and left front paws, respectively as compared to the contact times recorded for the age and sex-matched control mice ($p \leq 0.05$). For the rear paws, similar alterations were observed, with increases in the initial contact times of the right ($46.0 \pm 5.2\%$ increase) and left ($46.8 \pm 3.6\%$ increase) initial contact times as compared to the contact times of the control-infected mice ($p \leq 0.05$). Representative time course data are shown for the right hind paws of control and TMEV-injected C57BL/6J mice (Fig. 2B). The maximal contact times of both front and rear paws were also increased in TMEV-infected versus control-treated mice ($p \leq 0.05$). Time course data for the right front and hind paws are shown (Figs. 2C,D). Similar data were obtained for the left front and hind paws (data not shown). At week 5 p.i., the mean maximal contact time of the left front paw was 0.985 ± 0.0848 sec in the control mice compared with 2.358 ± 0.105 sec in the TMEV-injected mice. Similarly, the final mean maximal contact time in the left hind paw was 1.106 ± 0.08950 sec in the control animals. The maximal contact time increased to 2.722 ± 0.08861 sec in the virus injected mice. An animal's gait is typically divided into three phases – the brake, propulsion, and swing (36). Together our data thus far demonstrate that infection of C57BL/6J mice with TMEV-DA induce alterations in both the brake and propulsion. The changes in gait parameters examined thus far were consistent with the observation that TMEV-injected C57BL/6J mice walk significantly more slowly across the walkway than mock-injected mice.

The swing is the final phase of the gait. To analyze the affects of TMEV infection on this segment of the gait, the swing duration, defined as the number of seconds between two consecutive initial contacts of the paw with the walkway, and the swing speed were assessed. Both swing duration and speed were affected in the hind limbs of TMEV-infected mice relative to controls ($p \leq 0.05$). The hind swing duration increased from 0.104 ± 0.002 seconds (control mice) to 0.119 ± 0.003 seconds (infected mice) on

the left side and from 0.106 ± 0.00322 seconds (control mice) to 0.113 ± 0.00416 seconds (infected mice) on the right side by the end of the study. Congruent with these data, the swing speed of the hind limbs of the TMEV-infected mice also decreased relative to the controls ($p \leq 0.05$). The mean speed decreased from 0.536 ± 0.0198 m/s (controls) to 0.452 ± 0.0256 m/s ($p \leq 0.05$) on the left side. The mean for the right hind limb paw swing speed decreased from 0.526 ± 0.0301 m/s in the controls to 0.465 ± 0.0389 m/s in the TMEV-injected animals. The bilateral changes in swing speed indicate that damage due to viral replication does not preferentially damage (or preserve) the function on the side of the CNS either ipsilateral or bilateral to the injection site.

Because a mouse's level of coordination can impact the speed at which it walks as well as its foot placement, we therefore analyzed different gait parameters that measure an animals' degree of coordination. We measured the regulatory index (RI), an indication of interlimb coordination which reflects the normal step sequence. No significant differences in the RI of control and TMEV-injected mice were observed over the course of the study. The RIs of control and TMEV-injected mice were 96.47 ± 0.6355 and 93.01 ± 1.679 respectively at 5 weeks p.i. ($p \leq 0.05$). Additionally, the base of support (a measure of the distance between the two front or two rear limbs) of the front and the rear paws was unchanged in the TMEV-injected mice compared to the control mice (data not shown). It would be expected that the base of support would be altered to compensate for the instability if an animal were experiencing an unstable gait. Together, these data do not support a hypothesis that animals which experience TMEV-induced encephalitis, have impaired coordination compared to control animals.

Another parameter that was assessed was the print area, defined as the surface area of a complete footprint; this characteristic is affected in some models of CNS injury

(20) due to an animal compensating for an injury, which may be manifested by dragging a limb or altering their paw placement and/or shifting their weight on a particular paw. The print areas of all four paws were significantly increased in TMEV-infected mice in the current study ($p \leq 0.05$). The increase in print size ranged from $7.2 \pm 0.42\%$ (left front) to $12.2 \pm 0.52\%$ (right hind). Print size increased $9.5 \pm 0.54\%$ and $9.8 \pm 0.61\%$ in the right front and left hind paws, respectively. These modest changes in paw size cannot be attributed to aging of the animals, as age-matched controls were used as a comparison. The observation that the print areas increased by similar amounts for all four paws, does not support the hypothesis that mice have altered weight-bearing capacities [as observed in some models of CNS damage (18) or pain (36)]. Rather, the data suggest that animals which have experienced TMEV-induced encephalitis have equal alterations in the weight-bearing abilities of all four paws. This conclusion is further supported by the results of analyzing the stand (the time that a paw is in contact with the walkway) in the infected mice. By the end of the study, the contact time of the left front paws of virus-infected mice increased by $38.4 \pm 5.7\%$ and the right front paws increased by $38.4 \pm 5.3\%$. The increase in the stand of the right and left hind paws was $45.6 \pm 4.7\%$ and $44.7 \pm 5.2\%$, respectively ($p \leq 0.05$).

In summary, inoculation of C57BL/6J mice with TMEV-DA results in measurable clinical deficits as defined using the CatWalk Automated Gait Analysis System. Speed of movements, but not coordination, was impaired. The data presented herein support the presence of a stable gait in TMEV-infected C57BL/6J mice. Gait parameters usually altered in order to compensate for an unstable gait include the base of support (unaffected, data not shown) and decreased swing duration (in our studies, swing duration was increased) (21). Furthermore, the regulatory index and step pattern were unaffected by TMEV replication in the brain. These studies define, for the first time, the

gait deficits resulting from TMEV infection of immunocompetent mice in the absence of demyelination, and provide a basis for further delineating the defects due to the encephalitic versus demyelinating phases of TMEV infection in mice susceptible to demyelination.

List of abbreviations

CNS: central nervous system; DA: Daniels strain of TMEV; i.c.: intracerebral; MS: multiple sclerosis; pfu: plaque forming unit; p.i.: post-infection; TMEV: Theiler's murine encephalomyelitis virus

Competing Interests

The authors have no competing interests to declare.

