THE ROLE OF DECORIN AND BIGLYCAN IN CNS ANGIOGENESIS AND EAE

RECOVERY

by

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DEDICATION

To my incredibly patient and unconditionally loving husband, Jacob, and my beautiful children, Gavin, and Evalynn. To my mom, who was the main driving force behind my motivation to pursue higher education. To my grandma, who helped me in so many ways, who I know is watching me from up above.

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ABSTRACT

Multiple Sclerosis (MS) is a severe demyelinating disease of the Central Nervous System (CNS) associated with an autoimmune response directed against myelin antigens. Angiogenesis, the formation of new blood vessels from preexisting ones, is a vital process for tissue regeneration and wound healing but is a pathological hallmark of both MS and an MS mouse model, experimental autoimmune encephalomyelitis (EAE). This type of aberrant angiogenesis is a fundamental event during an inflammatory injury, which is mutually dependent upon the intrusion of inflammatory molecules, disruption of the blood-brain barrier (BBB), and immature vessel formation. In the EAE recovery phase, this process is induced to repair tissue damage. Unfortunately, aberrant angiogenesis can lead to chronic lesion formation and slower recovery due to underdeveloped endothelial barrier integrity. A role for small leucine-rich proteoglycans (SLRP) in angiogenesis has been established in the periphery. However, their abundance in the CNS is typically low. Recently, the type I SLRPs, Decorin (DCN) and Biglycan (BGN), have been identified in MS and EAE brains, but their cellular source and functional role still need to be fully understood. Therefore, this study aimed to investigate the role of endothelial-derived DCN and BGN in EAE-induced aberrant angiogenesis. The hypothesis tested was that CNS endothelial knockout of SLRPs is associated with faster recovery in EAE-induced angiogenesis, BBB dysfunction, and clinical paralysis. We show that using endothelial-specific SLRP double knockout mice (BGN/DCN$^{\text{BBB/KO}}$) reduces aberrant angiogenesis and significantly enhances the recovery of EAE. In addition, using *in vitro* assays, primary

brain microvascular endothelial cells (BMVEC) lacking BGN/DCN were shown to have inhibited angiogenesis. Collectively, our data indicate that BGN/DCN$^{BBB/KO}$ mice had faster recovery of EAE associated with reduced angiogenic progression. Future studies, potentially using genetic interventions, are needed to explore SLRP inhibition as a therapeutic approach to inhibiting aberrant angiogenesis in MS recovery.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AF488   AlexFluor 488

BBB   Blood-Brain Barrier

BBB-EC   Blood-Brain Barrier Endothelial Cell

BBB-KO   Blood-Brain Barrier – Knock Out

BGN   Biglycan

Bgn-floxed   Mice carrying the Bgntm1.1Debi allele

BM   Basement Membrane

BME   Basement Membrane Extract

BMVEC   Brain microvascular endothelial cells

BSA   Bovine Serum Albumin

CD31   Cluster of Differentiation 31

CD4   Cluster of Differentiation 4

CM   Conditioned Media

CNS   Central Nervous System

CNS-EC   Central Nervous System Endothelial Cell

COL1   Collagen Type I

COL4   Collagen Type IV

CS	Clinical Score
d.p.i.	Days post-induction
d.p.i.	Days post-induction
d.p.o.	Days post-onset
d.p.s.	Days post-seeding
DCN	Decorin
Dcn-floxed	Mice carrying the Dcntm1.1Debi allele
DMEM	Dulbecco's Modified Eagle's Medium
EAE	Experimental Autoimmune Encephalomyelitis
EC	Endothelial Cell
ECIS-TEER	Electric Cell-Substrate Impedance Sensing Transendothelial Electrical Resistance
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
HBSS	Hanks Buffered Salt Solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
Hz	Hertz
i.p.	Intraperitoneally
IFN-β	Interferon Beta
IFN-γ	Interferon Gamma

IL-12	Interleukin-12
IL-17	Interleukin-17
kHz	kiloHertz
KO	Knockout
MBP	Myelin Basic Protein
MOG	Myelin Oligodendrocyte Glycoprotein
MOG35-55	Myelin Oligodendrocyte Glycoprotein (residues 35-55)
MS	Multiple Sclerosis
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PPMS	Primary Progressive Multiple Sclerosis
PRMS	Progressive-relapsing Multiple Sclerosis
Rb	Cell-Cell Adhesion Parameter for ECIS-TEER Modeling
RRMS	Relapsing-remitting Multiple Sclerosis
SB	Slco1c1-Cre x Bgn-floxed Mice
SBD	Slco1c1-Cre x Bgn-floxed x Dcn-floxed Mice
SD	Slco1c1-Cre x Dcn-floxed Mice
siRNA	Small interfering RNA
Slco1c1-Cre	Mice carrying the Tg(Slco1c1-iCre/ERT2)1Mrks transgene

SLRP	Small Leucine-rich Proteoglycan
SPMS	Secondary Progressive Multiple Sclerosis
TAT-Cre	Recombinant Cre recombinase (Cre) with a cell-penetrating peptide tag from HIV(TAT)
TAT-HA2	Fusogenic peptide from influenza hemagglutinin
TCR	T Cell Receptor
TEER	Transendothelial Electrical Resistance
TGF-β	Transforming Growth Factor Beta
Tie2-Cre	Mice carrying the Tg(Tek-cre/ERT2)1Arnd transgene
TL	Tomato lectin
TNF-α	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor
WM	White Matter
WT	Wild-type

CHAPTER ONE: MULTIPLE SCLEROSIS AND THE ROLE OF ANGIOGENESIS

Introduction

Multiple Sclerosis

" Now a new disease began to show itself: every day, I found gradually my strength leaving me. A torpor or numbness and want of sensation became apparent about the end of my back-bone... At length, about the 4th of December my strength of legs had quite left me… I remained in this extreme state of weakness for about 21 days".[1]

To truly understand the history of Multiple Sclerosis (MS), you can travel back to the first known personal and possibly the first patient's description of MS, which is found in the diary of Sir Augustus D'Este, grandson of King George III.[2] Aspects of the pathology of this debilitating disease were described as early as the mid-1800s, yet there is still no known cure or promising treatment.

Multiple Sclerosis is a severe demyelinating disease of the Central Nervous System (CNS) associated with an autoimmune-mediated response directed against myelin-specific antigens. The pathological signature of MS is the white matter plaque, a circumscribed area of demyelination.[3] It is characterized by damage to the myelin sheaths surrounding the axons of neurons. This myelin damage disrupts the conduction of action potentials along these axons, which is thought to cause many of the symptoms of the disease.[4] The brains

of MS patients can contain evidence of this damage in the form of plaques or visible lesions with some types of neuroimaging.[5] However, the disease's causes are not fully understood and are thought to involve genetic and environmental factors. The deposition of antibodies and activation of complements associated with vesicular disintegration of the myelin membrane is present in most lesions in MS.[6, 7]

A recent report estimated that approximately one million U.S. adults live with MS. The burden of MS in the United States has historically been underestimated, but the total economic burden in 2022 was estimated at $85.4 billion.[8] MS patients suffer a broad range of neurological functions, including loss of vision, gait, motor function, cognition, coordination, balance, and bladder and bowel functions.[9, 10] Despite enormous advances in our understanding of MS pathology and clinical care, treatment options are non-curative and do not prevent or repair neurodegeneration. Meanwhile, MS remains a severe and debilitating condition.[11, 12]

Pathophysiological Features of Multiple Sclerosis

The critical element of diagnosis is a neurologic event and its subsequent resolution.[13] There are four main clinical forms of MS: relapsing remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), and progressive relapsing MS (PRMS).[14, 15] The most common form of MS, RRMS, is associated with acute inflammatory episodes resulting in reduced neurological function.[16] At the beginning of this lifelong and disabling RRMS, several neurological symptoms develop over several days, then improve over a period of days to weeks.[17] These relapses are usually characterized by a combination of symptoms such as sensory disorders, optic dysfunction, limb weakness,

and fatigue.[18] In addition, infiltration by the immune cells and demyelination in brain white matter usually worsen the severity of these clinical symptoms.[19]

