Schwann cells promote peripheral neuropathy through upregulation of extracellular matrix protein periostin and novel population of dedifferentiated Schwann cells

By
Rebecca A. Notini
Su Lab

Senior Honors Thesis
Department of Biology
University of North Carolina at Chapel Hill

03/20/2018

Thesis Readers:

Dr. Mark Peifer

Dr. Jonathan Berg

Abstract

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a debilitating autoimmune disease in humans characterized by sensorimotor impairment, demyelination, and inflammation via macrophage infiltration into the peripheral nervous system (PNS). The dysfunction of Schwann cells, as a major component of the PNS, is thought to contribute heavily to the observed motor impairment in CIDP. Here we investigate the role of Schwann cells in the development of CIDP. Since extracellular matrix (ECM) proteins are known to play a role in the inflammatory response, we examined the differences in ECM protein expression in the sciatic nerves of wild-type (WT) mice and mice with spontaneous autoimmune peripheral polyneuropathy (SAPP), a mouse model for human CIDP. We found that the ECM protein periostin (Postn) is upregulated by Schwann cells in mice with SAPP. Immunofluorescence showed that Postn expression is increased in the endoneurium, the interior of the nerve, in mice with SAPP whereas it is only expressed on the periphery of the endoneurium in WT mice. We also found that Postn elicits the migration of macrophages into the PNS, suggesting that Postn upregulation indirectly causes inflammation and nerve damage via macrophage recruitment. Additionally, we identified a novel population of cells in the PNS of mice with SAPP that express the marker CD49b along with classical markers for Schwann cells. qPCR and immunofluorescent staining show that these CD49b+ cells are a form of dedifferentiated Schwann cells, exhibiting lack of myelination, which is a possible explanation for the motor impairment seen in CIDP patients. Understanding the mechanisms behind the upregulation of Postn and CD49b+ cells and inhibiting their

upregulation and/or signaling could lead to treatments that suppress inflammation and neurodegeneration for CIDP patients.

Introduction

Chronic inflammatory demyelinating polyneuropathy (CIDP) is one of the most common inflammatory, autoimmune diseases of the PNS, with a prevalence of 1-9 in 100,000 (1, 2). This disease is characterized by demyelination and secondary axonal damage in the peripheral nervous system (PNS), which manifests as severe sensory and motor impairment (2). The underlying mechanisms of CIDP pathogenesis, however, are not well defined. To investigate the mechanisms of this disease, our lab used a mouse model for human CIDP. The mouse strain develops spontaneous autoimmune peripheral neuropathy (SAPP) due to a dominant, negative G228W mutation in the Autoimmune Regulator Gene (*Aire*$^{GW/+}$ mice). Experimentation with the SAPP model could provide further treatment options for CIDP patients. Current treatments for CIDP include corticosteroids, intravenous immunoglobulin, and plasmapheresis (3). However, patients exhibit variable levels of responsivity to such treatments and most patients must receive continuous treatment, which can be both physically and monetarily taxing (3). Therefore, a deeper understanding of the mechanisms behind the causation of CIDP is necessary for the development of novel treatments.

Schwann cells, which are crucial to the synthesis and maintenance of myelin in the PNS, may contribute to the development of peripheral neuropathies through loss of myelination. Schwann cells produce myelin along the neuronal axon, providing the insulation necessary for efficient signal propagation throughout the nervous system. The

Schwann cells themselves reside within the endoneurium, which consists of everything within the perineurium of the peripheral nerve (Supp. Fig. 1). In CIDP, some Schwann cells alter their morphology by exhibiting marked demyelination, inhibiting proper nerve signal propagation and causing patients to experience motor impairment and sensory deficits (4).

Schwann cells, in conjunction with extracellular matrix (ECM) proteins, have also been implicated in the cause of inflammation, although their role in inflammatory neuropathies has not been defined. The inflammatory response is normally part of the healing process, but can lead to nerve damage, and neuropathy, if it persists and the response is not dissipated (5). It has been shown that Schwann cells can secrete certain cytokines that act as pro-inflammatories through the recruitment of macrophages, which are classical inflammatory activators (6). However, the signal inducing macrophage recruitment into the PNS is not clear.