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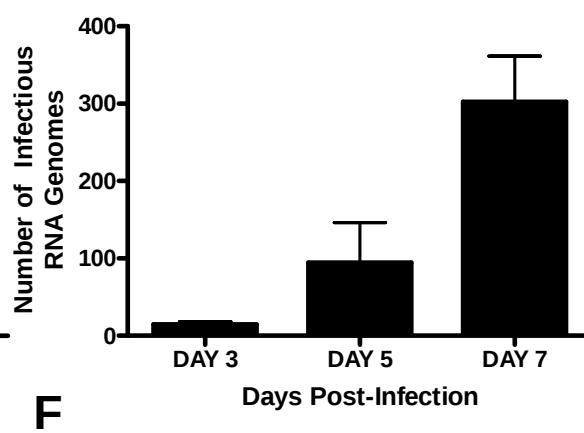
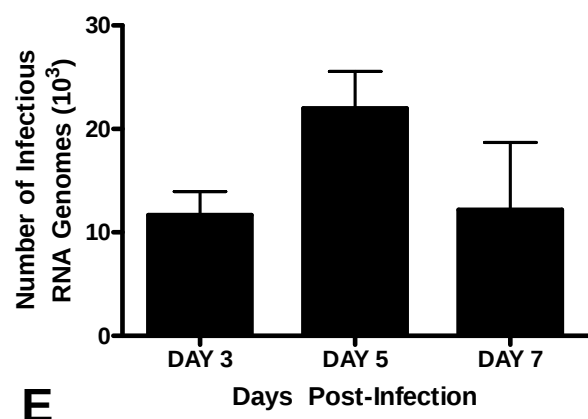
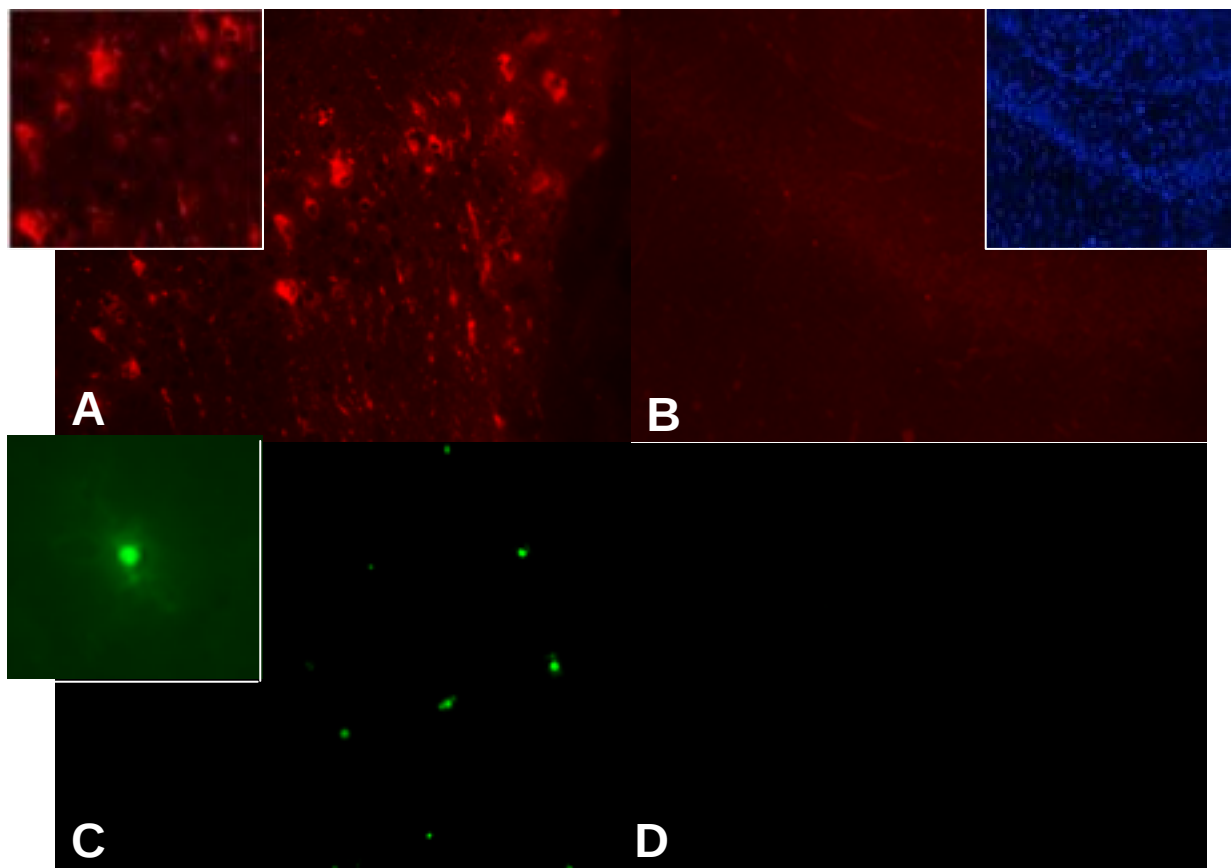
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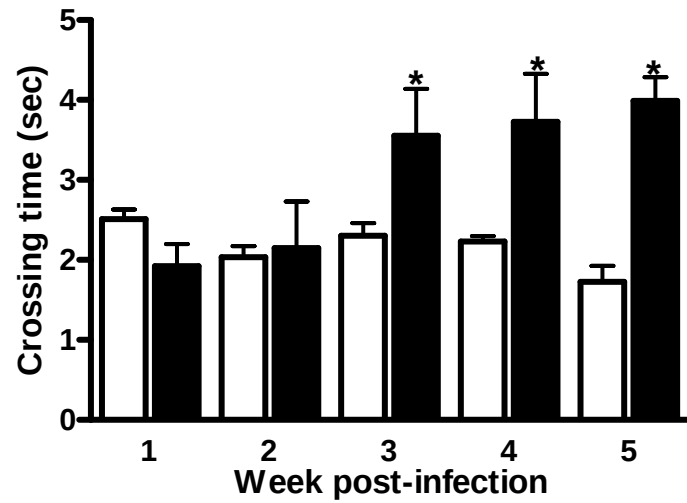
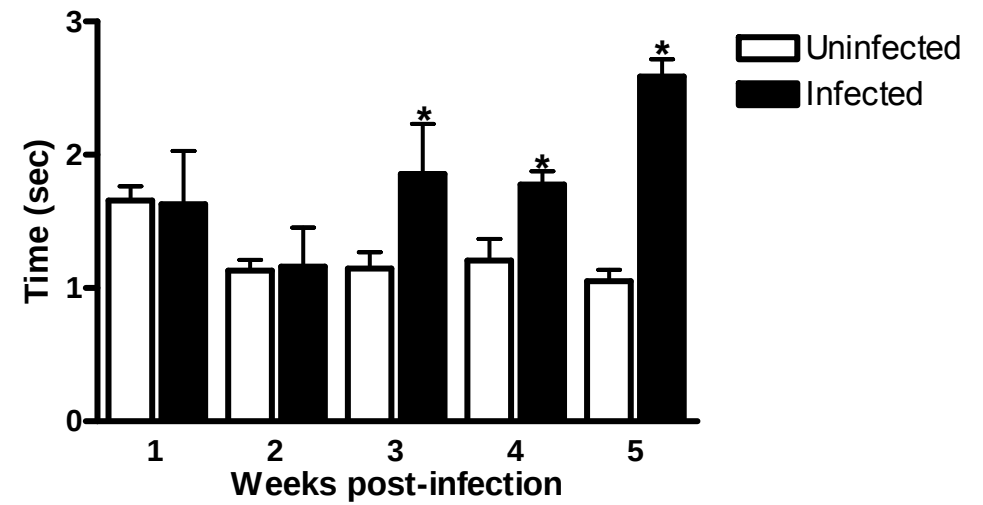
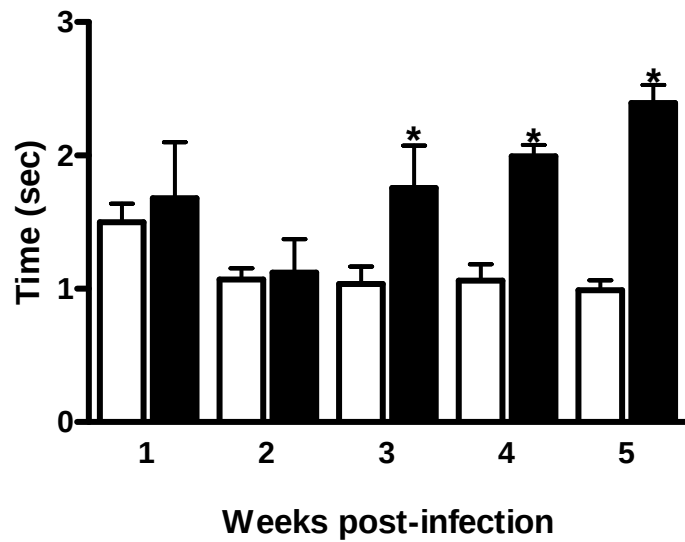
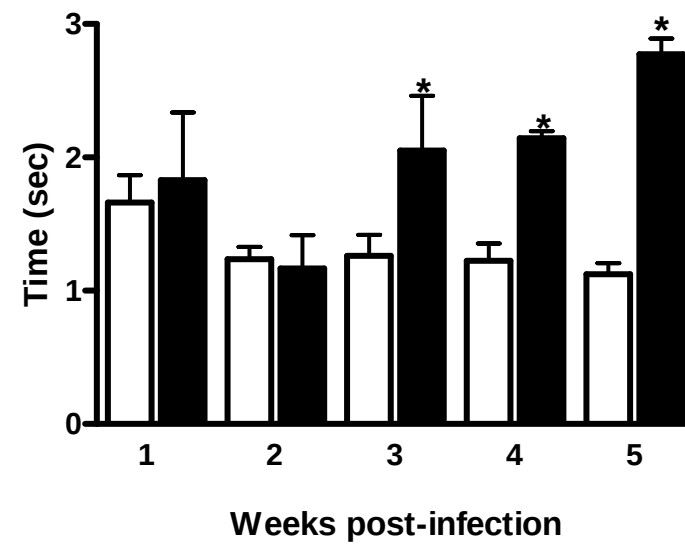
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Figure Legends

Figure 1. TMEV replicates to high levels in the CNS gray matter during the acute encephalitis phase of the disease. A) Using a polyclonal antisera to TMEV and an Alexa Fluor-labeled secondary antibody, large amounts of immunoreactivity to viral antigen were observed in the hippocampus of C57BL/6J mice at day 3 p.i. Positive staining is indicated by the red color. B) Immunostaining of the hippocampus of a C57BL/6J mouse using a polyclonal antisera to TMEV at day 17 p.i. detected no positive staining. The inset shows the DAPI-stained image represented in the larger photo. C) Apoptotic cells (green) were also observed in the hippocampus of mice early in infection (day 3 p.i.). D) By day 17 p.i., apoptotic cells could no longer be detected in the hippocampus. E-F) High levels of TMEV mRNA were localized to the gray matter of the brain in the first week after infection with TMEV. The hippocampus and brainstem were dissected out of the brain and quantitative RT-PCR performed. E) Large amounts of TMEV-specific mRNA were detected in the hippocampus over the first week of infection. F) Low levels of TMEV-specific RNA were detected in the brainstem of mice over the first week of infection.

Figure 2. Impact of TMEV infection on gait parameters in C57BL/6J mice. A) The time required for TMEV-infected C57BL/6J mice to cross the CatWalk was increased compared to that observed in age-matched control mice. B) The initial contact time of the right hind paw was similarly increased in the TMEV-infected C57BL/6J mice. The maximal contact time of both the right front (C) and right hind (D) paws was increased in TMEV-infected mice.



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5 ErbB2 expression increases in Theiler's murine encephalomyelitis virus-infected FVB/nJ mice

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Abstract:

Previous studies, using the *Catwalk Automated Gait Analysis System*, demonstrated improved motor function in Theiler's murine encephalomyelitis virus (TMEV)-infected female FVB/nJ mice treated with an adenovirus vector expressing a cDNA clone of human glial growth factor (GGF). GGF is a splice variant of the neuregulin-1 (NRG1) gene. Neuregulins (NRGs) are a family of epidermal growth factors that are necessary for nervous system development. Isoforms of the NRG1 gene interact with epidermal growth factor receptors EGFR, ErbB2, ErbB3, and ErbB4. ErbB3 and ErbB4 bind these proteins with high affinity. In order to transduce an effective signal ErbB3 or ErbB4 must heterodimerize with EGFR or ErbB2 after ligand is bound. ErbB2 is implicated to be the primary receptor with which ErbB3 and ErbB4 heterodimerize. Our goal was to determine the level and distribution of EGFR and ErbB2 in female FVB/nJ mouse spinal cords throughout a time course of TMEV-infection. Positive immunoreactivity for ErbB2 was found in demyelinating lesions in TMEV-infected mice. Macrophages, B cells, CD4⁺ T cells, and CD8⁺ T cells express ErbB2, indicating that GGF's effects are not limited to resident central nervous system cells. Together, these data suggest that GGF may alter the phenotype of infiltrating cells, which are participants in the pathology observed in TMEV-induced demyelination.

Introduction

Multiple sclerosis (MS) is defined as an inflammatory demyelinating disease of the central nervous system (CNS). The etiology of the disease is unknown, but it is hypothesized that environmental factors play a role in the onset of MS. Viral infections are proposed environmental factors that may induce MS through bystander activation of autoreactive immune cells^{1,2}. An inflammatory response initiated by a viral infection in the CNS can lead to the breakdown of immune privileged sites, allowing autoreactive immune cells to become primed against CNS-specific proteins^{3,4}. Subsequent demyelination can lead to deficits in motor, sensory, and neurocognitive function.

Theiler's murine encephalomyelitis virus (TMEV) is an extensively studied mouse-specific virus that induces a MS-like disease in susceptible strains of mice^{5,6}. Mice with susceptible genotypes (H-2^{f,p,q,r,s,v})⁷⁻⁹ develop a biphasic disease characterized by acute and chronic phases when the virus is injected intracerebrally¹⁰. Rapid virus replication in the gray matter of the brain produces an encephalomyelitis approximately 1 week post-infection. Approximately 2 weeks post-infection, the virus progresses into the white matter of the spinal cord. By 21 days post-infection, axonal degeneration and inflammatory demyelination is observed in the cervical and upper thoracic regions of the spinal cord, demarcating the beginning of the chronic phase of disease¹⁰⁻¹².