Periods of clinical remissions vary in length and last several years but are not always permanent. Roughly fifteen percent of patients begin the disease course by experiencing gradually progressive neurological function, typically a slowly worsening myelopathy or PPMS.[18] Similarly, two-thirds of patients with RRMS eventually undergo a similar fate; as relapse frequency lessens over time, progressive neurological dysfunction emerges, signaling the development of SPMS.[20] Patients may experience some recovery between relapses, but most patients with RRMS evolve to a more progressive form, SPMS. Some patients who transform to a secondary progressive course experience superimposed relapse. Finally, a steadily worsening neurologic function from the beginning with occasional relapses will result in PRMS.[16] Increasing attention is being paid to the role of axonal injury and loss, the likely correlate of progressive and irreparable injury in MS.[3]

MS pathogenesis - Immune cell regulators

The breakdown of the BBB is an early step in the onset of the disease, which causes demyelination and axonal loss, leading to neurodegeneration and irreversible neurological impairment[21] **(Figure 1.1).** Collective evidence suggests that a significant hallmark of MS is the disease onset due to a T-cell-mediated aberrant immune response directed against several CNS-specific myelin antigens. During an inflammatory event, an immune response occurs where T cells, B cells, and myeloid cells infiltrate the CNS and lesions of MS patients.[22] Lymphocyte activation, extravasation, and recruitment, involve the turning on and off of several genes, thus triggering specific transcriptional pathways.[23] Additionally,

neutrophils have a crucial role in the pathogenesis of chronic inflammation, and when extravasated, they release multiple signaling compounds, and form extracellular traps for circulating pathogens.[24] During neuroinflammatory injury, however, these processes can worsen disease progress and outcome.

Both B and T cells accumulate in active white matter lesions of the MS brain.[25] In diagnostic biopsy studies, T-cell-dominated inflammation is a characteristic of all lesion types observed.[26] Also in post-mortem MS lesions, white matter MS lesions with active demyelination are associated with increased T cell numbers.[27] T cells move from the blood into the brain and attack their target. It has been shown that a robust MHC class II up-regulation suggests that antigen is being presented locally to activated T cells.[28] Once T cells are activated, they release special proteins called cytokines. Again, it has been shown that a robust MHC class II up-regulation suggests that antigen is being presented to activated T cells locally. Cytokines destroy the myelin sheath of neurons. Cytokines also signal other white blood cells called macrophages, release toxins that destroy myelin, and cut nerve fibers.[29]

Vascular Disease and Endothelial Dysfunction in MS/EAE

Endothelial cells, which form the inner lining of blood vessels and compose the blood–brain barrier (BBB), play a critical role in the CNS by controlling the exchange of biological substances essential for the brain's metabolic activity and neuronal function. [30-32] The functional and structural integrity of BBB endothelial cells is critical to maintain the homeostasis of the brain.[33] During an inflammatory injury, an increased BBB permeability occurs in an ongoing vascular and brain injury site.[34, 35]

Under normal physiological conditions, the CNS has been considered an immune-privileged environment and consists of only a limited number of lymphocytes that cross the BBB.[36] The main question concerning the infiltration of activated T cells into the brain is associated with the molecular nature of the interactions between the membrane of T cells and the endothelial cells in health and disease. Unfortunately, there do not appear to be any tissue-specific answers addressing what governs the process.[37]

Since it cannot reasonably be assumed that the endothelial cells in a healthy brain can change during the arrival of T cells, they must constitutively express the ligands necessary for activated T cell passage. Thus, the molecular ligands leading to T cell traffic must be themselves dictated by the activation of the T cell itself. However, what those molecules are is still unknown.

Mounting evidence has implicated the role of vascular disease in contributing to the MS pathogenesis and disease progression. Indeed, BBB compromise, microhemorrhages, reduced blood flow, and aberrant angiogenesis has all been reported in MS histopathology.[38] As the gatekeeper of blood-to-brain entry of immune cells and plasma solutes, vascular endothelial cells (EC) endure the difficult task of monitoring the inflammatory injury. During an aberrant inflammatory response, however, BBB dysfunction can be triggered by mediators from circulation, which act upon endothelial cells (ECs) to reduce their cell–cell adhesive forces.[39]

Angiogenesis in MS/EAE

Angiogenesis, the process of new blood vessels forming from pre-existing ones, is induced by CNS inflammation. For angiogenesis to occur, endothelial cells need to proliferate, migrate and undergo tubulogenesis.[40] These progressions require growth factors, intracellular signaling, as well as interactions with basement membrane (BM) extracellular matrix components.[41] The mechanism by which endothelial cells (ECs) contribute to the purpose of angiogenesis is yet unclear.

It has been shown that despite the initial beneficial effect of angiogenesis, in an uncontrolled environment, aberrant angiogenesis can potentially cause more harm[42], acting as a "double edge sword" in clinical MS. In healthy conditions, EC functions are tightly regulated by angiogenic factors needed for development and regeneration or tissue repair.[43] In MS disease progression, however, excess synthesis of pro-angiogenic factors has been reported, which leads to increased endothelial cell proliferation and migration.[44]

Angiogenesis, during inflammatory injury, depends upon intrusion of inflammatory molecules and endothelial cells (EC) and results in the formation and infiltration of new vessels into damaged hypoxic CNS environments, to provide struggling affected cells with needed nutrients and oxygen.[45] Some cells induce angiogenesis via their ability to produce and secrete various pro-angiogenic factors VEGF.[46]

In contrast, very little is known concerning the role of ECs during this process, particularly their ability to remodel vascular microenvironments during angiogenesis. Although ECs are known to remodel their microenvironment by secreting various extracellular proteases, a thorough understanding of how these molecules mediate angiogenesis activation or recovery remains unclear.

Identifying and characterizing novel proteins secreted by angiogenic ECs will offer essential insights into the role of endothelial cells in remodeling the vascular microenvironment during angiogenesis, as well as the potential to therapeutically target novel mechanisms to prevent angiogenesis.

Identification of SLRPs in Angiogenic Pathologies during MS/EAE

There has been a great deal of effort devoted to characterizing MS/EAE lesions. Recent studies have shown an alteration in the CNS ECM during MS, including basement membrane-related ECM proteins.[47] Interestingly, two genes/proteins that are critical for angiogenesis were identified. The two ECM proteoglycans Biglycan (Bgn) and Decorin (Dcn) belong to the small leucine-rich proteoglycan (SLRP) family of proteins and are known to decorate fibrillar collagens.[47, 48] A disease-related phenomenon has been observed in MS lesions, and these proteins, have been identified in the extended perivascular space and in chronic active and acute lesions, where the infiltrating immune cells in the perivascular space were in close contact with these proteins.[47] Both, Bgn and Dcn were also found between the endothelium and astrocytic glia in the perivascular space where they formed a meshwork closely associated with infiltrating immune cells.[47] In addition, increased levels of Bgn and Dcn have been reported to be present as molecular components of the perivascular fibrosis in MS lesions and fibrous structures around blood vessels in chronic CNS inflammation. Interestingly, in healthy ECs, including those of the BBB, Bgn is present at the mRNA and protein level while DCN is completely absent.[49-53] Conversely, high levels of Bgn and Dcn have been observed around angiogenic vessels located within inflamed tissues, including lipid-rich/necrotic cores of athlerosclerotic

plaques,[54] granulomatous tissues,[55] granulomas,[56] and granulation tissues of healing dermal wounds.[56] Most recently, in support of our hypothesis, Dcn and Bgn were identified in the perivascular space of vessels in inflammatory lesions of MS patients as well.[47] As it turns out, Dcn can play stimulatory and inhibitory roles as in the processes of wound repair, angiogenesis, tumorigenesis, and autophagy.[57-59]

Their cellular sources and functional significance are still a mystery, which prompted me to analyze the role of these SLRP proteins in angiogenesis and to analyze whether or not they might be accountable for reduction in the rate of recovery and ideally prevent the severeness of the chronic remission stage of MS and EAE. As their roles in inflammation and angiogenesis are poorly understood, I investigated whether they could influence barrier function and endothelial cell proliferation.