Our lab chose to investigate certain ECM proteins based on their involvement with Schwann cells and the PNS. The ECM protein thrombospondin (TSP) is present throughout the developing PNS and has been shown to have a role in nerve regeneration of the sciatic nerve (7). Thus, TSP could be important in peripheral neuropathies, where there is a great presence of nerve damage. Laminins are a family of ECM proteins residing in the PNS and have been found to play a large role in peripheral nerve myelination (8). Mouse mutants lacking certain laminins display severe hypomyelination in peripheral nerves (9). Finally, our lab has found that the ECM protein periostin (Postn) can induce macrophage chemotaxis, linking Postn to inflammation. Postn is, therefore, a perfect candidate to investigate the link between Schwann cells,

ECM proteins, and inflammation. We investigated the expression of these proteins within the sciatic nerve of WT and SAPP mice.

In addition, we investigated a novel population of cells in the PNS of SAPP mice that express the CD49b marker, a classical marker for natural killer (NK) cells. However, our findings suggest that this population of CD49b+ cells does not express typical markers for NK cells and instead expresses classical Schwann cell markers (10). We hypothesized that this novel population of cells may be a form of dedifferentiated Schwann cells, exhibiting a lack of myelination and offering a possible explanation for the loss of motor function exhibited in CIDP patients.

Methods

Animals

All experiments were performed in compliance with the Animal Welfare Act and the National Institute of Health (NIH) guidelines for the ethical care and use of animals in research. *Aire*$^{GW/+}$ mice were generated as previously described (11). SAPP mice were evaluated as previously described (12). SAPP was confirmed through electromyography (EMG), performed as previously described (13, 14).

qPCR

Primers: We used the following primers: Lama2 (Mm00550083_m1), Lama4 (Mm01193660_m1), Lamb1 (Mm00801853_m1), Lama5 (Mm01222029_m1), Postn (Mm01284919_m1), Thbs2 (Mm01279240_m1), Itga2 (Mm00434371_m1) and the primer probe set for Cyclophilin as an internal control.

Whole sciatic nerve tissue was homogenized by mortar and pestle followed by QIAshredder. RNA was isolated from whole nerve using Qiagen RNeasy Plus Micro kit. SuperScript II (Invitrogen) was used for cDNA synthesis. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for PCR. The Applied Biosystems QuantStudio 6 Flex Real Time PCR System and Quantstudio 6-7 software were used for data acquisition and analysis respectively (12).

Perfusion and Sciatic Nerve Harvest

In order to obtain fixed sciatic nerves, blood perfusion with 4% paraformaldehyde (PFA) in PBS fixative was performed on both WT and *Aire*$^{GW/+}$ mice. The thoracic cavity was cut open; the right atria was cut and a blunt needle administering the PFA was stuck into the left ventricle in order to pump the PFA to the rest of the body. The perfusion was stopped once the abdominal organs began to appear white in color. The sciatic nerves were then immediately harvested from the mice's legs.

Nerve Section Preparation for Immunofluorescence

Frozen (-80°C) sciatic nerve sections were allowed to air-dry at RT for 30 min. Sections were fixed in -20°C acetone for 10 min. Slides were dried at RT for an additional 60 min and then washed three times for 5 min (3x5min) in PBS containing 0.1% Tween (PBST). Sections were blocked with 10% GOAT serum in PBST. Each section was covered with 50µL of 10% GOAT serum and incubated in a humidified chamber for 60 min.

Immunofluorescent Staining

Primary antibodies were diluted in 3% bovine serum albumin (BSA) in PBST. Fixed nerve sections were stained with rat anti-mouse CD49b (clone DX5; BioLegend;

1:100) or rabbit anti-P75 (Abcam; 1:500). The slides were stored in a Parafilm sealed humidified chamber overnight at 4°C. Sections were washed 3x5min in PBST and incubated in a humidified chamber with DyLight594-conjugated goat anti-rat IgM (Thermo Fisher; 1:500) or DyLight 488-conjugated goat anti-rabbit IgG (Thermo Fisher; 1:500) for 2 hr at RT. Slides were washed in PBST and mounted with DAPI-Fluoromount-G.

Microscope

All images of sciatic nerve sections were taken on a Zeiss LSM 780 confocal microscope.

Results

To investigate extracellular matrix (ECM) protein expression in neuropathic *Aire*$^{GW/+}$ mice and age matched WT mice, we performed qPCR with primers from multiple ECM proteins associated with Schwann cells. While there was no difference in the mRNA levels of Lamb1, Lama2, or Lama5 between *Aire*$^{GW/+}$ and WT (Fig. 1C,D,F), Lama4 was significantly upregulated in *Aire*$^{GW/+}$ compared to the WT (Fig 1E). The increased expression of Lama4 was expected because it has been shown to be upregulated in the endoneurium of other mutant mouse models that exhibit severe hind limb paralysis (15). Thbs and Postn mRNA levels were very significantly upregulated in *Aire*$^{GW/+}$ compared to the WT (Fig. 1A,B). The increased expression of Thbs was expected because it has been previously shown to be upregulated following nerve injury and may be involved in nerve regeneration (7). However, the increased expression of Postn was surprising, as it has not been previously linked to peripheral nerve injury.