Neuregulins (NRGs) are a family of epidermal growth factors that are required for nervous system development. Studies have shown that NRGs are necessary for neuronal growth, plasticity, and myelin production and repair in the peripheral and central nervous systems¹³. Glial growth factor 2 (GGF2), a splice-variant of the neuregulin-1 (NRG1) gene, has been

61 implicated to activate both myelin repair pathways and suppress pro-inflammatory processes in
62 the CNS during the chronic phase of an animal model of multiple sclerosis, experimental
63 autoimmune encephalomyelitis (EAE) ¹⁴. It is proposed that other splice variants of NRG1 also
64 regulate myelin-repair and inflammatory pathways, but the mechanisms are poorly understood <sup>14-
65 17</sup>.

66 Previous studies, utilizing the *Catwalk Automated Gait Analysis System*, demonstrated
67 improved motor function in TMEV-infected female FVB/nJ mice treated with an adenovirus
68 vector expressing a cDNA clone of human glial growth factor (GGF). The mechanisms by
69 which GGF affects clinical function are poorly understood. Studies show that NRG1 isoforms
70 interact with the family of epidermal growth factor tyrosine kinase receptors (EGFR, ErbB2,
71 ErbB3, and ErbB4) ¹⁸⁻²¹. These isoforms bind to ErbB3 and ErbB4 which heterodimerize with
72 EGFR and ErbB2 to transduce and activate intracellular signaling pathways ^{22, 23}. ErbB2 is the
73 primary receptor with which ErbB3 and ErbB4 heterodimerize with upon NRG1 binding ²². We
74 are interested in studying the expression of EGFR and ErbB2 throughout a time course of TMEV
75 infection in susceptible strains of mice. We hypothesize that EGFR and ErbB2 are upregulated
76 in spinal cords of TMEV-infected FVB/nJ mice during the demyelinating stages of disease.

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Materials and Methods

Theiler's murine encephalomyelitis virus (TMEV)

The Daniel's strain (DA) was used for all experiments. Baby Hamster Kidney (BHK) (ATCC; Manassas, VA) cells were infected with 2.3×10^6 pfu/mL of TMEV in Hanks Balanced Salt Solution (HBSS) (Life Technologies; Carlsbad, CA). Infected BHK cells were incubated for 1 hour in 5% CO₂ at 37°C and then diluted into Eagle's Minimal Essential Medium (EMEM) (Life Technologies; Carlsbad, CA) with 2% heat inactivated Fetal Bovine Serum (FBS). Cells were incubated for 3 days in 5% CO₂ at 37°C. Cells were cleaved and frozen and thawed twice to purify the virus. Supernatants were collected and titer was measured utilizing a viral plaque assay. L2 cells (ATCC; Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies; Manassas, VA) with 5% heat inactivated FBS, 100 µg/mL L-Glutamine (L-GLU), and 100 U/mL Penicillin/Streptomycin (Pen/Strep). L2 cells were split into 12 well plates and incubated in 5% CO₂ at 37°C until 90% confluency. Cells were infected with various dilutions of TMEV collected from BHK supernatant. Plates were incubated in 5% CO₂ at 37°C for 1 hour. Each well received a 50:50 mixture of 4% Sea Plaque Agar and L2 media and incubated for approximately 5-6 days. Visible plaques were counted and PFU/mL were calculated. All experiments were performed with the same preparation of virus.

Mice

Female FVB/nJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 3-5 weeks of age. Mice were housed in sterile polycarbonate Thoren Maxi-Miser System cages (Hazelton, PA) in a specific pathogen free colony and fed autoclaved food (Teklad 2018S 18% Rodent Pellets; Harlan Laboratories, Indianapolis, IN) and water. A 12 hours on/12 hours off

light/dark cycle was maintained in the animal housing room. Room temperatures were maintained at $21.2 \pm 2^{\circ}\text{C}$. All experiments were performed in accordance with the Creighton University's Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Intracerebral injection of TMEV

One week after receipt, female FVB/nJ mice were intracerebrally (i.c.) injected with 2×10^5 plaque-forming units (PFUs) of TMEV. The animals were anesthetized with Isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL) and i.c. injected with $10\mu\text{l}$ of TMEV using a 27-gauge needle attached to a Hamilton syringe. Virus infection occurs over 98% of the time following i.c. injection and fatalities are rare.

Tissue collection

Mice were sacrificed with an intraperitoneal (i.p) injection of $200\mu\text{l}$ of sodium pentobarbital (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA). Spinal cords were harvested and either snap frozen on dry ice for protein and RNA isolation or placed in OCT Frozen Embedding Medium (Miles Inc., IN) for immunostaining. Tissues were stored at -80°C until time of use.

Protein and RNA Isolation

Protein was isolated from whole spinal cords using the mirVANA Protein and RNA Isolation System (Life Technologies; Carlsbad, CA). Spinal cords were homogenized in $400\mu\text{l}$ of ice-cold Cell Disruption Buffer using a TissueMiser (Fisher Scientific; Waltham, MA). $200\mu\text{l}$ of lysate was set aside for protein analysis. The other $200\mu\text{l}$ of lysate was mixed with $200\mu\text{l}$

of 2X Denaturing Solution and incubated on ice for 5 minutes for total RNA isolation. 200 µl of Acid-Phenol:Chloroform was added to the sample lysate and vortexed for 1 minute. Sample was centrifuged for 5 minutes at 8,000RPM at 4°C. The aqueous phase was transferred to a separate tube and 250 µl of 100% ethanol was added. Tube was shaken vigorously and the sample was added to a Filter Cartridge. Sample was centrifuged for 1 minute at 8,000RPM at 4°C. Filter Cartridge was washed with 700 µl of Wash Solution 1 and centrifuged for 1 minute at 8,000RPM at 4°C. Filter Cartridge was then washed twice with 500 µl of Wash Solution 2/3 and centrifuged for 1 minute at 8,000RPM at 4°C. RNA was eluted from Filter Cartridge with 20 µl of 95°C Elution Solution. Protein lysates were diluted into 50 µl of Cell Disruption Buffer and 25 µl of each sample was pipetted into a 96-well polystyrene plate. Protein concentrations were measured using the BCA Protein Assay Kit (Fisher Scientific; Waltham, MA). Solution A was mixed with Solution B at a 50:1 (Working Solution) concentration and each well received 200 µl of the Working Solution. Plates were incubated for 30 minutes at 37°C and read by an Enspire 2300 Multilabel Reader (Perkin Elmer; Waltham, MA). RNA concentrations were measured using Nanodrop 2000c technology (Thermo Scientific; Waltham, MA).

Western Blotting

A concentration of 25µg of spinal cord protein lysate was loaded at 20 µl per well in NuPage Novex 4-12% Bis-Tris Gels (Life Technologies; Carlsbad, CA). Gels were run in the XCell Surelock Mini-Cell Electrophoresis Chamber System (Life Technologies; Carlsbad, CA) and transferred to a 0.45µm polyvinylidene fluoride (PVDF) membrane (Pall Corporation; Port Washington, NY) using the Trans-Blot Cell System (Bio-Rad; Hercules, CA). Blots were probed with rabbit anti-Neu (C-18) antibody specific to the C-terminus of Neu of human origin

(Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-EGF receptor antibody specific to Tyr1068 of human EGF receptor (Cell Signaling Technology; Danvers, MA), and normalized with mouse anti- β -actin (C4) antibody raised against gizzard Actin of avian origin (Santa Cruz Biotechnology; Santa Cruz, CA). All antibodies are reactive with mouse epitopes. Blots were cut horizontally to probe top half with anti-Neu (C-18) or anti-EGF and bottom half with anti- β -actin receptor. Blots were visualized with HRP-conjugated secondary antibodies and were imaged and analyzed with the Kodak Image Station 4000R (Carestream; Atlanta, GA). Data were analyzed by two-way ANOVA with a Bonferroni's test as a post-test. p values of ≤ 0.05 were considered statistically significant.

RT qPCR

Reverse transcription (ImProm II Reverse Transcription System; Promega; Madison, WI) followed by realtime quantitative PCR analysis was performed in 20 μ l volumes containing 1 μ l of cDNA corresponding to 40 ng of DNase-treated RNA, gene specific internal primers (0.5 μ M of each primer), and 1X SsoFast EvaGreen Supermix (Bio-Rad; Hercules, CA). Primers were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) and have 100% sequence homology to the murine genome. Primer sequences are as follows: ErbB2: forward (5' $\rightarrow$ 3'): TGT AAA TCT CGG TGA CCC CAC; ErbB2: reverse (5' $\rightarrow$ 3'): GTA CGC TCG TGC AGA AAG AG; EGFR: forward (5' $\rightarrow$ 3'): TTT GGT GCC ACC TGT GTG AA; EGFR: reverse (5' $\rightarrow$ 3'): ACA CTT GCG GAT GCC ATC TT. Transcript levels were measured using a CFX96 Real Time System (Bio-Rad; Hercules, CA). The PCR cycle is as follows: 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 57°C for 5 sec. Each biological sample was run in triplicate. All data were reported as fold-increase (or decrease) compared to control. Expression levels were

determined using the $\Delta\Delta C_q$ method²⁴. Data were analyzed by two-way ANOVA with a Bonferroni's test as a post-test. P values of ≤ 0.05 were considered statistically significant.