Conclusion

In conclusion, it is still unclear, how Dcn and Bgn affect angiogenesis or overall recovery in EAE and requires further investigation. This thesis examines the role SLRPs (Dcn and Bgn) play in angiogenic pathologies during MS and EAE and the affects they have on the BBB as ECM proteins, during chronic inflammation. Treatment of inflammatory diseases today is mainly based on interrupting the action of mediators that drive the host's response to injury.[60] Non-steroidal anti-inflammatories, steroids, and antihistamines, for instance, are developed and provide the main treatment for inflammatory diseases. We offer an alternative approach to the development of novel therapeutics based on the endogenous mediators and mechanisms that switch off acute

inflammation and bring about its resolution. It is thought that this strategy will open up new avenues for the future management of inflammation-based diseases.
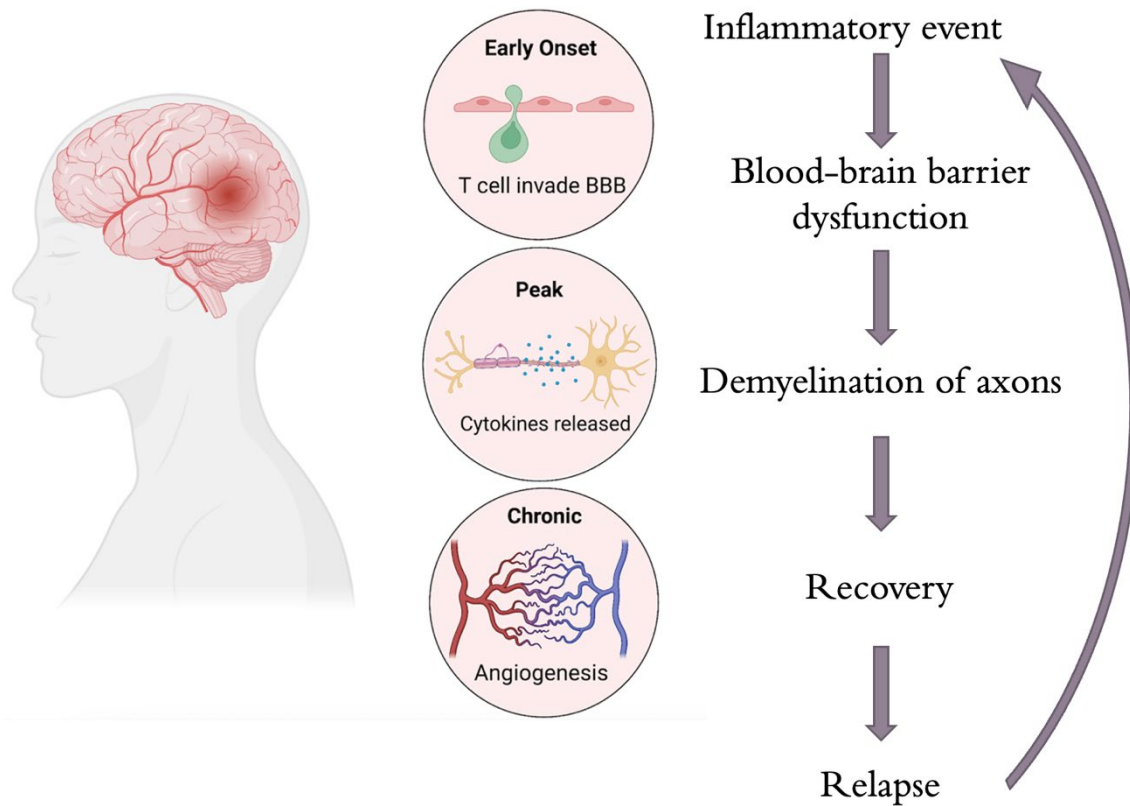
Figure 1.1   Pathological hallmarks related to MS and EAE.
Early-stage chronic neuroinflammation and neurodegeneration in the later stages of the disease: all of which include loss of blood-brain integrity, infiltration of immune cells, the release of cytokines, axonal dysfunction, recovery attempt through angiogenesis, and lastly, relapse of symptoms. Created with **BioRender.com**.

CHAPTER TWO: ANIMAL MODEL

Chapter 2A: Eradication of pathogens from transgenic mice colonies.

To begin testing our hypothesis and to determine the pathological role of Dcn and Bgn in MS EAE, we ordered an important specific Cre mouse line that is restricted to BBB-ECs - Slco1c1-iCreERT2, that was transferred to our vivarium from Dr. Markus Schwaninger (University of Lubeck). Fecal samples obtained during quarantine tested positive by PCR via an external vendor for murine Norovirus (MNV) and *Helicobacter spp*. The decision was made to try and eradicate the pathogens from these newly arrived mice before introducing them to our mouse colony so that future experiments are not altered by outside factors.

We implemented a comprehensive cross-fostering protocol together with a specialized drug diet, careful sanitary measures, and thorough fecal PCR testing to eradicate the MNV virus and *Helicobacter spp.* simultaneously from new mice that recently arrived at our animal facility. The validity and reliability of experiments that use *in vivo* models depend on controlling for variables that can influence the experimental results. Mouse colonies infected with pathogens are a significant burden to the scientific community because they introduce harmful variables to experimental outcomes, deeming them misrepresented and unreliable. Therefore, using infected mice in biomedical experimentation can have profound effects on research.[61]

Murine norovirus (MNV) is a newly discovered and extremely prevalent pathogen[62] infecting laboratory mice and causing persistent infections in immunocompetent mice.[63] In addition to MNV, infection of mouse colonies with *Helicobacter* spp. has also become an increasing concern for the research community[64], as it can confound cancer studies and alter inflammatory responses in diseased mice.[65, 66]

It has been previously shown that the use of pathogen-free animals is possible when proper protocols are set in place.[67] The goal for pathogen-free animal facilities should be driven by the efforts of all animal facility personnel to facilitate scientific research, with an intent on seeing accurate animal-based research conducted, and by investigators aware of the impact of pathogens on research results. Most modern animal facilities already incorporate health monitoring and quarantine procedures into their animal care program, however, protocols that successfully eradicate multiple pathogens concurrently from animal facilities are far and between.

Rederivation of mice can be achieved by processes that include embryo transfer, hysterectomy, or cross-fostering neonatal pups to surrogate mothers.[65] In our study, in particular, we proposed a first modified protocol that has successfully proven to eradicate MNV and *Helicobacter spp.* simultaneously from mice that were found positive for both of these pathogens, and we have demonstrated the effective use of cross-fostering in conjunction with a specialized drug diet, and strict quarantine procedure in the elimination of such pathogens.

Procedure

Cross-fostering one-day-old pups

MNV-negative breeders, from our MNV-negative holding room, were used as the foster dams. P1 litters from MNV-positive cages were separated from the contaminated dam and transferred to a new cage with foster dams in the designated surrogate room. All but 1 pup from a litter of the surrogate dam were culled and replaced with pups from the contaminated dam.

There were two technicians present during the transfer procedure, where technician (1) entered the quarantine room to exchange the newborn pups with technician (2) who is on the other side of the Dual Access Biosafety Cabinet was holding a new autoclaved cage, layered with a paper towel sprayed with Virkon S.

The technician (1) that was present in the quarantine room removed and properly disposed of all contaminated safety gear (gown, hat, mask) and did not enter the surrogate or the MNV-negative room until the following day. P1 pups were then transferred to a surrogate room by receiving technician (2). At all times while handling these mice, the highest level of aseptic techniques will be employed. The handling of surrogate cages was to be considered contaminated until proven otherwise.

The following detailed steps as shown in **Figure 2.1,** were taken to increase the success of foster dams accepting new pups:

1) Ideal foster dams were chosen from existing breeders who already have had two or three successfully weaned litters with no history of cannibalism.

2) To remove unrecognizable scents from pups, they were carefully rolled and dusted in debris from the MNV-negative foster cage.

3) Trio breeding (one male and two female mice) was used in the surrogate caging and the sire was removed after the dams were pregnant.

4) If both foster dams in the surrogate cage had litters, one litter was removed, and replaced with new pups. However, if only one litter was present, then all but one pup from that litter was culled and replaced with new pups.

5) After transferring the pups, the foster cage and mice were not disturbed until our standard toe and tail biopsies were performed for ID and genotyping in the surrogate room.

Methods

Mouse colony

All mice used in this case study were generic transgenic mice on the C57BL/6J background. Our mouse colony consists of three mouse holding rooms: one with stand-alone filter-equipped cages, one with quarantine cages exposed to MNV / *Helicobacter spp*, and lastly, with stand-alone surrogate cages containing surrogate mothers. The quarantine room is separate from the other two rooms and has a separate entrance. All animals coming into the BRV facility are quarantined before entry into the main mouse population. All experimental procedures involving animals were approved by an institutional animal care and use committee (IACUC). All animal use was approved by the

Institutional Animal Care and Use Committee at Boise State University and was performed following the Guide for the Care and Use of Laboratory Animals.