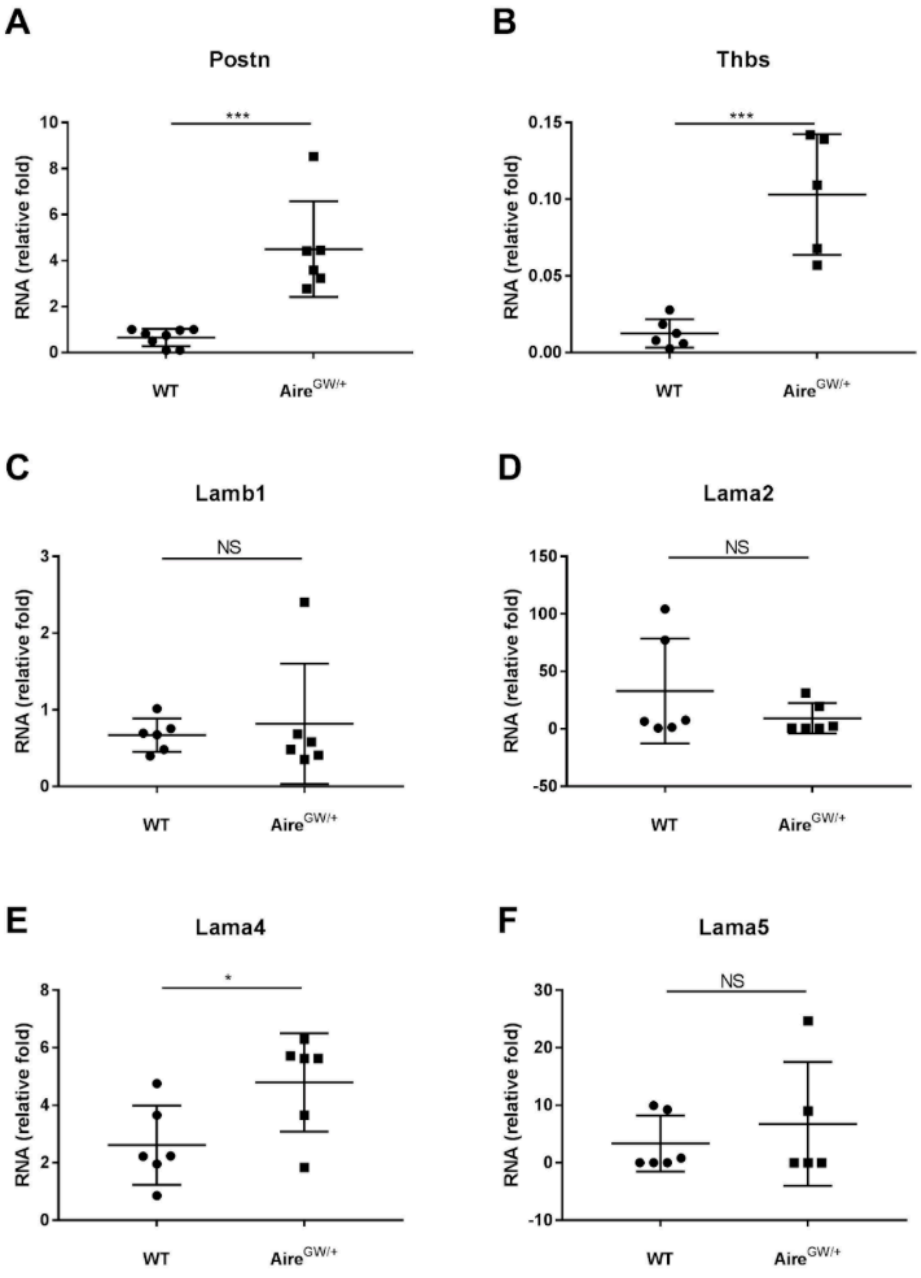


Figure 1. ECM proteins are upregulated during SAPP. RNA was taken from whole sciatic nerves of *Aire*$^{+/+}$ (WT) and *Aire*$^{GW/+}$ neuropathic mice. We measured expression of A) periostin (Postn), B) thrombospondin (Thbs), C) laminin β1 (Lamb1), D) laminin α2 (Lama2), E) laminin α4 (Lama4), and F) laminin α5 (lama5) relative to cyclophilin via qPCR.