Concanavalin A stimulation

Spleens were isolated from B6 x 129 mice and dissociated in chilled Hank's Balanced Salt Solution (HBSS) (Life Technologies; Carlsbad, CA). Splenocytes were washed and resuspended into RPMI 1640 media (Life Technologies; Carlsbad, CA) containing 5% heat inactivated FBS, 100 $\mu\text{g/mL}$ streptomycin, 100 U/mL penicillin, $5 \times 10^{-5}\text{M}$ 2-ME, and 2.5 μg of Concanavalin A (Sigma; St. Louis, MO). Control unstimulated splenocytes received media only. Both Con A stimulated and unstimulated splenocytes were incubated at 37°C and 5% CO₂ for 48 hours. After incubation, splenocytes were centrifuged at 1,000 RPMs at 4°C for 5 minutes and washed with PBS4 (PBS plus 4% FBS). Splenocytes were counted using a Coulter Particle Counter (Beckman-Coulter; Brea, CA), and resuspended in PBS4 at 1×10^6 cells per 50 μL . Splenocytes were incubated with PE-Texas Red-conjugated rat anti-mouse CD45R (BD Biosciences; San Jose, CA), PE-Cy 7-conjugated rat anti-mouse CD4 (BD Biosciences; San Jose, CA), or Alexa Fluor 700-conjugated rat anti-mouse CD8a (BD Biosciences; San Jose, CA). Splenocytes were additionally stained with rabbit anti-Neu (C-18) antibody specific to the C-terminus of Neu of human origin (Santa Cruz Biotechnology; Santa Cruz, CA) or rabbit anti-EGF receptor antibody specific to Tyr1068 of human EGF receptor (Cell Signaling Technology; Danvers, MA) for double staining analysis by flow cytometry (FacsARIA) (BD Biosciences; San Jose, CA). ErbB2 antibody and EGF receptor antibody were stained with Alexa Fluor 488-conjugated secondary antibodies for cell sorting. All antibodies are reactive with mouse

epitopes. Data were analyzed by a two-tailed T-test. p values of ≤ 0.05 were considered statistically significant.

Immunofluorescence

Six micron thick sections of spinal cord tissue embedded in OCT Frozen Embedding Medium (Miles Inc., IN) were cut and fixed in -20°C acetone. Sections were stained with rat anti-mouse F4/80 (AbD Serotec; Raleigh, NC), rat anti-mouse CD45R (BD Biosciences; San Jose, CA), rat anti-mouse CD4 (BD Biosciences; San Jose, CA), or rat anti-mouse CD8a (BD Biosciences; San Jose, CA). Sections were additionally stained with ErbB2 goat anti-Neu (C-18) antibody specific to the C-terminus of Neu of human origin (Santa Cruz Biotechnology; Santa Cruz, CA) and goat anti-EGF receptor antibody specific to Tyr1068 of human EGF receptor (Cell Signaling Technology; Danvers, MA) for double staining experiments. Visualization was performed using Alexa Fluor-conjugated secondary antibodies (Life Technologies; Carlsbad, CA) which were excited at wavelengths of either 488 (green) or 568 (red). Coverslips were applied using Vectashield Mounting Media with DAPI (Vector Labs, CA).

Results

ErbB2 levels are increased in TMEV-infected spinal cords

ErbB2 and EGFR levels were examined via western blotting in TMEV-infected spinal cords 21, 35, 45, 60 days post-TMEV infection (p.i.). ErbB2 levels increased: 1.59-fold $\pm$ 0.05 on day 21 p.i., 1.92-fold $\pm$ 0.05 on day 35 p.i., and 1.68-fold $\pm$ 0.09 on day 60 p.i. (Figure 1). EGFR levels remained unaltered throughout the time course (data not shown).

ErbB2 transcript levels are increased in TMEV-infected spinal cords

ErbB2 and EGFR transcript levels were examined via RT qPCR in TMEV-infected spinal cords 21, 35, 45, and 60 days p.i. ErbB2 transcript levels increased 1.36-fold $\pm$ 0.09 on day 21 p.i (Figure 2). ErbB2 transcript levels were not significantly different compared to HBSS controls on days 35, 45, and 60 p.i. EGFR transcript levels remained unaltered (data not shown).

ErbB2 expression is localized within demyelinating lesions

Western blotting and RT qPCR indicated that ErbB2 expression is increased in TMEV-infected spinal cords. To determine why there is an increase, immunohistochemistry experiments were performed on TMEV-infected spinal cords. Strong positive immunoreactivity for ErbB2 was localized within demyelinating lesions (Figure 3). ErbB2 immunoreactivity was seen in demyelinating lesions on days 21, 35, 45, and 60 p.i. EGFR staining was not localized within demyelinating lesions (data not shown).

Concanavalin A stimulation increased ErbB2 expression on splenocytes

The strong positive immunoreactivity for ErbB2 localized within demyelinating lesions suggested that immune cell infiltrates are expressing ErbB2. Flow cytometry analysis determined that ErbB2 is expressed on B cells, CD8⁺ T cells, and CD4⁺ T cells. ErbB2 expression increased 1.65-fold $\pm$ 0.29 on B cells and 1.89-fold $\pm$ 0.24 on CD4⁺ T cells after 48 hours of Con A stimulation (Figure 4), suggesting that the strong positive immunoreactivity in demyelinating lesions is due to the presence of activated immune cells.

ErbB2 is expressed on immune cells within demyelinating lesions

Flow cytometry results indicated that ErbB2 is expressed on B cells, CD8⁺ T cells, and CD4⁺ T cells. When these cells are stimulated, ErbB2 expression increases on B cells and CD4⁺ T cells. These results were further confirmed utilizing immunohistochemistry. Macrophages, B cells, CD4⁺ T cells, and CD8⁺ T cells express ErbB2 in demyelinating lesions located in the upper cervical levels (C1-C2) of the spinal cord (Figure 5). This phenotype is observed on days 21, 35, 45, and 60 p.i. EGFR staining was not observed in demyelinating lesions (data not shown).

Discussion

Current therapies for MS patients reduce immune responses or treat overbearing symptoms. No treatment has focused on promoting repair or preserving tissue, largely due to central nervous system (CNS) complexities. Biological mechanisms and signaling pathways necessary for CNS development are poorly defined, which causes setbacks in therapy advances for MS. Multiple studies have attempted to develop therapies targeting oligodendrocyte preservation and differentiation, but it is now understood that there are a variety of factors that influence such processes. NRG1 has been extensively studied in peripheral nervous system (PNS) myelination. Studies have demonstrated that type III isoforms of the NRG1 gene are necessary for Schwann cell development and myelination²⁵. Translating these studies into the CNS indicates that NRG1 is necessary for early development of oligodendrocytes. Utilizing NRG1 isoforms for MS therapy makes sense if it can functionally preserve oligodendrocytes and promote remyelinating responses^{13, 26}. What previous studies have failed to examine are the effects NRG1 isoforms have on cells not resident to the CNS, particularly immune cell infiltrates.

Many studies have focused on the role NRG1 plays in myelination in the CNS. One study demonstrated that human glial growth factor 2 (rhGGF2) treatment ameliorates disease progression in animals induced with experimental autoimmune encephalomyelitis (EAE) by enhancing remyelinating responses and modulating immune function *in vivo*¹⁴.

Our studies indicate that glial growth factor's (GGF) interactions are not limited to CNS-resident cells. We demonstrated that ErbB2 is expressed on immune cells in demyelinating lesions and is upregulated on activated B cells and CD4⁺ T cells. These data suggest that GGF

may change the phenotype of these immune cell infiltrates, elucidating one mechanism responsible for improved motor function seen in GGF treated TMEV-infected FVB/nJ mice. Future studies will examine how immune cell phenotypes change in the presence of GGF.

In terms of relevance to MS, the most significant findings of this study are that ErbB2 expression is seen on immune cell infiltrates, and it is upregulated on activated B cells and CD4⁺ T cells. Canella et al. demonstrated that GGF2 expression is increased around the borders of demyelinating lesions in MS patients¹⁵. Studies have concluded, that NRG1 is necessary for nervous system development and myelination, but various splice-variants of NRG1, such as GGF and GGF2, might also interact with immune cell infiltrates in attempt to change the inflammatory response and promote remyelinating mechanisms. Treatments that overexpress these variants *in vivo* might represent a useful addition to currently available MS therapies, either alone or in combination with immunomodulatory agents.

Figure Legends

Figure 1: ErbB2 abundance in spinal cords isolated from TMEV-infected female FVB/nJ mice. 25µg of spinal cords lysate was loaded per well. Values represent the mean abundance level $\pm$ SEM, relative to β -actin abundance levels. Statistical analysis was performed using a two-way ANOVA, with HBSS-injected female FVB/nJ mice as a control, and $n = 3$. * < 0.05 ; ** < 0.01 .

Figure 2: ErbB2 transcript levels in spinal cords isolated from TMEV-infected female FVB/nJ mice. ErbB2 mRNA levels were measured by RT qPCR. Values represent the mean transcript level $\pm$ SEM, relative to GAPDH transcript levels. Statistical analysis was performed using a two-way ANOVA, with HBSS-injected female FVB/nJ mice as a control, and $n = 3$. * < 0.05 .

Figure 3: Increased ErbB2 expression in demyelinating lesions in spinal cord sections from TMEV-infected female FVB/nJ mice. **Figure a** shows no positive immunoreactivity for ErbB2 in spinal cords from HBSS-injected mice. **Figure b** localizes ErbB2 within demyelinating lesions, suggesting immune cells infiltrates express ErbB2.

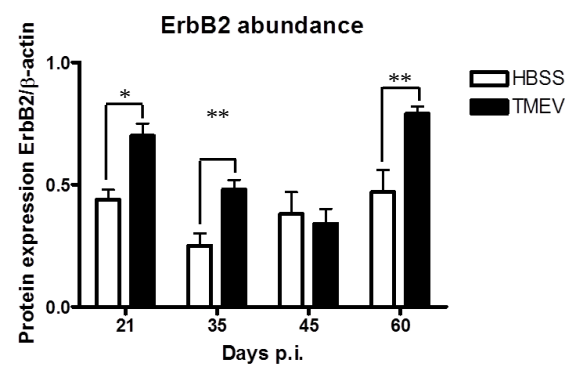
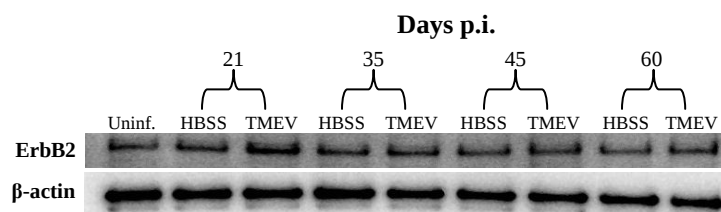
Figure 4: Splenocytes were treated with 2.5µg of Concanavalin A in RPMI media for 48 hours. Activated splenocytes were analyzed by flow cytometry (FacsAria). Values represent the mean % of positively stained cells $\pm$ SEM. Statistical analysis was performed using a two-tailed T-test, with unstimulated splenocytes as a control, and $n = 5$. *** < 0.001 .