Husbandry

All mice were kept under a 12-hour light/dark cycle with food (LabDiet Drug Helicobacter mouse diet) and water ad libitum regardless of which housing room they were housed within. Mice used for fostering were bred in-house and were housed in an MNV-negative holding room. Bedding mixed with nesting squares, rodent houses, and water mixed with (HCL) were used for quarantine cages in the MNV-positive holding room. Cages were changed every fourteen days, and water bottles were changed once they reached the 150 mL mark. Dirty cages were sanitized in a cage and rack washer using detergent. A changing hood was used for cage manipulations and exchange.

Quarantine

Mice entering the facility were housed in the quarantine room until pathogen status was confirmed. The quarantine room requires that all personnel wore protective clothing, this included a disposable gown, gloves, mask, hat, and double booties. All cage manipulations were performed in a Dual Access Biosafety Cabinet with soiled cages sprayed with disinfectant Virkon-S broad-spectrum disinfectant (Fisher Scientific) before exiting the room. After the 14 days quarantine period was completed, fecal samples from the mice were collected and shipped to Charles River Laboratories for independent PCR testing. If all screening results were negative, the mice were then able to be released to the main mouse holding rooms.

<u>Surrogate room</u>

The mice used as surrogates' mothers for cross-fostering were housed in a separate limited-access room in autoclaved caging with an irradiated diet, autoclaved municipal water in bottles, and autoclaved bedding. The cages were changed in a Class II Type A biosafety cabinet and dirty cage parts were reassembled in the hood before transfer to the cagewash area.

<u>Sample collection and MNV testing</u>

Fecal pellets were collected from mice by using autoclaved forceps and placed in a vial provided by Charles River. Fecal samples from the litter were sent to Charles River Laboratories for MNV and *Helicobacter spp.* testing 7, 14, and 30 days after fostering, using their Research Animal Diagnostic Services through PCR Rodent Infectious Agent (PRIA) Direct Sampling and TaqMan® PCR testing to identify any viral, bacterial, or parasitic agents in our animal colonies.

<u>Pathogen testing</u>

Lab animal infectious agent PCR testing was used to identify viral, bacterial, fungal, and parasitic agents in animal colonies. ("Lab Animal Infectious Agent Testing | Charles River") Charles River's PCR assays are available as panels that cover all commonly excluded or reported pathogens.

<u>Sanitary procedures</u>

Animal facility personnel wear dedicated work boots that do not leave the facility. Disposable gowns, gloves, hats, masks, and an additional set of booties are put on when personnel enter the animal room and are removed on leaving the room. The MNV-negative room was dealt with first, and the quarantine room was always dealt with last with no re-entry into any other mouse-holding rooms until the next day. Cages were changed in Dual Access Biosafety Cabinet that was wiped down with Virkon before and after use. All instruments were autoclaved or thoroughly wiped down with Virkon. All surfaces of the room were cleansed with Virkon.

Results

Here we report the results of a successful attempt to eradicate MNV and *Helicobacter spp.* simultaneously from mice that were found positive for both of these pathogens on arrival at our facilities. We pooled the samples into 1 sample for Charles River Diagnostic. The mice remained in quarantine until tests from Charles River were reviewed to decide if the mice would be cleared to enter the rest of the mouse colony. The initial results on newly arrived mice indicated that they tested positive for *Helicobacter spp*. and MNV as stated in **Table 1**. The presence of *Helicobacter spp* was determined by polymerase chain reaction (PCR) analysis of fecal samples by Charles River.

Since our mouse colony, as well as the rest of our colony in the holding room, has always tested negative, we decided to avoid the spread of pathogens within the mouse colony. These mice were held in quarantine and set up as breeder pairs, and the use of

cross-fostering in conjunction with a specialized *Helicobacter* drug diet, and strict quarantine procedure was performed as described previously.

A total of 3 litters were studied. Cross-fostered pups from these 3 litters were tested for the presence of *Helicobacter spp.* and MNV, utilizing fecal PCR testing at 7, 14, and 30 days old. The results proved the use of cross-fostering in conjunction with a specialized *Helicobacter* drug diet, and strict quarantine procedure was successful in the elimination of both pathogens simultaneously. Successful elimination of MNV infection was also diagnosed by PCR performed at Charles River as stated in **Table 1**. Therefore, we report that MNV does not appear to be transferred readily to neonatal litters. The *Helicobacter* treatment diet (21-day treatment) was also successful in eliminating the pathogen and breeding that occurred while on the diet was beneficial for all pups born. Limited access to the surrogate room was also critical for success. Dedicated and trained staff were used to ensure that standard operating procedures were followed.

We had no evidence of any additional disease transmission for any of the agents in the room that housed the surrogate mothers. Four years have passed since the last litter was tested in this report, and we have had no evidence of contamination with previous pathogens as shown in **Table 1**.

Discussion

Our biomedical research facility was aware of the profound effects that pathogens including the MNV virus can have on our research and therefore attempted, for the first time, to eliminate the MNV and *Helicobacter spp.* by creating new and improved

guidelines for cross-fostering, customized drug diet and strict sanitary procedures before assimilating the new transgenic line into the main mouse colony.

Previous studies offer several methods available to perform to remove certain pathogens from existing mouse colonies.[68] The methods available at this time include rederivation by embryo transfer, as is performed by large mouse vendors such as Jackson Laboratories and Charles River. This method has the advantage of the likely removal of unknown, as well as known, pathogens. However, this procedure is costly if many strains of mice need to be decontaminated.

There is also the method of total depopulation and decontamination of the room with chlorine dioxide solution. However, this method required the identification of alternative housing for hundreds of mice and the euthanasia of nonessential mice.[66] Another alternative method is the identification and removal of infected mice which is ultimately ineffective as stated by a previous case study, where it required extensive and expensive testing as well as precise coordination between the veterinarian and technicians to identify positive cages and test all cages surrounding the infected one. This finding as well as other previous case studies published did not describe the sanitary measures[68] used to prevent contamination from room to room or cage to cage and suggested that a test-and-removal eradication system without environmental decontamination was ineffective in eliminating MNV and other pathogens simultaneously[66]

This further demonstrates the reasoning behind our modifications and the creation of new guidelines that offer sanitary procedures to be a crucial part of the eradication of the MNV virus, as well as *Helicobacter spp*. Our case study reveals that MNV is not easily

eradicated using a test and removal procedure and further strengthens why our approach is better and how it can be beneficial to other research facilities.

The primary advantage of using cross-fostering as a means of rederivation is that it is a viable low-cost method for the rederivation of mouse colonies contaminated with pathogens[69] and it is also less invasive and less demanding than embryo transfer. Studies on cross-fostering have reported that fostered pups, in particular, test consistently negative for *Helicobacter spp.*[70] however literature lacks reports of eliminating this pathogen together alongside MNV and other viruses.

The efficacy of embryo transfer in the elimination of viral diseases has also been examined as one of the techniques to be used to replace the rederivation of animals.[71] However, the disadvantages of embryo transfers include the inability to obtain mice with the desired genotype. As it was reported by Van Keurin et. al (2004), the pregnancy rate was only 45% and none of the 135 pups carried the desired genotype. Although they successfully eliminated the pathogens in all transfers, they were unable to obtain pups with the desired genotype in 15 of 111 mouse lines.[72] Multiple factors affect the efficiency of rederivation by embryo transfer and affect the overall yield in the numbers of embryo transfers and desired genotypes. In addition, the possibility of other common murine virus diseases being transmitted during embryo transfer remains unresolved, prompting our decision to eliminate embryo transfer as the method used in our facility.

In our study, we evaluated the reliability and likelihood of using cross-foster rederivation in combination with a drug diet customized to *Helicobacter spp* as a means to eliminate not only *Helicobacter spp*. but also MNV from new mice before entering the rest of our mouse colony.

Our additional modifications to the cross-fostering procedure included very careful quarantine steps taken. All potentially contaminated items, including biological safety hoods, pens, measuring devices, mouse procedure cages, and so forth, need to be decontaminated by autoclaving or with appropriate disinfectants such as Virkon S. Another important potential problem is the contamination of the newborn pups, which can infect the foster mother and then the litter in turn. We included an additional step in which pups were placed on a paper towel sprayed with Virkon S before transfer to the foster mother. Two extra rooms are also desirable, one for the newly fostered mice and one used only for mice that test negative after repeat testing. Furthermore, PCR fecal testing may be positive before seroconversion, and this early warning may be important in stemming an outbreak. Although PCR assays may be more expensive, obtaining fecal samples for PCR testing may be easier to accomplish than would survival bleeding of mice for serology.