The ECM protein Postn has been previously linked to inflammatory diseases (16, 17) and our lab has shown that Postn can induce macrophage chemotaxis (Supp. Fig. 2). We, therefore, further investigated the role of Postn in neuropathic mice. The expression of Postn was analyzed on a protein level in the PNS of neuropathic mice. Immunofluorescence showed that Postn is normally expressed on the periphery of the sciatic nerve (perineurium) in WT mice, but the protein is markedly increased in the interior of the nerve (endoneurium) in *Aire*$^{GW/+}$ mice (Fig. 2). Thus, Postn is upregulated in the PNS of neuropathic *Aire*$^{GW/+}$ mice.

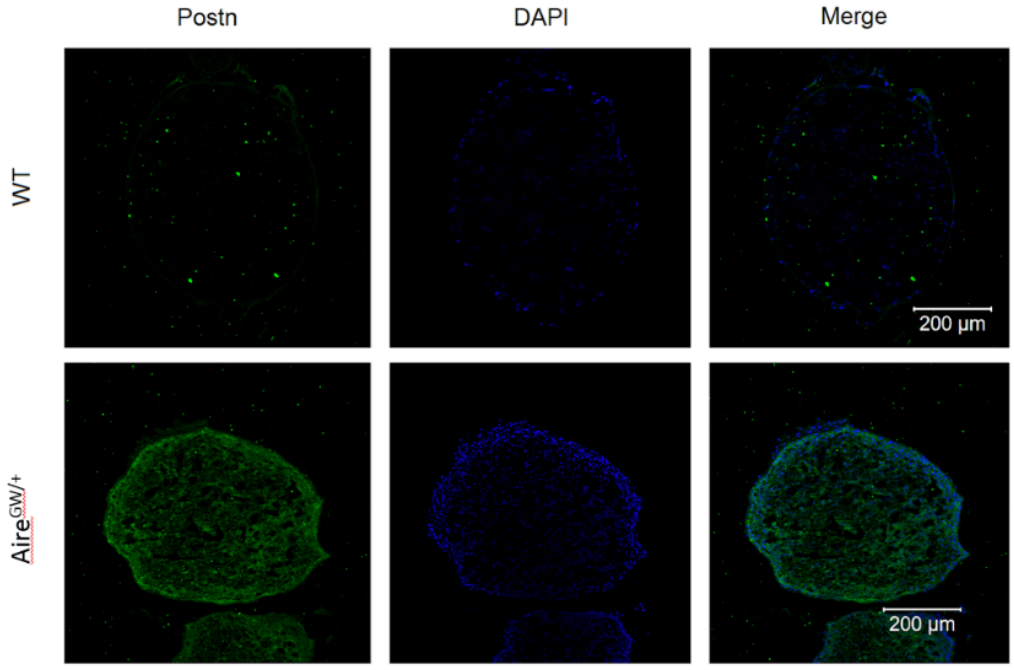


Figure 2. ECM protein periostin is upregulated in the endoneurium during SAPP. Immunofluorescent staining of cross sectional sciatic nerves of *Aire*$^{+/+}$ (WT) and *Aire*$^{GW/+}$ mice. The ECM matrix protein periostin (Postn) is shown in green.

Additionally, a novel population of CD49b+ cells was discovered to be upregulated in *Aire*$^{GW/+}$ mice. Mice with the *Aire*$^{GW/+}$ genotype develop SAPP, during which immune cells, such as macrophages, infiltrate the PNS. On the level of mRNA,

there was a significant upregulation of CD49b in *Aire*$^{GW/+}$ mice compared to WT mice (data not shown). CD49b is typically a marker for NK cells, thus our lab examined whether these CD49b+ cells were NK cells. We examined staining of both *Aire*$^{GW/+}$ and WT mice with NKp46, a more specific marker for NK cells, before and after the onset of neuropathy. We found that the sciatic nerves of *Aire*$^{GW/+}$ and WT mice both lacked expression of NKp46, suggesting that the CD49b+ cells in the sciatic nerve were not NK cells (data not shown). However, microarray analysis revealed that CD49b+ cells expressed classical markers for Schwann cells, implicating these cells are a form of Schwann cell (Supp. Fig. 3). Colocalization revealed that cells expressing CD49b in *Aire*$^{GW/+}$ mice also express p75, a dedifferentiated Schwann cell marker, suggesting that CD49b+ cells are a form of dedifferentiated Schwann cells located within the sciatic nerve of *Aire*$^{GW/+}$ mice (Fig. 3).