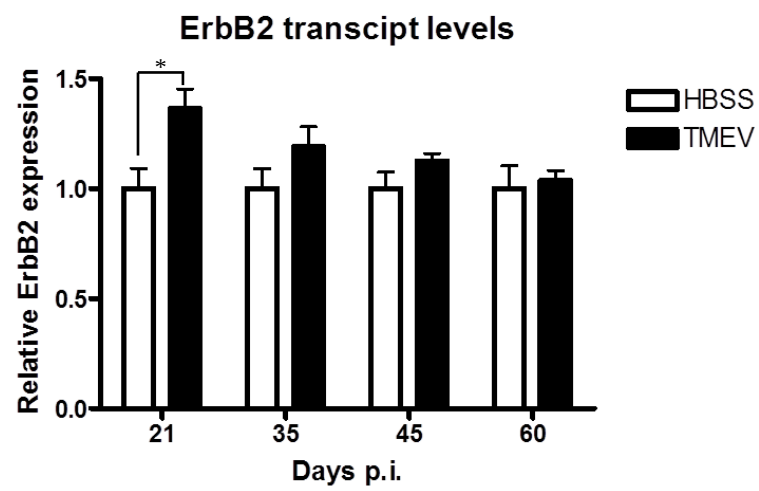
Figure 5: ErbB2 staining (red) is seen on macrophages, B cells, CD4⁺ T cells, and CD8⁺ T cells (red) within demyelinating lesions in spinal cords isolated from TMEV-infected female FVB/nJ mice.

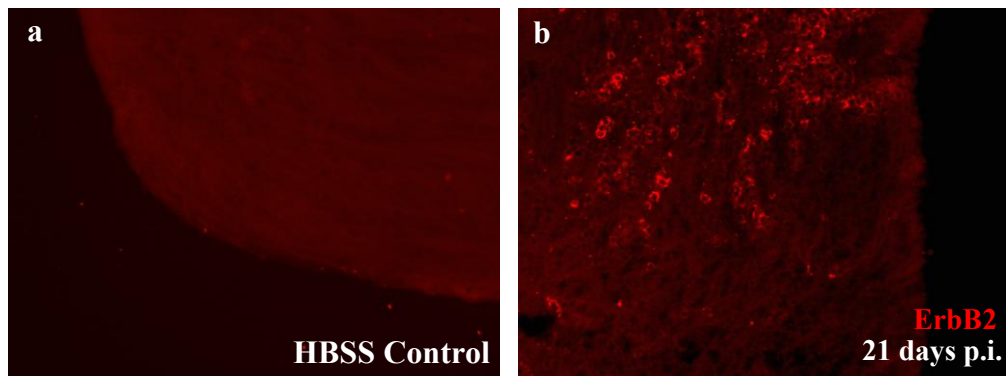
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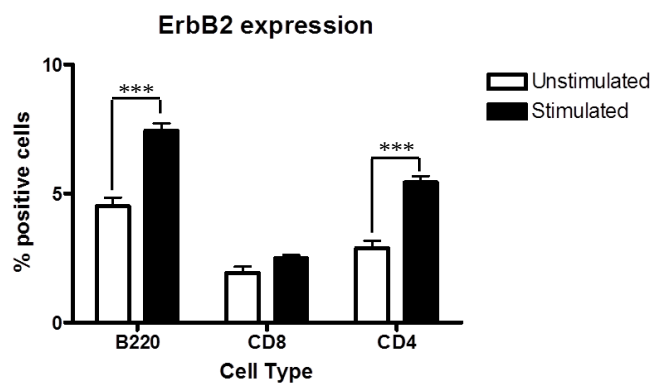
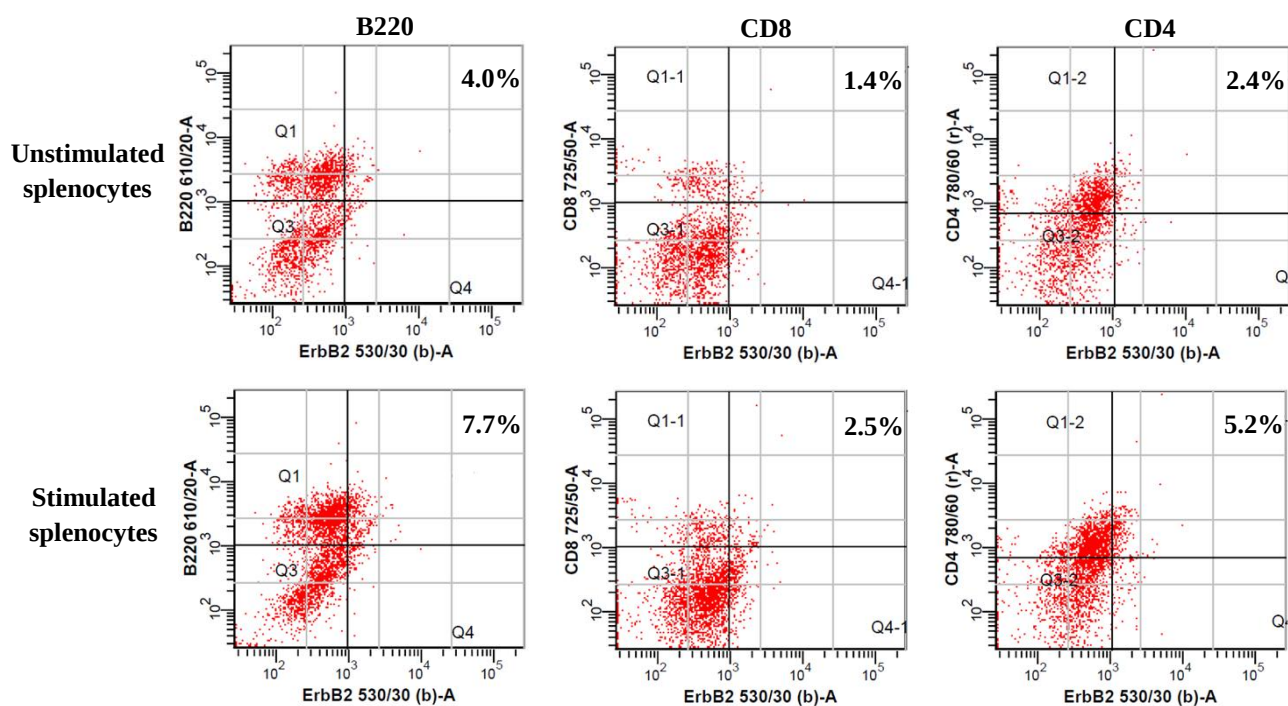
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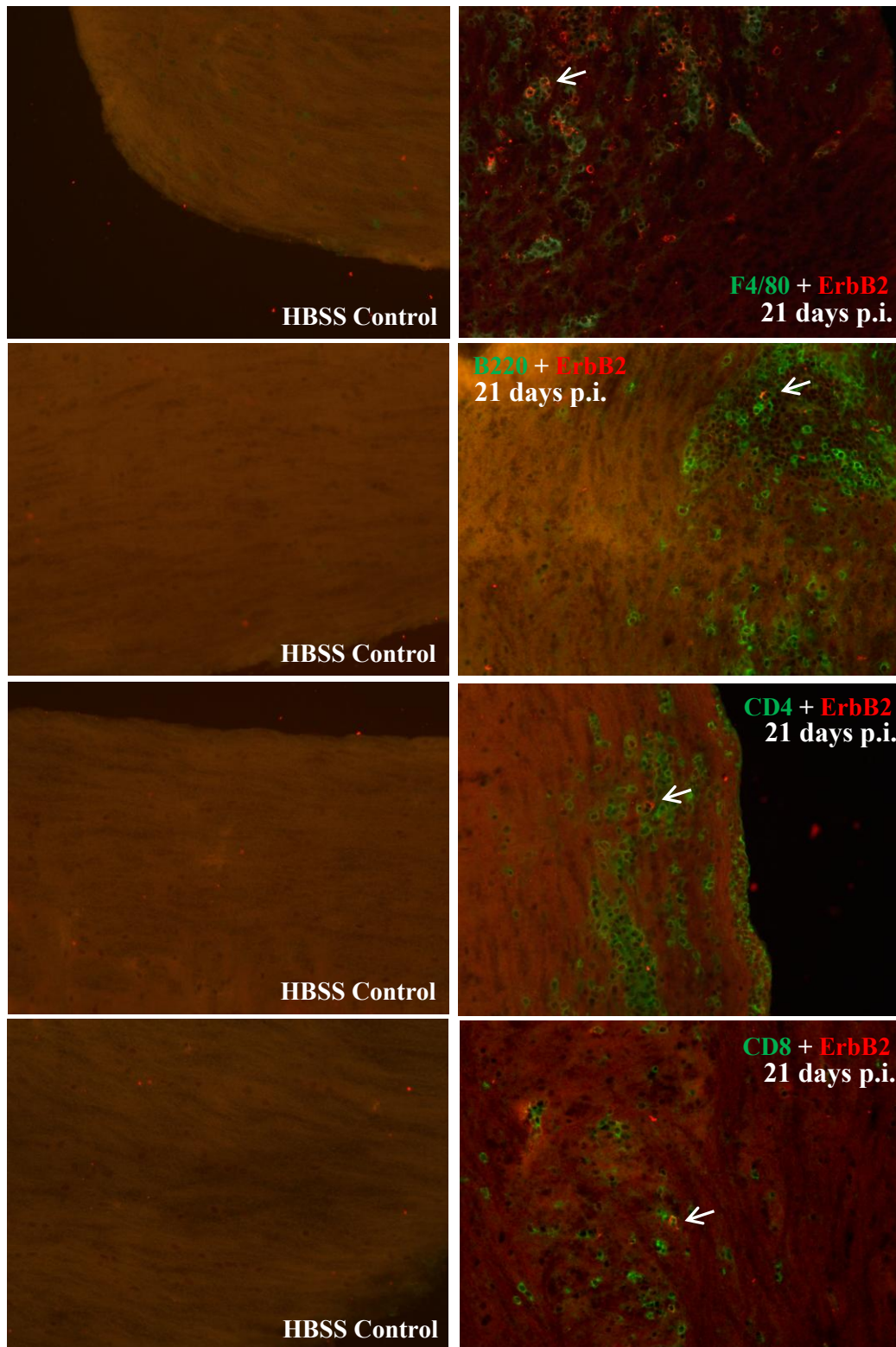
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Curriculum Vitae
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I. Professional Experience