Conclusion

In conclusion, this case study reports the results of the first successful attempt to eradicate MNV and *Helicobacter spp.* simultaneously from mice that were found positive for both of these pathogens on arrival. *Helicobacter* infection was eliminated in 100% of strains of mice treated with the medicated diet in conjunction with cross-fostering on MNV / *Helicobacter*-free foster mothers. The mouse colony has been maintained pathogen-free, as determined by PCR analysis, and has remained pathogen free from April 2018 to March 2023. It has been four years since the removal of pathogens from our mouse colony, and no infection with the two pathogens has been confirmed to date.

Our findings are consistent with other reports on the effective use of cross-fostering in the elimination of *Helicobacter spp.* and MNV, as we have demonstrated that a cross-fostering technique in combination with drug diet and early testing and removal of contaminated mice after cross-fostering can successfully eliminate MNV and *Helicobacter spp.* simultaneously, from contaminated mice.

Biomedical research facilities should be aware of the overwhelming effects that many of these agents can have on research and are encouraged to not only follow the strict guidelines in this case study but also incorporate early testing and immediate quarantine of the mice in a holding room until results come back, into their mandatory standard operating procedure, upon receipt of new transgenic strains.

Our study had no evidence of any additional pathogen contamination after mandatory semi-annual testing performed by the vivarium. With the ever-increasing use of transgenic mice and increasing cost to produce desired strains, more scientists attempt to acquire mice via material transfers from other researchers that already have them. With such an increase in transfers among facilities, the widespread MNV transmission among colonies may drastically increase if strict guidelines are not followed. Thus, every research facility involved in murine research must incorporate this protocol into their SOP, in hopes to continue towards a pathogen-free murine-research community.

Chapter 2B: Generation of Transgenic Mice

As mentioned in Chapter 1, we proposed to evaluate the role of endothelial-derived *Bgn* and *Dcn* in inflammatory-mediated BBB dysfunction and to determine the overall impact this endothelial-specific matrix pathobiology has on the development and

progression of EAE. Given the potential for both SLRPs to play interrelating or opposite roles in MS, we proposed to use individual transgenic single-floxed mice for each of these genes and double-floxed mice. To determine the specific role of BBB-EC-derived SLRPs, we have crossed these mice with a well-characterized inducible BBB-specific Cre line to produce inducible SLRP $^{BBB/KO.}$ (**Figure 2.2**)

We acquired Bgn$^{fl/0}$ and Dcn$^{fl/fl}$ mice from Dr. David Birk (University of South Florida) in late 2017 and crossed these floxed mice with recently acquired Cre-line (Slco1c1-iCreERT2), which was now pathogen free. This line has been shown to produce high levels of recombination in the endothelium of the brain and spinal cord of adult mice. **(Figure 2.3)**

Chapter 2C: Adoptive Transfer EAE as a model for MS

The role of animal models, especially mouse models, is essential in modern biomedical research. Mouse models can closely mimic most human diseases, including inflammation, and BBB function, and play a critical role in the development of diagnosis and monitoring of symptoms.

There are several common MS corresponding animals models – experimental autoimmune encephalomyelitis (EAE), viral-induced Theiler's murine encephalomyelitis (TMEV), and the cuprizone toxin-mediated model.[3-5] The traditional view of the pathophysiology of MS has held that inflammation is principally mediated by CD4+ type 1 helper T cells.[73] Therefore, EAE, which was our primary choice of an animal model, shares many clinical and pathologic features with multiple sclerosis (MS), and is a commonly used animal model of this human autoimmune disease. In the classic EAE

model, animals are immunized with CNS tissue homogenates or myelin protein-derived peptides to produce a CD4+ T cell-driven disease.[74] Immunization of C57BL/6 mice with a peptide corresponding to residues 33-55 of myelin oligodendrocyte glycoprotein (MOG_{35-55}) yields a disease course that is chronic in nature.[75] It is induced by immunizing antigens derived from myelin, such as myelin oligodendrocyte (MOG)[13], provoking an acute demyelinating process driven by T cells and macrophages which can have a chronic relapsing stage similar to MS.[7] Strong evidence supports that CNS-specific CD4+ T cells are central to the pathogenesis of multiple sclerosis and EAE.[8] T cells are activated in the periphery and translocate into the CNS, followed by permeabilization of the BBB. EAE model clearly demonstrates a key role of T cells in initiating disease and therefore was our choice for the MS animal model.

EAE can also be induced by the adoptive transfer (AT) of activated myelin-specific CD4+ T cells from mice with EAE into naive recipient mice, imitating mediated T cell response identical to MS.[73] (**Figure 2.4**). This model isolates the induction phase of EAE from the effector phase and is more vigorous than other EAE models. In addition, adoptive transfer EAE allows the study of diseases specifically targeting Th1 or Th17 cells. In the classic EAE model, the induction phase continues during the effector phase, which makes it impossible to isolate the phases. The advantage of using the adoptive transfer EAE, however, is that the induction phase occurs in donor mice, while the effector phase occurs in the recipient mice, isolating the phases. AT-EAE enables us to focus on variables associated with the effector phase of disease specifically and to avoid its induction phase altogether.[76]
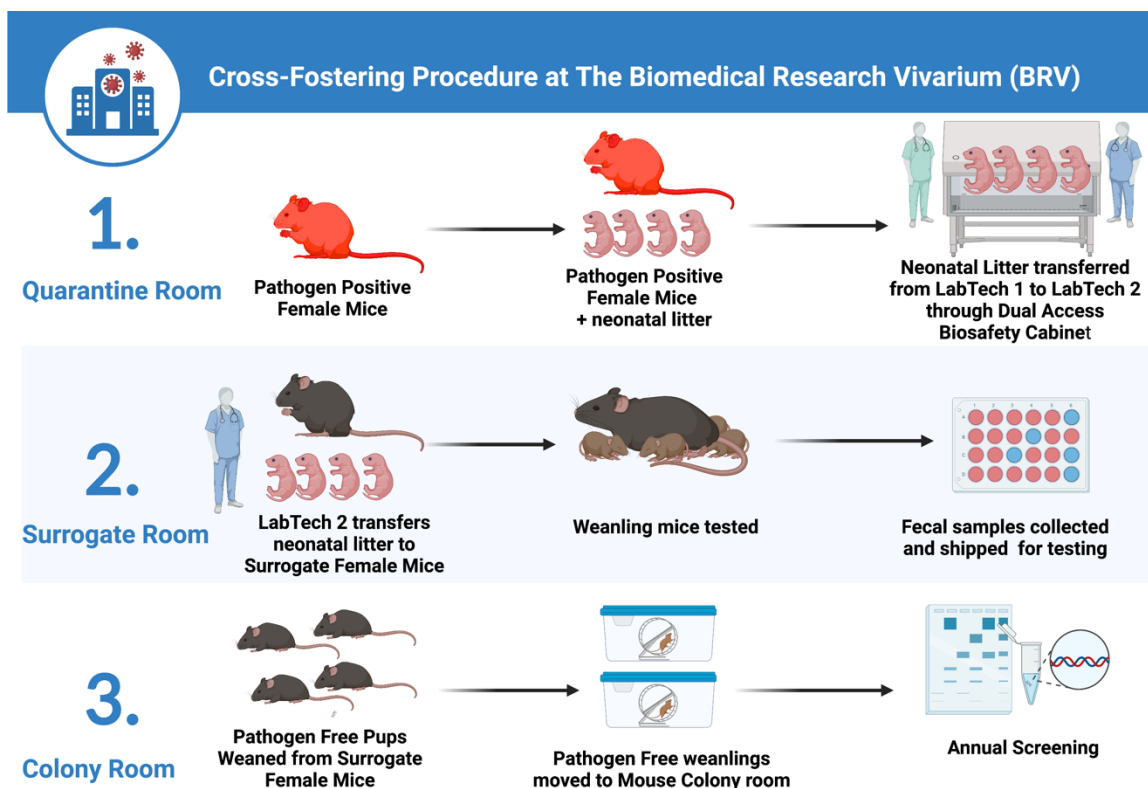
Figure 2.1   Eradication of pathogens from Transgenic Mice colonies.
A cross-fostering technique and early testing and removal of contaminated mice after cross-fostering can successfully eliminate MNV and *Helicobacter spp*. simultaneously, from contaminated mice. Created with **BioRender.com**.