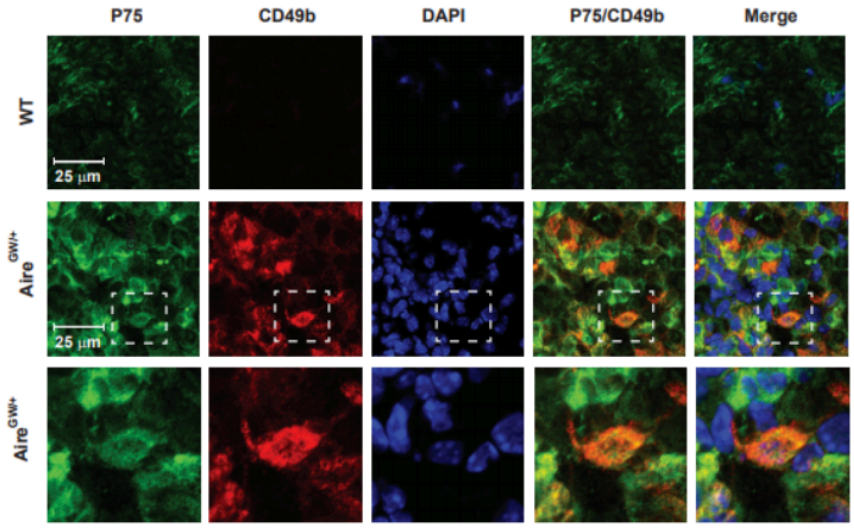


Figure 3. CD49b+ cells are upregulated and colocalize with P75 during SAPP. Immunofluorescent staining of cross-sectional sciatic nerves of *Aire*$^{+/+}$ (WT) and *Aire*$^{GW/+}$ mice. P75 (green) is a marker for Schwann cells, CD49b is a marker for Cd49b+ cells (red). Third row is a magnified image of the boxed area of second row, highlighting a single Schwann cell.

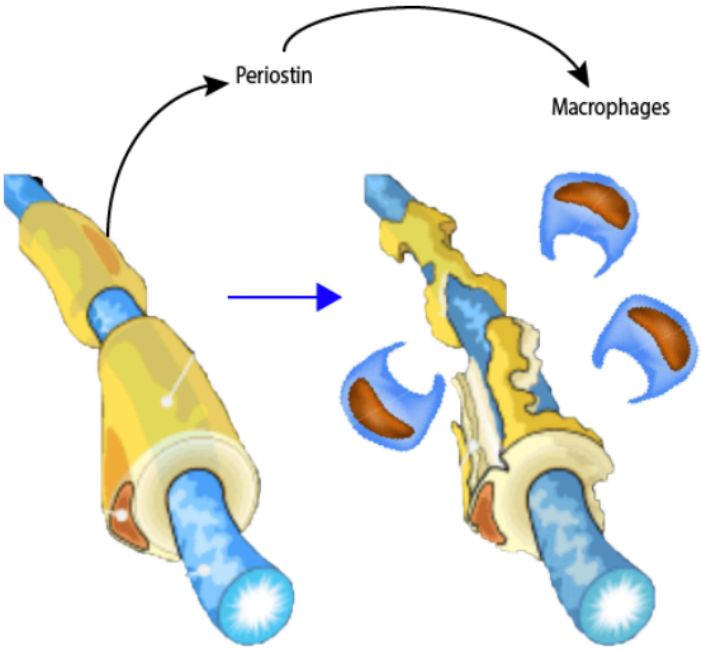


Figure 4. Schwann cells upregulated the ECM protein periostin during SAPP. Diagram of proposed mechanism for Schwann cell autoimmunity. Schwann cells upregulate the extracellular matrix protein periostin in the endoneurium. Periostin recruits macrophages, which break down the myelin of Schwann cells.

Discussion

Inflammation plays a large role in the development of peripheral neuropathies, although the underlying mechanisms are not defined. Here we propose that Schwann cells promote neuropathy by inducing the upregulation of the ECM protein periostin (Postn). Postn is upregulated in the endoneurium of *Aire*$^{GW/+}$ mice exhibiting spontaneous autoimmune peripheral neuropathy (SAPP). *Aire*$^{GW/+}$ mice with SAPP show severely decreased motor function in the nerve compared to the WT. However, our lab has demonstrated marked improvement in motor function of *Aire*$^{GW/+}$, Postn deficient mice, validated through electromyography (EMG), a test that demonstrates muscle response to nerve stimulation (data not shown). The upregulation of Postn, coupled with the pro-inflammatory role of Schwann cells and the link between Postn and

inflammation, suggests that Schwann cells may modulate autoimmunity in peripheral neuropathies through the upregulation of the ECM protein Postn.