July 2011-present	Professor, Department of Medical Microbiology and Immunology, Creighton University School of Medicine
July 2005-2011	Associate Professor, Department of Medical Microbiology and Immunology, Creighton University School of Medicine Tenure Granted
October 2004-present	Secondary Appointment, Department of Medicine Creighton University School of Medicine
July 1999-June 2005	Assistant Professor, Department of Medical Microbiology and Immunology, Creighton University School of Medicine

II. Education

June 1995 to July 1999	Mayo Clinic / Foundation, Rochester, MN Departments of Immunology and Neurology Post-doctoral Fellowship in Neuroimmunology
September 1990 to June 1995	Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD Department of Molecular Microbiology and Immunology, Doctor of Philosophy “Glial Cells of Müller as Intraretinal Immunomodulators”
January 1988 to August 1990	University of Massachusetts – Lowell, Lowell, MA Department of Biological Sciences, Master of Science “Dose Response of Four Antischistosomal Agents Against Four Strains of <i>Schistosoma mansoni</i> ”
September 1979 to May 1983	University of New Hampshire, Durham, NH Department of Animal Science, Bachelor of Science

III. Publications

Papers in Peer-Reviewed Journals

1. McDonald T, **Drescher KM**, Weber A, Tracy S. Creatinine inhibits bacterial replication. *J Antibiot (Tokyo)*, 2012; Feb 1.
2. Zhou R, Li X, Hu G, Gong AY, **Drescher KM**, Chen XM. miR-16 Targets Transcriptional Corepressor SMRT and Modulates NF-kappaB-Regulated Transactivation of Interleukin-8 Gene. *PLoS One*, 2012; 7: e30772.
3. Tracy S, **Drescher KM**, Chapman NM. Enteroviruses and type 1 diabetes. *Diabetes Metab Res Rev*, 2011; 27: 820-3.
4. Leland KM, McDonald TL, **Drescher KM**. Effect of creatine, creatinine, and creatine ethyl ester on TLR expression in macrophages. *In press, International Immunopharmacology*
5. **Drescher KM**, Sharma P, Lynch HT. Current Hypotheses on How Microsatellite Instability Leads to Enhanced Survival of Lynch Syndrome Patients. *Clinical and Developmental Immunology*, 2010; 170432.
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17. Tracy SM, **Drescher KM**. Coxsackievirus infections and NOD mice: Relevant models of protection from, and induction of, type 1 diabetes. How Do We Best Employ Animal Models for Type 1 Diabetes and Multiple Sclerosis. *Annals of the New York Academy of Sciences*, 2007; 1130: 143-151.
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Book Chapters

1. "T-Cell Development " by **KM Drescher** in Immunology for Medical Students. Nairn and Helbert. C.V. Mosby Co, 2002.
2. "Constitutive Defenses" by **KM Drescher** in Biotest Study Aids, Microbiology and Immunology. F.C. Knoop, Biotest Publishing Company, Ventura, CA, 2004.
3. "Complement" (Chapter 35) by **KM Drescher** in Biotest Study Aids, Microbiology and Immunology. F.C. Knoop, Biotest Publishing Company, Ventura, CA, 2004.
4. "MHC Antigen Processing and Presentation" by **KM Drescher** in Biotest Study Aids, Microbiology and Immunology. F.C. Knoop, Biotest Publishing Company, Ventura, CA, 2004.
5. "T-Cell Development " by **KM Drescher** in Immunology for Medical Students. Nairn and Helbert. C.V. Mosby Co, 2nd edition, 2006.
6. "The CVB and etiology of type 1 diabetes" by **KM Drescher** and SM Tracy in Current Topics in Microbiology and Immunology 323, The Group B Coxsackieviruses. Tracy S, Drescher KM, and Obserte S, eds. Berlin, Springer-Verlag, 2008.
7. "Group B coxsackieviruses" Preface. by Oberste S, **Drescher K**, Tracy S. Current Topics in Microbiology and Immunology 323 v-vi, The Group B Coxsackieviruses. Tracy S, **Drescher KM**, and Obserte S, eds. Berlin, Springer-Verlag, 2008.

8. "MicroRNAs in epithelial anti-microbial immunity" by Liu J, Hu G, Zhou R, **Drescher KM**, Chen XM. in: RNA Technologies and Their Applications. Erdmann, VA and Barciszewski, J (Eds.). Springer. 2009.

IV. Funding

Complete:

1. IL-4 Upregulation in Theiler's virus-infected mice (K. Drescher, Principal Investigator), Health Futures Foundation, direct/total costs, July 01, 2000-June 30, 2002
2. Upgrading the Animal Resource Facility for Mouse Genetic Research: Specific Pathogen-Free (SPF) Unit (K. Drescher, Co-Principal Investigator), Health Futures Foundation, direct costs, total costs, July 01, 2000-June 30, 2001
3. Burroughs Wellcome Fund Visiting Professorship (K. Drescher, Co-Principal Investigator), Burroughs Wellcome Fund, direct/total costs, September 01, 2000-August 31, 2001.
4. Nebraska Center for Viral Pathogenesis: Role of Glial Cells in Prion Diseases (K. Drescher, Co-Principal Investigator), National Institutes of Health, direct costs, total costs, September 30, 2000-August 31, 2002.
5. Use of Coxsackieviruses as TMEV Vectors (K. Drescher, Principal Investigator), State of Nebraska-LB692, direct/total costs, September 01, 2001-August 31, 2003.
6. Group B Coxsackievirus Vectors (K. Drescher, Collaborator; S. Tracy Principal Investigator), National Institutes of Health, direct costs of sub-contract, total costs of sub-contract, October 01, 2002-September 30, 2003.
7. Characterization of TILs in Colon Cancer (K. Drescher, Principal Investigator), State of Nebraska-LB595, direct/total costs, November 01, 2002-June 30, 2004.
8. Antibody-Mediated Mechanisms of CNS Viral Clearance (K. Drescher, Principal Investigator). American Cancer Society, direct costs, total costs, July 01, 2001-December 6
9. Inhibiting Demyelination by Immunization Using Coxsackievirus Vectors (K. Drescher, Principal Investigator). National Multiple Sclerosis Society direct costs, total costs, April 01, 2003-March 31, 2007
10. Role of Neuregulins in Myelin Repair in the CNS and PNS (K. Drescher, Principal Investigator of Project II; Part of Program Project "Center for Neurosensory Systems" S. Smith, Program Project Principal Investigator), National Institutes of Health, direct costs of Project II, total costs of Project II, October 1, 2003-September 30, 2008

11. GlaxoSmithKline, Assessment of PPAR agonists on cognition, Abeta deposition and metabolic markers in Tg2576 APP mice, 07/01/07-06/30/08
Role on Project – PI
12. Replacing an Animal Resource Facility Cage Washer. National Institutes of Health – G20, May 15, 2007 – April 30, 2009. PI: Kristen Drescher, Ph.D.
13. TILS in Colon Cancer (K. Drescher, Principal Investigator of Project III; Part of Program Project ‘Hereditary Cancer’ H. Lynch, Program Project Principal Investigator), LB 595, State of Nebraska, direct/total costs of Project III, July 1, 2004-June 30, 2009
14. National Multiple Sclerosis Society – Research Grant, “Role of erbB in limiting TMEV-induced damage to the Central Nervous System.” October 1, 2006 – September 30, 2010, Total costs: . PI: Kristen Drescher, Ph.D.
15. Department of Defense- “Impact of Erb-B Signaling on Myelin Repair in the CNS Following Virus-Induced Damage.” March 1, 2007 – February 28, 2011, total costs. PI: Kristen Drescher, Ph.D.
16. LB692, State of Nebraska – “Treatment of Mice with SMDF Ameliorates Clinical Deficits in a Model of MS” July 1, 2009 – June 30, 2010. PI: Kristen Drescher, Ph.D.
17. LB-595, State of Nebraska - “Impact of Immune System Gene Polymorphisms on Colon Cancer.” (K. Drescher, Principal Investigator of Project; Part of Program Project ‘Hereditary Cancer’ H. Lynch, Program Project Principal Investigator), direct/total costs of Project III, July 1, 2009-June 30, 2011

IV. Research Presentations

National / International Meetings

1. **Drescher, KM**, Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Poster Presentation. Military Health Research Forum. Kansas City, Missouri. September 1, 2009
2. **Drescher, KM**, Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Oral Presentation. Military Health Research Forum. Kansas City, Missouri. September 2, 2009
3. **Drescher KM**, Schemmel A, and Leland K. American Association for Immunology Meeting, Seattle, WA, May 2009 (poster).
4. **Drescher KM** and Tracy SM. Development of a model allowing study of the early stages of demyelination. International Society of Neuroimmunology, Ft. Worth, TX, October, 2008 (oral presentation).
5. Tracy S, **Drescher KM**, and Carson SD. Suppression of CVB3 replication by murine or

human sera possessing anti-enterovirus antibodies but lacking B3 type-specific neutralizing antibodies: a role for non-neutralizing antiviral antibodies in protection from disease. American Society for Virology, Ithaca, NY, July 2008 (poster).

8. **Drescher KM** and Tracy SM. Injection of the sciatic nerve with TMEV: A new model for peripheral nerve demyelination. Positive Strand Virus Meeting, Washington, DC, May 2007 (poster).

9. **Drescher KM** and Tracy SM. Injection of the sciatic nerve with TMEV: A new model for peripheral nerve demyelination. Positive Strand Virus Meeting, Washington, DC, May 2007.

10. **Drescher KM** and Tracy SM. Establishment of a model to examine the early events involved in the development of virus-induced demyelinating lesions. Animal Models of Type 1 Diabetes and Multiple Sclerosis. San Francisco, CA, November 2006 (poster). Received Jr. Investigator Award

11. Tracy SM and **Drescher, KM**. Coxsackievirus infections and NOD mice Animal Models of Type 1 Diabetes and Multiple Sclerosis. San Francisco, CA, November 2006

12. **Drescher KM** and Tracy S (2006) Coxsackie B virus replication in pancreatic islets: virus associates with the four major islet cell types, not merely beta cells American Society of Virology, Madison, WI.