Dcn-floxed BBB-ECs

| Decorin Expression | Recombination | No Decorin Expression |

Bgn-floxed BBB-ECs

| Biglycan Expression | Recombination | No Biglycan Expression |
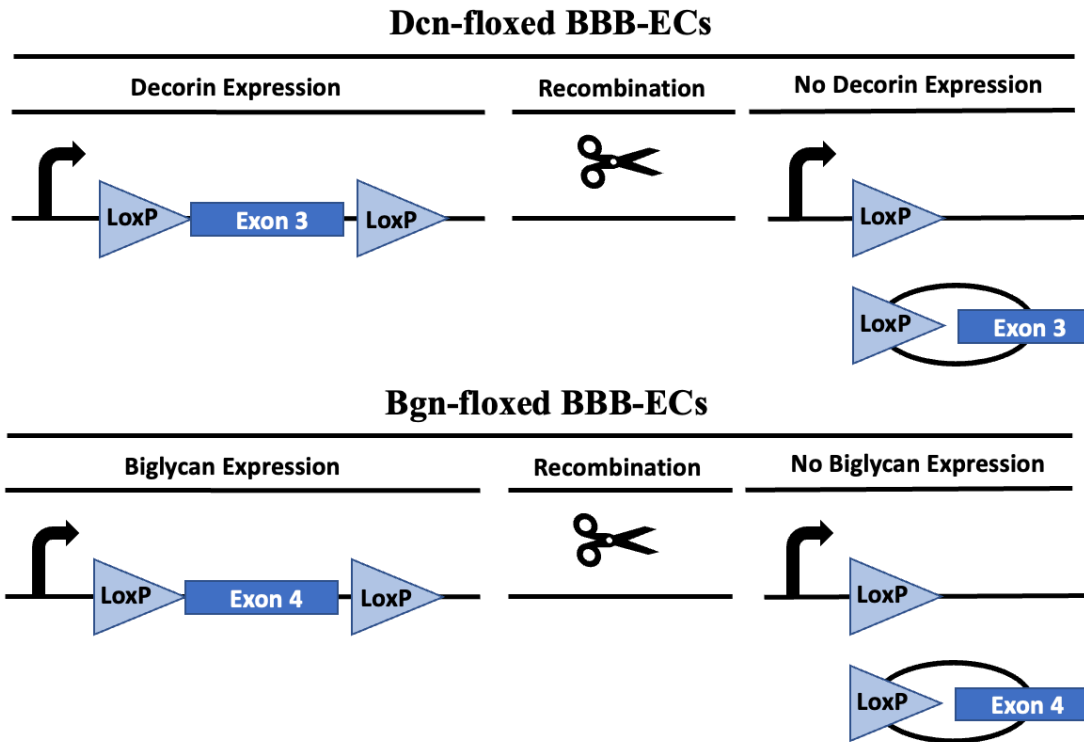
Figure 2.2   Principle of Cre-mediated Recombination.
The Dcn-floxed and Bgn-floxed lines contain loxP sites upstream and downstream of gene specific site to Dcn or Bgn. In the presence of Cre, the gene sequence and one of the loxP sites are excised, thereby ablating Decorin and Biglycan expression.

$Slco1c1\text{-}CreER^{T2}$

+   +   +

$Bgn^{fl/0} / Dcn^{fl/fl}$ $Bgn^{fl/0}$ $Dcn^{fl/fl}$

$Slc1c1\text{-}CreER^{T2} / Bgn^{fl/0} / Dcn^{fl/fl}$ $Slc1c1\text{-}CreER^{T2} / Bgn^{fl/0}$ $Slc1c1\text{-}CreER^{T2} / Dcn^{fl/fl}$

+ Tamoxifen   + Tamoxifen   + Tamoxifen

= Bgn/Dcn $^{BBB/KO}$ = Bgn $^{BBB/KO}$ = Dcn $^{BBB/KO}$
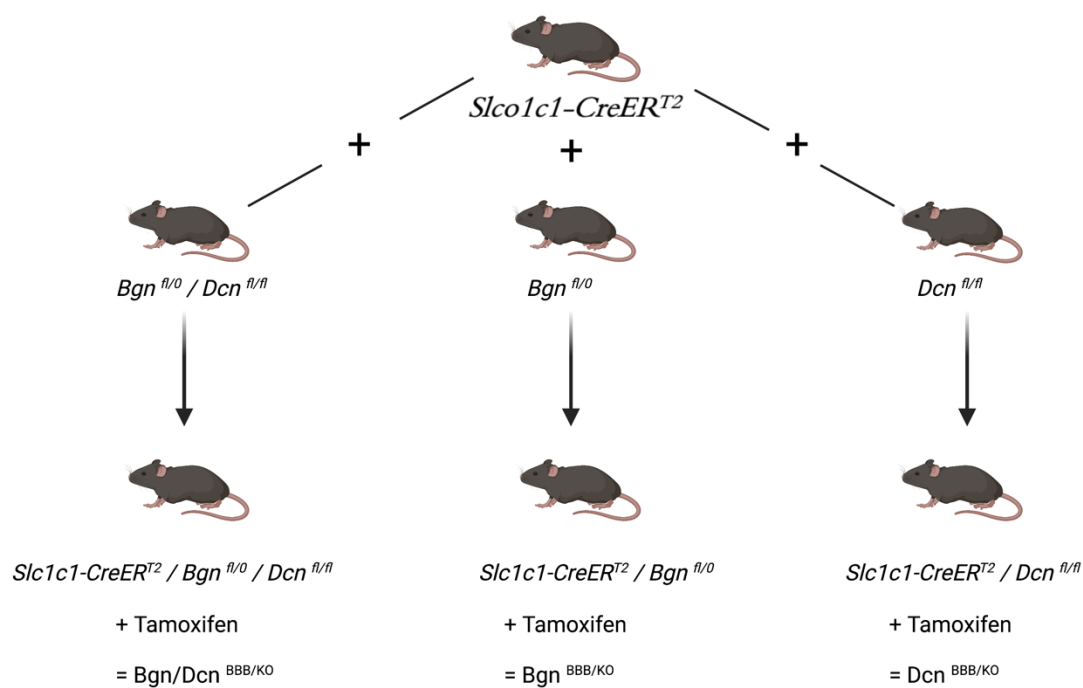
Figure 2.3   Cross-breeding of individual transgenic single-floxed and double-floxed mice.
To determine the specific role of BBB-EC-derived SLRPs, these mice were crossed with a well-characterized inducible BBB-specific Cre line to produce inducible SLRP $^{BBB/KO}$. In the presence of Cre, following injections with tamoxifen, the gene sequence and one of the loxP sites are excised, ablating Decorin and Biglycan expression. Created with **BioRender.com**.

Adoptive Transfer EAE

Antigen (MOG$_{35-55}$) Injection (s.c)   Spleen cell culture with antigen + IL12 + anti-INFλ   T cells Injection (i.p.)   EAE Paralysis

10-11 days   10-11 days   6-10 days

Pleura
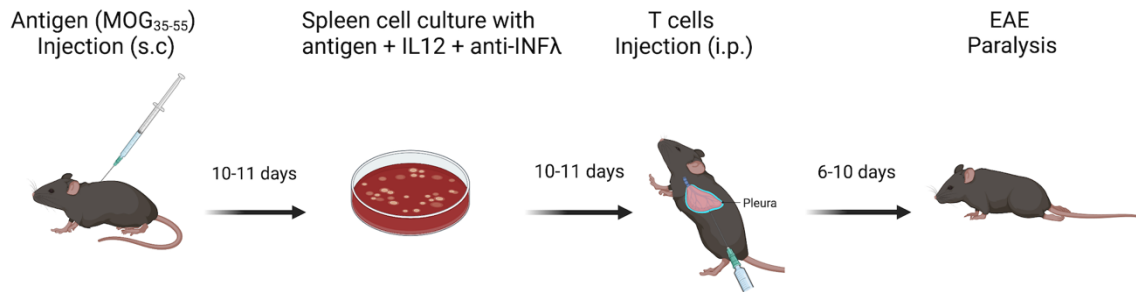
Figure 2.4   Adoptive transfer EAE.
EAE induced by adoptive transfer of activated myelin specific CD4+ T cells from mice with EAE into naive recipient mice, imitating mediated T cell response identical to MS. Created with **BioRender.com**.