Macrophages are also important to inflammatory neuropathies. Acting as phagocytes in the innate immune response, macrophages can lead to tissue destruction (18). Thus, macrophage over-activation causes tissue damage, leading to inflammatory chronic and autoimmune diseases, such as Crohn's disease, rheumatoid arthritis, and multiple sclerosis (19-21). Macrophage recruitment has also been shown to lead to hyperalgesia, a disease characterized by peripheral nerve damage (22). Depletion of macrophages in this disease reduces axonal damage (23). We have shown that Postn can recruit macrophages via transwell assay, corroborating that Postn promotes peripheral neuropathy through macrophage recruitment. We have also shown that Postn deficiency in mice "rescues" the *Aire*$^{GW/+}$ phenotype, exhibiting delayed onset of neuropathy and marked motor improvement (12). These results suggest that Postn works both downstream of Schwann cells and upstream of macrophages. We therefore postulate that Schwann cells upregulate the ECM protein Postn, which subsequently recruits macrophages, leading to autoimmune peripheral neuropathy (Fig. 4).

Additionally, our lab identified a population of CD49b cells that expand during autoimmune peripheral neuropathy. Although CD49b is a classical marker for NK cells, we found that this marker was not expressed by NK cells during SAPP but was expressed by a nerve-resident cell population. The CD49b+ population was found to co-express markers of precursor Schwann cells, suggesting that CD49b+ cells may be dedifferentiated, precursor Schwann cells. This possibility could help explain the observed demyelination and decreased motor function in *Aire*$^{GW/+}$ mice with SAPP.

Schwann cells may contribute to inflammatory peripheral neuropathy by upregulating the ECM protein Postn, subsequently promoting inflammation via macrophage chemotaxis, and through a dedifferentiated population of Schwann cells that exhibit decreased myelination. Further characterization of the mechanisms behind the upregulation of Postn and CD49b+ cells could lead to treatments that suppress inflammation and/or neurodegeneration. Targeting Postn, for example, could lead to delayed onset and improved motor function for CIDP patients.

Acknowledgments

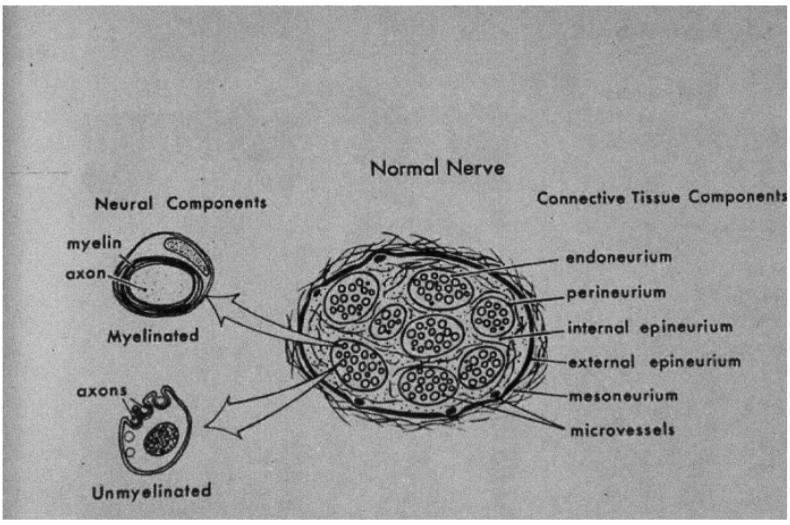
Special thanks to Dr. Maureen Su, MD and Denise Allard for their continual mentorship and guidance throughout my research project and for designing the study. Thanks to Yan Wang and Xiaopei L. Zeng for assisting with experiments. Microscopy was performed at the Neuroscience Center Microscopy Core Facility, supported, in part, by funding from the NIH-NINDS Neuroscience Center Support Grant P30 NS045892 and the NIH-NICHD Intellectual and Developmental Disabilities Research Center Support Grant U54 D079124. Research reported in this paper was supported by the National Institute of Neurological Disorders and Stroke Grant 5R01NS079683-02.