13. Kim, K., Kanno, T., **Drescher, K.M.**, Haas, E., Tracy, S. (2006) Enterovirus receptor expression in human pancreas tissue. American Society of Virology, Madison, WI.

14. Kanno, T., Kim, K., Kono, K., **Drescher, K.M.**, Chapman, N.M., Tracy, S. (2006) Coxsackie B virus diabetogenic phenotype: definition by replication rate in pancreas tissue and beta cells. American Society of Virology, Madison, WI.

15. Tracy, S.M., **Drescher, K.M.**, Jackson, J.D., Sanderson, S. Vaccinating NOD mice against CVB-induced rapid T1D onset: type-specific immunity is not required to lower T1D incidence American Society of Virology, Madison, WI.

16. **Drescher KM**, Sunde J, Tracy SM. (2005) Neuregulin-1 Is Upregulated in the Sciatic Nerve Following Infection with TMEV, International Congress of Virology, August, San Francisco, CA.

17. Tracy S and **Drescher KM** (2005) Group B coxsackievirus infections in NOD mice: towards clarifying a role of enteroviruses in human type 1 diabetes onset. International Congress of Virology, San Francisco, CA.

16. **Drescher KM**, Kocakova P, Chapman NM, Tracy SM. Use of CVB-based vaccines to modify an immune-response to self. Americas Conference on the Treatment and Research in Multiple Sclerosis (ACTRIMS), Toronto Canada, October 2004.

17. Tracy S, **Drescher KM**, Kono K, Bopegamage S, Carson S. Coxsackie B virus replication within inflamed islets of older, prediabetic NOD initiates type 1 diabetes. Second European Congress of Virology, Madrid Spain, September 2004.
18. **Drescher KM**, Kocakova P, Chapman NM, Tracy SM. Inhibition of cellular immune response to self-epitopes in TMEV-Infected mice. 12th International Congress of Immunology, Montreal Canada, July 2004.
19. Tracy SM, **Drescher KM**, Kono K, Carson SD, Bopegamage S. Coxsackievirus replicates in islets of old NOD mice and initiates diabetes. Seventh International Symposium on Positive Stranded RNA Viruses, San Francisco, California, May 2004.
20. Kocakova P, **Drescher KM**. C3 is required for efficient clearance of virus from brains of TMEV-infected mice. Meeting of the Federation of American Society of Experimental Biology, Washington DC, April 2004.
21. Oshunleti O, **Drescher KM**. Use of CVB chimeric viruses as vaccine vectors in an animal model of multiple sclerosis. National Medical Fellowship Meeting, Princeton, New Jersey, March 2004.
22. **KM Drescher**. Modulation of activin / inhibin mRNA levels following TMEV infection. XIIth International Congress of Virology, Paris France, July 2002.
23. Tracy S, Chapman NM, Kim KS, **Drescher KM**, Lane PH, Pirruccello S, Leser JS. Infection of young NOD mice with group B coxsackievirus lowers development of type 1 diabetes. XIIth International Congress of Virology, Paris France, July 2002
24. Tracy S, Chapman NM, Kim KS, **Drescher KM**, Lane PH, Pirruccello S, Leser JS. Vaccination of young nonobese diabetic (NOD) mice with group B coxsackievirus lowers type 1 diabetes incidence. 26th International Congress of Internal Medicine, Kyoto Japan, May 2002.
25. **Drescher KM**, Rodriguez M. Mice with severe immunodeficiencies upregulate IL-4 in the spinal cord gray matter after TMEV infection. Meeting of the American Association of Immunologists. Seattle, Washington, May 2000.
26. Murray PD, **Drescher KM**, Leibowitz J, Rodriguez M. Extensive myelin repair in a virus model of multiple sclerosis. Meeting of the Federation of the American Society of Experimental Biologists. Washington, DC, April 1999.
27. **Drescher KM**, Hogancamp W, Johnston SL, David CS, Wettstein PJ, Rodriguez M. Paradoxical role of V_β8.2 T cells in the pathogenesis of demyelination in a viral model of multiple sclerosis. Meeting of the Federation of the American Society of Experimental Biologists. Washington, DC, April 1999.
28. Das P, **Drescher KM**, Bradley DS, Rodriguez M, David CS. Complementation between specific HLA-DR and HLA-DQ genes in transgenic mice determines susceptibility to experimental autoimmune encephalomyelitis. Keystone Symposia. Immunogenetics of Human Disease – MHC/TCR and Peptide. Taos, New Mexico, January 1999.

- 29 Pavelko KD, McGavern DB, **Drescher KM**, David CS Rodriguez M. HLA-DQ8 enhances demyelinating disease relative to DQ6 in a murine viral model of multiple sclerosis. Keystone Symposia. Immunogenetics of Human Disease – MHC/TCR and Peptide, Taos, New Mexico, January 1999.
- 30 Das P, **Drescher KM**, Bradley DS, Rodriguez M, David CS. HLA-DR is critical for the induction of EAE, while HLA-DQ modulates severity of disease. 5th International Congress of the International Society of Neuroimmunology, Montreal Canada, July 1998.
- 31 **Drescher KM**, Murray PD, Pease LR, Rodriguez M. CNS cell populations are protected from virus-induced pathology by distinct arms of the immune system. 5th International Congress of the International Society of Neuroimmunology, Montreal Canada. July 1998.
- 32 Das P, **Drescher KM**, Bradley DS, Rivera-Quinones C, Rodriguez M, David CS. (1997). HLA transgenic mice as humanized models of MS: Studies on EAE. Human Immunology 55 (Supplement 1): 4.
- 33 **Drescher KM**, Nguyen LT, Coenen MJ, Leibowitz JL, David CS, Rodriguez M. The HLA-DR3 transgene reduces the severity of demyelination in Theiler's virus infected mice. 1st International Conference on Neurovirology. Philadelphia, Pennsylvania, September 1997.
- 34 Das P, Bradley DS, **Drescher KM**, Rodriguez M, David CS. (1997). T cell responses to myelin basic protein (MBP) and proteolipid protein (PLP) in HLA-DR2 and HLA-DQ6 transgenic mice. Journal of Clinical Immunology 99: S10.
- 35 Bureau JF, **Drescher KM**, Pease LR, Vikoren T, Delcroix M, Brahic M, Rodriguez M. Predisposition genetique de la souris a la demyelination induite par le virus de Theiler. Association pour la recherche sur la sclerose en plaques (ARSEP), Paris, France, June 1996.
- 36 **Drescher KM**, Murray PD, Pease LR, Rodriguez M. T cells and immunoglobulins protect mice from Theiler's virus-induced encephalitis whereas Class I MHC / CD8+ T cells protect against demyelination. Meeting of the American Neurological Association, Miami, Florida, October 1996.
- 37 Rodriguez M, **Drescher KM**, David CS. Human leukocyte antigen-DR3 transgene reduces the severity of virus-induced demyelinating disease in mice. Europe's Conference on the Treatment and Research in Multiple Sclerosis (ECTRIMS). Copenhagen, Denmark, September 1996.
- 38 **Drescher KM**, Whittum-Hudson JA. Retinal astroglia transcribe proinflammatory and antiviral cytokines. Meeting of the Federation of the American Society of Experimental Biology. Atlanta, Georgia, April 1995.
- 39 **Drescher KM**, Whittum-Hudson JA. Type I interferon production by retinal glia. Meeting of the American Researchers in Vision and Ophthalmology, Fort Lauderdale, Florida, April 1995.
- 40 **Drescher KM**, Whittum-Hudson JA. Upregulated cytokine transcription in vitro by retinal

Müller cells: in vivo correlates. International Immunology and Immunopathology of the Eye Meeting, Washington, DC, June 1994.

41 **Drescher KM**, Whittum-Hudson JA. Müller cells are an intraretinal source of immunological and antiviral cytokines. Meeting of the American Researchers in Vision and Ophthalmology, Sarasota, Florida, April 1994.

42 **Drescher KM**, Whittum-Hudson JA. Immunohistochemical evidence for interferon γ – mediated activation of retinal astroglial cells. Meeting of the American Association of Immunologists, Denver, Colorado, May 1993.

V. Professional Service

Reviewer for Grants

2011-present	American Heart Association, Basic Science 1, Immunology Study Section
2003-present	National Alzheimer's Disease Association
2003-2004	Creighton University Summer Medical Student Research Fellowships
2003-2005	<i>Ad hoc</i> Reviewer: American Cancer Society
2001, 2007	Health Futures Foundation Individual Investigator <i>Ad Hoc</i> Grant Reviewer
2000	Health Futures Foundation Program Project <i>Ad Hoc</i> Reviewer
1999	National Institutes of Health Study Section National Institute of Allergy and Infectious Diseases Autoimmunity Centers of Excellence

Reviewer of Abstracts

2009	Served on Abstract Selection Committee for the Military Health Research Forum held in Kansas City, Missouri (August 31 – September 3, 2009).
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Reviewer for Journals

Ad hoc reviewer for the following scientific journals:

Canadian Journal of Pharmacology and Physiology
Investigative Ophthalmology and Visual Sciences
Journal of Immunology
Journal of Neuroimmunology
Journal of Virology
Neuroscience Letters
Gastroenterology
Virology

Antiviral Research
 Science Signaling
 Lancet Oncology
 Autoimmunity
 Journal of Innate Immunity
 Journal of Neuroinflammation
 Cellular and Molecular Life Sciences

Other Professional Activities

2000, 2001, 2004, 2006	Judge	
Midwest Omaha,		Medical Student Research Forum Nebraska
2000, 2001	Panel	Discussion
Expanding Omaha,		Your Horizons Nebraska
2001	Session	Moderator
Omaha,		Regional American Society of Microbiology Meeting Nebraska
2002-2012		Member, Scientific Advisory Panel Southeast Nebraska Alzheimer's Disease Association
2002-2004	Women's	Liaison Officer
American		Association of Medical Colleges

VI. Professional Societies and Organizations

2000 - present International Society for Neuroimmunology, Regular Member
 2000 - present International Society of Neurovirology, Founding Member
 2000 - present American Association of Immunologists, Regular Member

VII. Awards and Honors

1980 L.V. Tirrell Scholarship
 University of New Hampshire

1981 H.P. Hood Memorial Award
 University of New Hampshire

1990 – 1992 Pre-Doctoral Fellowship
 National Eye Institute
 Wilmer Eye Institute
 John Hopkins University