Table 2.1 **Results report from Charles River Research Animal Diagnostic Services.**

Test results show initial results on newly arrived mice tested positive for *Helicobacter spp.* and Murine Norovirus (MNV) (2018). The years following show negative results for all pathogens, demonstrating that a cross-fostering technique in combination with drug diet and early testing and removal of contaminated mice after cross-fostering can successfully eliminate MNV and *Helicobacter spp.* simultaneously, from contaminated mice.

TEST RESULTS FOR:	2/20/2018	4/24/18	5/8/18	2019	2020	2021	2022
LCMV PCR	-	-	-	-	-	-	-
MAV 1 & 2 PCR	-	-	-	-	-	-	-
MHV PCR	-	-	-	-	-	-	-
MNV PCR	+	-	-	-	-	-	-
Mousepox (Ectromelia) PCR	-	-	-	-	-	-	-
Mouse Parvovirus (MPV/MVM)	-	-	-	-	-	-	-
PCR	-	-	-	-	-	-	-
MRV (EDIM) PCR	-	-	-	-	-	-	-
PVM PCR	-	-	-	-	-	-	-
REO PCR	-	-	-	-	-	-	-
SEND PCR	-	-	-	-	-	-	-
TMEV/GDVII PCR	-	-	-	-	-	-	-
Beta Strep Grp A PCR	-	-	-	-	-	-	-
Beta Strep Grp B PCR	-	-	-	-	-	-	-
Beta Strep Grp C PCR	-	-	-	-	-	-	-
Beta Strep Grp G PCR	-	-	-	-	-	-	-
B. bronchiseptica PCR	-	-	-	-	-	-	-
B. pseudohinzii PCR	-	-	-	-	-	-	-
Helicobacter genus	+	-	-	-	-	-	-
H. hepaticus +	+	-	-	-	-	-	-
H. typhlonius +	+	-	-	-	-	-	-
Campylobacter Genus PCR	-	-	-	-	-	-	-
C. bovis PCR	-	-	-	-	-	-	-
CAR Bacillus (F. rodentium)	-	-	-	-	-	-	-

CHAPTER THREE: SLRPS IN ANGIOGENESIS / EAE

Introduction

In Multiple Sclerosis (MS), following an inflammatory event, brain endothelial cells (EC) play an important role in recovering damaged tissue. They elicit a robust angiogenic response necessary for tissue revascularization and repair.[77] However, once activated, vascular permeability increases and so does angiogenesis. Inflammatory signals endorse interactions between leukocytes and endothelial cells, leading to extravasation of neutrophils and monocytes.[78] In attempt to suppress the inflammatory response, the activation of cells responsible for tissue repair takes place. Thus, after an initial inflammatory response, a recovery phase follows, resulting in fibroblast proliferation and neovascularization via angiogenesis.[77] Evidence suggests that neutrophils, endothelial cells and pericytes contribute to the resolution of the inflammatory reaction. Moreover, alterations in the composition of the extracellular matrix also facilitates in the repression of inflammatory signals.[79]

MS lesion repair results from a finely orchestrated and complex series of events initiated by extreme inflammation and immune cell infiltration during the onset phase, followed by a recovery phase with a suppression of inflammation, endothelial cell proliferation, and angiogenesis.[44] As mentioned previously, undoubtedly angiogenesis is a well-regulated process intended for tissue regeneration and wound healing.[80] Regrettably,

dysregulated angiogenesis is a pathological hallmark of both MS and an MS mouse model, experimental autoimmune encephalomyelitis (EAE). This type of aberrant angiogenesis is one of the primary consequences of an inflammatory injury, which is mutually dependent upon the intrusion of inflammatory molecules, disruption of the blood-brain barrier (BBB), and immature vessel formation.

As in human patients of MS, EAE is characterized by paralysis caused by CNS inflammation, demyelination of neurons, axonal damage, and neurodegeneration.[76] EAE models exhibit similar remission and relapse stage (relapsing-remitting EAE), however, disease induced by the immunodominant MOG_{35-55} peptide in C57BL6/J mice tends to be of a chronic nature.[81]

We postulate that angiogenesis is particularly active in proliferating brain endothelial cells during recovery phase of chronic MS / EAE, where growth factors such as VEGF are continually released to stimulate tissue repair. In the EAE chronic recovery phase, this process is induced to repair tissue damage in CNS. Unfortunately, aberrant angiogenesis can lead to chronic lesion formation and slower recovery due to underdeveloped endothelial barrier integrity.

Chronic tissue inflammation has been linked to a heightened risk for malignant transformation,[82] therefore, inflammation can also be considered an enabling characteristic of cancer.[83] The small leucine-rich proteoglycans (SLRPs), which are expressed and highly abundant in the extracellular matrix (ECM),[84] after being secreted into the pericellular space, are incorporated into the ECM.[83] Inflammation associated with chronic inflammatory response significantly increases the SLRPs release by endothelial cells and alters their function within the basement membrane. Recently, the type I SLRPs, Decorin

(Dcn) and Biglycan (Bgn), have been identified in MS and EAE brains, but their cellular source and functional role still need to be fully understood.

Bgn, for example, is strongly expressed in inflammatory and fibrotic tissue,[85, 86] and has been shown be upregulated in tumor angiogenesis in EC, which exhibit a higher migratory potential than normal EC[87], at both the mRNA and protein levels.[88] Dcn is mainly known for its functions in inflammation, innate immunity, wound healing, fibrotic diseases, angiogenesis, autophagy and cancer[89], and was the first SLRP associated with regulating the cell cycle by inhibiting transforming growth factor β (TGF-β) signaling.[90] Dcn has the ability to act as an inhibitor of vascular endothelial growth factor receptor 2 (VEGFR2) and the mesenchymal-epithelial transition factor (Met) receptor and can induce degradation of the RTKs, thus restraining angiogenesis[82] and able to oppose pro-tumorigenic as well as the process of angiogenesis.[91]

My study aimed to investigate the role of endothelial-derived Dcn and Bgn in EAE-induced aberrant angiogenesis. The hypothesis tested was that CNS endothelial knockout of these SLRPs is associated with faster recovery of EAE-induced angiogenesis, BBB dysfunction, and clinical paralysis.

Methods

Animal Use

Animals were maintained under a 12-hour light-dark schedule with food, water, and libitum. All experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at or Boise State University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

<u>Adoptive transfer EAE</u>

Adoptive transfer-induced experimental autoimmune encephalomyelitis (AT-EAE) was used to determine the effects of SLRP$^{BBB/KO}$ on the recovery phase specifically. Fresh spleen and lymph node cells isolated from wild-type C57BL/6J mice induced with MOG$_{35-55}$-based EAE were purchased from Hooke Laboratories (US), then activated according to the provider's instructions. Briefly, cells were pooled and suspended at a final concentration of ~3.5 million cells per mL in RPMI 1640 media supplemented with 10% HEPES, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1X penicillin-streptomycin solution, 1X MEM non-essential amino acids solution, 1 mM sodium pyruvate, and 55 µM 2-mercaptoethanol. Next, MOG$_{35-55}$ peptide, recombinant mouse interleukin-12 (IL-12), and anti-mouse IFN-γ antibodies were added to a final concentration of 20 µg/mL, 20 ng/mL, and 10 µg mL, respectively. Cells were cultured for 72 hours before being pelleted via centrifugation for 10 minutes at 300 x g for determination of cell count. The supernatant was removed, and the cells were washed with additional media, filtered, and centrifuged two additional times. The resultant cell pellet was suspended in T cell culture media (RPMI 1640, 2 mM L-glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1x MEM non-essential amino acids, 1 mM sodium pyruvate, 5.5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES) on ice during counting. Counting was completed using Molecular Probes NucRed and NucGreen stains on an BD Accuri C6 flow cytometer. Cells were diluted to 3.5 x 10^6 cell/mL, plated to T175s, then stimulated with 20 µg/mL MOG$_{35-55}$, 20 ng/mL IL-12, and 10 µg/mL anti-interferon γ antibodies. Cells were incubated at 37 °C, at 5% CO2 in a humidified incubator for 70 hours. Following stimulation, cells suspensions were centrifuged for 10 minutes at 300 RCF at 4 °C. The supernatant was discarded, and cell

pellets were combined in HBSS. After counting again, cells were diluted and 12-week-old female mice (5 wildtype, 5 SLRP $^{BBB/KO}$) were injected with 5.92 million cells i.p. each, and returned to their cage, and evaluated using scoring as described below. Beginning on day 5, scoring was completed as follows: 0 = no signs of paralysis, 0.5 = 1 = tail paralysis, 2 = unsteady gait/hind-limb weakness, 3 = hind-limb paralysis, 4 = hindlimb and forelimb paralysis, and 5 = death.