References

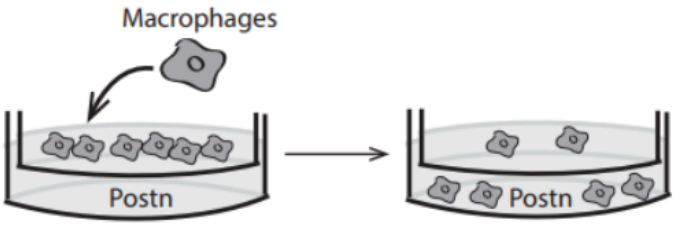
1. McLeod JG, Pollard JD, Macaskill P, et al.1999. Prevalence of chronic inflammatory demyelinating polyneuropathy in New South Wales, Australia. *Ann Neurol*, 46:910–13.
2. Mathey, E. K., Park, S. B., Hughes, R. A. C., Pollard, J. D., Armati, P. J., Barnett, M. H., … Lin, C. S.-Y. 2015. Chronic inflammatory demyelinating polyradiculoneuropathy: from pathology to phenotype. *Journal of Neurology, Neurosurgery, and Psychiatry*, 86(9):973–985.
3. Dalakas, M.C., and Medscape. 2011. Advances in the diagnosis, pathogenesis and treatment of CIDP. *Nat Rev Neurol,* 7:507-517.
4. Joshi, A.R., Holtmann, L., Bobylev, I., Schneider, C., Ritter, C., Weis, J., and Lehmann, H.C. 2008. Loss of Schwann cell plasticity in chronic inflammatory demyelinating polyneuropathy (CIDP) *Journal of Neuroinflammation,* 13:255
5. Medzhitov R. 2008. Origin and physiological roles of inflammation. *Nature*, 454:428-35.
6. Koski C.L. 1997. Mechanisms of Schwann cell damage in inflammatory neuropathy. *Journal of Infectious Diseases*, 176(6):S169–S172.
7. Hoffman, J.R., and O'Shea, K.S. 1999. Thrombospondin expression in nerve regeneration I. Comparison of sciatic nerve crush, transection, and long-term denervation. *Brain Res Bull* 48:413-420.
8. Sunada, Y., Bernier, S.M., Utani, A., Yamada, Y., Campbell, K.P. 1995. Identification of a novel mutant transcript of laminin alpha 2 chain gene responsible for muscular dystrophy and dysmyelination in dy2J mice. *Hum Mol Genet* 4:1055–1061
9. Chernousov, M. A., Yu, W.-M., Chen, Z.-L., Carey, D. J. and Strickland, S. 2008. Regulation of Schwann cell function by the extracellular matrix. *Glia,* 56:1498–1507.
10. Allard, D.E. *et al.* 2018a. CD49b (DX5) is expressed by Schwann cells and not NK or NKT cells during spontaneous autoimmune peripheral polyneuropathy. Unpublished manuscript, Department of Pediatrics, University of North Carolina at Chapel Hill, NC.
11. Su, M.A. et al. 2008. Mechanisms of an autoimmunity syndrome in mice caused by a dominant mutation in Aire. *J Clin Invest,* 118:1712-26.
12. Allard, D.E., *et al.* 2018b. Schwann cell-derived periostin promotes autoimmune peripheral polyneuropathy via macrophage recruitment. Manuscript submitted for publication.
13. Zeng, X.L., Nagavalli, A., Smith, C.J., Howard, J.F. & Su, M.A. 2013. Divergent effects of T cell costimulation and inflammatory cytokine production on autoimmune peripheral neuropathy provoked by Aire deficiency. *J Immunol,* 190:3895-904.
14. Xia, R.H., Yosef, N. & Ubogu, E.E. 2010. Dorsal caudal tail and sciatic motor nerve conduction studies in adult mice: technical aspects and normative data. *Muscle Nerve,* 41:850-6.
15. Yu, W.M., Yu H., Chen, Z.L. 2007. Laminins in Peripheral Nerve Development and Muscular Dystrophy. *Molecular Neurobiology,* 35(3):288-297.