- 1992 Sigma Xi Grant-in-Aid of Research
- 1992 Eleanor Bliss Fellowship
Johns Hopkins University
- 1995 - 1996 National Institutes of Health Post-Doctoral Fellowship
Department of Immunology
Mayo Clinic
- 1996 – 1999 Fellow of the National Multiple Sclerosis Society
- 1999 Dale McFarlin Travel Award
National Multiple Sclerosis Society
- 1999 Basic Science Research Award
Department of Neurology
Mayo Clinic / Foundation
- 2000 Charles A. Monasee Faculty Development Award
Health Futures Foundation
Creighton University School of Medicine
- 2004 Abstract Award
Americas Committee on Research and Treatment in Multiple Sclerosis
National Multiple Sclerosis Society
- 2006 Jr. Investigator Award
NY Academy of Sciences
Animal Models of Type 1 Diabetes and Multiple Sclerosis
- 2010 Golden Apple, Class of 2013

VIII. Teaching

Graduate Students:

- 2002 Cellular and Molecular Immunology (course developer, co-director; 15
contact hours)
- 2004 Cellular and Molecular Immunology (18 contact hours)
- 2004 Research Ethics (1 contact hour)
- 2005 Research Ethics (1 contact hour)
- 2006 Research Ethics (1 contact hour)
- 2006 Advanced Immunology (20 contact hours)
- 2006 Cellular and Molecular Immunology (18 contact hours)
- 2007 Research Ethics (1 contact hour)
- 2007 Host Defense (course director, 33 contact hours)
- 2008 Research Ethics (1 contact hour)
- 2008 Cellular and Molecular Immunology (18 contact hours)

2008	Host Defense (course director, 33 contact hours)
2009	Research Ethics (1 contact hour)
2009	Current Topics in Microbiology and Immunology (1.5 contact hours)
2009	Host Defense (course director, 33 contact hours)
2010	Research Ethics (1 contact hour)
2010	Cellular and Molecular Immunology (18 contact hours)
2011	Host Defense (course director; 33 contact hours)
2012	Host Defense (course director; 36 contact hours)

Professional Students:

2002	Medical Microbiology (5 contact hours); School of Pharmacy
2002	Tutoring; Suzie Krauss, Dental Student
2004	Microbiology (2 contact hours); School of Dentistry

Medical Students:

2001	Host Defense (1 contact hour)
2002	Host Defense (2 contact hours)
2003	Host Defense (course co-director; 10 contact hours)
2003	Neuroscience (1 contact hour)
2004	Host Defense (course director, 31 contact hours)
2004	Neuroscience (1 contact hour)
2005	Host Defense (course director, 31 contact hours)
2005	Neuroscience (1 contact hour)
2006	Host Defense (course director, 31 contact hours)
2006	Neuroscience (1 contact hour)
2006	Host Defense (summer) 30 contact hours
2007	Host Defense (course director, 31 contact hours)
2007	Neuroscience (1 contact hour)
2007	Host Defense (course director, 33 contact hours)
2007	Neuroscience (1 contact hour)
2008	Host Defense (course director, 33 contact hours)
2008	Neuroscience (1 contact hour)
2009	Host Defense (course director, 33 contact hours)
2009	Neuroscience (1 contact hour)
2010	Host Defense (course director, 33 contact hours)
2010	Neuroscience (1 contact hour)
2011	Host Defense (course director, 33 contact hours)
2011	Neuroscience (1 contact hour)

Ph.D. Student Committees:

2001-2006	Abdo Berro, Ph.D. Candidate
Department	of Medical Microbiology and Immunology

2002-2006 Department	Ashraf Hassaballa, Ph.D. Candidate of Medical Microbiology and Immunology
2002-2006t Department	Jehad Edwan, Ph.D. Candidate of Medical Microbiology and Immunology
2000-2003 Department	Marianne Mannion, Ph.D. Candidate of Medical Microbiology and Immunology
2003-2006 Department	Arpita Bharadwaj, Ph.D. Candidate of Medical Microbiology and Immunology
2003-2004 Department	Sarath Dhananjayan, Ph.D. Candidate of Medical Microbiology and Immunology
2003-2007 Department	Kriti Rakesh, Ph.D. Candidate of Medical Microbiology and Immunology
2003- 2005 Department	Julia Vent, Ph.D. Candidate of Biomedical Sciences
2005-2010 Department	Jacob Ayers, Ph.D. Candidate of Medical Microbiology and Immunology
2005-present Department	Michelle Kramer, Ph.D. Candidate of Medical Microbiology and Immunology
2005-2006 Department	Bharti Chaudhari, MS. Candidate of Biomedical Sciences
2006-2007 Department	Joseph Franzee, MS. Candidate of Biomedical Sciences
2005-2007 Department	Ajeeth Ramanathan, MS. Candidate of Biomedical Sciences
2004-2009 Department	Halvor McGee, PhD Candidate of Biomedical Sciences
2004-2008 Department	Edward Moran, M.S. Candidate of Biomedical Sciences
2005-2009 Department	Benjamin Moore, M.S. Candidate of Biomedical Sciences
2005-2009	Toluwalope Mankinde, Ph.D. Candidate Department of Biomedical Sciences

2006-2009 Department	Zhifei Shao, Ph.D. Candidate of Biomedical Sciences
2007-present Department	Vincent Ngana, Ph.D. Candidate of Medical Microbiology and Immunology
2008	Sarah Brumfield, M.S. Candidate Department of Biomedical Sciences
2009-present	Jun Liu, Ph.D. Candidate Department of Medical Microbiology and Immunology
2008-2009	Brati Das, M.S. Candidate Department of Biomedical Sciences
2009-2010	Dalia Youssef, M.S. Candidate Department of Biomedical Sciences
2009-present	Rohit Gaurav, Ph.D. Candidate Department of Biomedical Sciences
2009-present Department	Kelsey Kokoburn, M.S. Candidate of Biomedical Sciences
2009-2010 Department	Jon Roxas, M.S. Candidate of Biomedical Sciences
2009-present Department	Cynthia Bangura, Ph.D. Candidate of Biomedical Sciences
2009-present Department	Tanupryia Agrawal, Ph.D. Candidate of Biomedical Sciences

Summer Students:

2000	Norma Flores Creighton University School of Medicine Minority Student Program College Student
2001	Odey Ukpo Creighton University School of Medicine Minority Student Program College Student
2001	Stacey Hopkins Creighton University School of Medicine Minority Student Program High School Student
2002	Tiffany Johnson

- Creighton University School of Medicine Minority Student Program
College Student
Third Place, Poster Competition
- 2003 Sola Fasusi
Creighton University School of Medicine Minority Student Program
College Student
First Place, Poster Competition
- 2003 Olufunke Oshunleti
Creighton University Medical Student
Recipient of National Medical Fellowship
- 2003 Lee Ann Vaughan
North High School Teacher
Recipient of American Association of Immunologists Teacher Award
- 2008 Jon Lindquist
Creighton University School of Medicine, M1 Student
Recipient of a fellowship from the Nebraska Medical Foundation
"The Roles of ErbB2 and EGFR in the Development of TMEV-Induced Lesions"
- 2008 Allison Rasband Lindquist
Creighton University School of Medicine, M1 Student
Recipient of a research fellowship from the Dean's Research Fund
"Role of Neuregulins in Myelin Preservation and Repair in the Central Nervous System"
- 2008 Andrew Schemmel
Creighton University School of Medicine, M1 Student
- 2010 Thomas Hendricks
Creighton University School of Medicine, M1 Student
Recipient of a fellowship from the Nebraska Medical Foundation
"miRNAs in a Mouse Model of Multiple Sclerosis"

IX. Committees

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| 2011-present | Committee on Rank and Tenure
Creighton University School of Medicine |
| 2010-present | Distinguished Lecture Committee
Creighton University School of Medicine |
| 2010-present | Executive Committee
Creighton University School of Medicine |
| 2010-present | Committee on Committees |

	Creighton University School of Medicine
2009-present	Advisory Committee, Clinical and Translational Research Creighton University School of Medicine
2009-present	Crisis Committee Creighton University
2009-present	Research and Compliance Committee Creighton University
2008-2011	Educational Policy Committee Creighton University School of Medicine
2008-present	Advancement Committee (Chair 2011-present) Creighton University School of Medicine
2008-present	Graduate and Post-Doctoral Program Task Force Creighton University School of Medicine
2006-2009	Continuing Medical Education Committee
2006-2009	Continuing Medical Education Committee Strategic Planning Subcommittee
2006-2007	Mentoring Committee: Women in Medicine and Science
2005-2007	Research and Development Committee, VA Medical Hospital, Omaha
2005-2006	Program Committee: Women in Medicine and Science
2005-2006	Pharmacology Chair Search Committee
2004 – 2006	Medical Student Research Advisory Committee Creighton University School of Medicine
2004	Dean of Research Search Committee Creighton University School of Medicine
2004	Basic Science Departments Organization Task Force Creighton University School of Medicine
2003 - 2007	Immunology Search Committee Department of Medical Microbiology and Immunology
2003 – 2006	Distinguished Lecture Committee Creighton University School of Medicine

2003	LCME	Accreditation Task Force Creighton University School of Medicine
2002 - 2004		Animal Resource Facility Emergency Planning Committee Creighton University School of Medicine
2000 – 2005		Virology Search Committee Department of Medical Microbiology and Immunology
2001 - 2002		Research Strategic Planning Committee: C06 Creighton University School of Medicine
2000 - present		Curriculum Committee Department of Medical Microbiology and Immunology

X. Community Service

2007-present	Board of Directors, National Multiple Sclerosis Society, Nebraska Chapter
2007, 2008	District 2 Representative, American Cancer Society, Lobby Day
2006-2010	District 2 Captain, American Cancer Society, Celebration on the Hill
2006-2010	Legislative Action Committee, American Cancer Society
2006	Speaker, American Cancer Society, Relay for Life, Fremont, NE, June
2006	Volunteer, American Cancer Society, Cancer Survivors Day, June 2006
2004-2011	Alzheimer's Disease Association, Medical Scientific Panel
2000-2001	Volunteer Conservation Corps
2000 – present	Opera Omaha Wardrobe