16. Masuoka, M., Shiraishi, H., Ohta, S., Suzuki, S., Arima, K., Aoki, S., Toda, S., Inagaki, N., Kurihara, Y., Hayashida, S., et al. 2012. Periostin promotes chronic allergic inflammation in response to Th2 cytokines. *J Clin Invest,* 122:2590-2600.
17. Yokota, K., Kobayakawa, K., Saito, T., Hara, M., Kijima, K., Ohkawa, Y., Harada, A., Okazaki, K., Ishihara, K., Yoshida, S., et al. 2017. Periostin Promotes Scar Formation through the Interaction between Pericytes and Infiltrating Monocytes/Macrophages after Spinal Cord Injury. *Am J Pathol,* 187:639-653.
18. Wynn T.A., Chawla A., Pollard J.W. 2013. Macrophage biology in development, homeostasis and disease. Nature. 496:445–455.
19. Smith A.M., Rahman F.Z., Hayee B., Graham S.J., Marks D.J.B., Sewell G.W., et al. 2009. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med,* 206:1883–1897.
20. Kinne R.W., Bräuer R., Stuhlmüller B., Palombo-Kinne E., Burmester G.R. 2000. Macrophages in rheumatoid arthritis. *Arthritis Res*, 2:189–202.
21. Furlan R., Cuomo C., Martino G. 2009. Animal models of multiple sclerosis. *Methods Mol Biol*, 549:157–173.
22. Myers, R.R., Yamamoto, T., Yaksh, T.L., Powell, H.C. 1993. The role of focal nerve ischemia and Wallerian degeneration in peripheral nerve injury producing hyperesthesia. *Anesthesiology*, 78:308-316
23. Liu, T., van Rooijen, N., Tracey, D.J. 2000. Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain*, 86(1-2):25-32,.
24. Evans, G.R.D. 2001. Peripheral nerve injury: A review and approach to tissue engineered constructs. *Dev. Dyn.* 263(4):396-404.

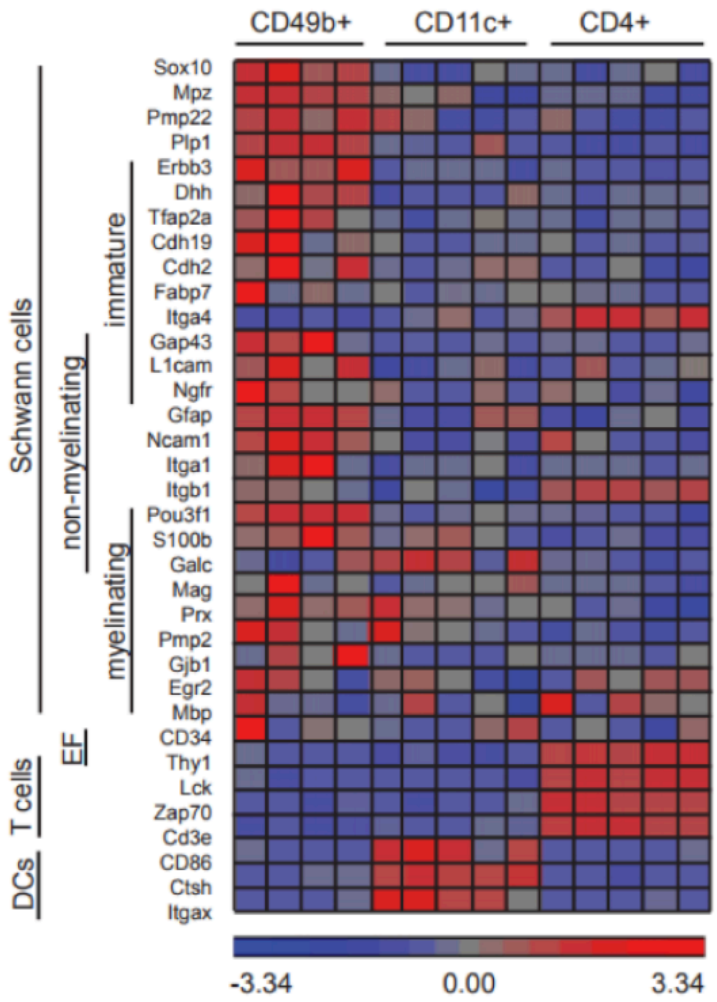
Appendix



Supplemental Figure 1. Anatomical components of the nerve fascicle (24).



Supplemental Figure 2. Diagram of a transwell assay. Macrophages were placed in the upper chamber of a transwell separated by a porous membrane; Postn was placed in the lower chamber of the well. Following 18 hr in culture, the number of cells in the bottom chamber was enumerated (12).



Supplemental Figure 3. Heat map showing expression of genes associated with Schwann cells, endoneurial fibroblasts (EF), T cells, and dendritic cells (DCs) in CD49b+ cells compared to CD11c+ and CD4+ cells isolated from *Aire*$^{GW/+}$ nerve (10).