Generation of SLRP$^{BBB/KO}$ mice

To determine the specific role of BBB-EC-derived SLRPs, we have crossed the two single floxed lines (Bgnfl/0 and Dcnfl/fl) of mice acquired from Dr. Birk (University of South Florida, US) with a well-characterized inducible BBB-specific Cre line, (Slco1c1-iCreERT2), from Dr. Markus Schwaninger (University of Lubeck) to produce inducible BBB-EC-restricted knockout lines - SLRP $^{BBB/KO.}$ All experimental mice were genotyped by Transnetyx (US) **(Figure 2.2)**

Tamoxifen-induced Cre-mediated Recombination *in vivo*

SLRP$^{BBB/KO}$ Cre(+) and Cre(-) littermates were aged to 90 days. A 20 mg/mL solution of tamoxifen was prepared in a 10% ethanol-90% corn oil mixture. Animals were injected i.p. at 50 mg per kg body weight every 12 hours for five consecutive days for a total of 10 injections. For the data presented in **Figure 3.2**, animals were then induced with adoptive transfer EAE and scored as described above. For the data presented in **Figure 3.3**, animals were then anesthetized with isoflurane (2%) and processed for TomatoLectin/ Biotin staining and confocal microscopy.

In vivo Brain Microvessel Angiogenesis assay

Following the adoptive transfer EAE, to define the role of SLRPs in aberrant angiogenesis during EAE recovery, the mice were deeply anesthetized, and perfused with Biotin and 2% PFA transcardially, at 24 days post transfer. The brain was sectioned into 100μm slices with a vibratome. Brains were post-fixed by immersion in a 1% paraformaldehyde (PFA) solution overnight at 4°C. They were then washed with PBS, embedded in low-melting-point agarose, and sectioned into 100 μm thick sections with a vibratome. For the data presented in **Figures 3.3**, slices were permeabilized with 0.5% Triton X-100, then blocked with 2% bovine serum albumin (BSA). Free-floating sections from each genotype were post-stained with tomato lectin labeled with DyLight649 and Dylight555 overnight at 4°C. Sections were washed six times with PBS (1 hour-long washes), mounted with Vectashield on PFTE-coated microscope slides, and coverslipped. Prepared slides were examined with a Zeiss LSM 510 Meta inverted confocal microscope (Zeiss, Germany) using the 488 and 549 nm laser lines and appropriate filter settings. Confocal images were analyzed using the ImageJ imaging software.

Primary BBB-EC Isolation

Primary mouse BBB-ECs were isolated from 7-to-10-day-old pups using a combination of density centrifugation, enzymatic digestion, and positive and grown on a T25 flask. First, mice were anesthetized with isoflurane (4%), then decapitated. Whole brains were excised, and the meninges and pial vessels were removed, to avoid contamination of cell culture with meningeal fibroblasts. Second, the remaining tissue was homogenized with a 40 mL Dounce homogenizer in 25 mL of chilled isolation buffer

containing Hanks Buffered Salt Solution (HBSS) supplemented with 10 mM HEPES and 0.1% BSA. The homogenate was centrifuged at 4°C for 5 minutes at 500 x g, then the supernatant was discarded. Third, the pellet was suspended in warmed digestion buffer containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with collagenase/dispase and DNAse I at 2 mg/mL and 20 µg/mL, respectively, then slowly rotated for 30 minutes at 37°C using a tube rotator. The mixture was centrifuged at 4°C for 5 minutes at 400 x g, then the supernatant was discarded. Fourth, the pellet was suspended in a warmed separation buffer containing 22% BSA prepared in HBSS, centrifuged at 4°C for 7 minutes at 3000 x g, and the supernatant discarded. The pellet was then washed with DMEM prior to centrifugation at 4°C for 5 minutes at 500 x g. Fifth, the pellet was suspended in a warmed digestion buffer and slowly rotated for additional 15 minutes at 37°C using a tube rotator. The mixture was centrifuged at 37°C for 5 minutes at 500 x g, then the supernatant was discarded. The pellet was then washed with DMEM prior to centrifugation at 37°C for 5 minutes at 500 x g. Sixth, the pellet was suspended in a warmed complete mouse endothelial cell medium (Cell biologics, US) and seeded into T25 flasks coated with collagen type I (10 µg/cm2). For the first three days post-seeding (d.p.s.), wells were rinsed with 1X HBSS prior to the addition of fresh complete mouse endothelial cell medium supplemented with 3 µM puromycin. Daily media changes were continued with puromycin-containing media until ready for passaging (5-7 d.p.s.).

TAT-Cre-mediated Recombination in Primary BMVEC

Recently, our lab developed a protocol for using recombinant TAT-Cre in combination with TAT-HA to induce excision between loxP flanked Dcn and Bgn. To recombine floxed genes in primary mouse BBB-ECs, we adapted a cell-penetrating peptide-based Cre recombinase-delivery system. Primary mouse BBB-ECs isolated from *Slc BBB KO* mice were cultured in black, glass-bottom 16-well plates coated with collagen type IV (10 μg/cm^2). Following maturation (5-7 *d.p.s.*), 5 μM TAT-HA$_2$ and 50 U/cm^2 TAT-Cre was added for one hour of incubation with serum-free media (Cellbiologics, US). For the data presented in Figure 3.2, *Dcn*-floxed BBB-ECs were cultured 5-7 *d.p.s.*, incubated with serum-free mouse endothelial cell medium (Cellbiologics, US) containing 5 μM TAT-HA$_2$ with or without 50 U/cm^2 TAT-Cre for one hour, and then allowed to recover for 24 hours. Readings were collected at a single frequency (4 kHz) to monitor barrier integrity.

In vitro Primary BMVEC Angiogenesis assay

Standard Matrigel tube formation assays were employed to determine the effect of Dcn and Bgn in angiogenic microvessel formation. To determine SLRP involvement in angiogenesis through a quantitative in vitro method, we isolated primary BMVECs from transgenic SLRP floxed mice and performed exogenous TAT-Cre-mediated excision. BMVEC knockdown and control cells were then used in standardized angiogenesis assays in Matrigel.[92] Prior to seeding on Matrigel, cells were >90% confluent. Briefly, 24 hours following gene excision, P2 BMVECs (1 x 10^5) were serum starved (Basal Media + 2% FBS), loaded with 1g/mL Calcein AM, trypsinized, and passaged to Matrigel imbedded

culture ware. Growth factor reduced Matrigel (Corning, catalog no. 356231) was thawed overnight on ice at 4°C. To initiate the assay media was exchanged with growth factor-supplemented media containing 5% FBS. The media was then exchanged with an equivalent volume of Live Cell Imaging Solution (Invitrogen, US), then monolayers were visualized with an EVOS FL Auto Imaging System equipped with a green fluorescent protein (GFP)-based filter set. Fluorescent microscopy (EVOS) was used to visualize the formation of microvessels mesh networks via 10X magnification. Cells were then monitored and imaged every 3 hours for 24 hours. Morphometric analyses were performed on fluorescent micrographs using NIH ImageJ software (v2.0.0-rc-43/1.52n), with the Angiogenesis Analyzer Plug-in (32665552).

Endothelial barrier integrity analyses

Barrier integrity and resistance of primary mouse BBB-ECs was assessed using electric cell-substrate impedance sensing (ECIS) Zθ station (Applied Biophysics, US). Primary BBB-ECs were seeded at confluence into 8W10E+ PET arrays coated with collagen type I ($1\mu g/cm^2$), pre-treated with TAT-Cre, and allowed to mature. To initiate "wounding / healing" assay, cells were subjected to increase in Voltage 60kHz, 5V amplitude for 30s duration resulting in cellular detachment from electrodes allowing unaffected cells to reform the monolayer over the injured area. Following the injury, the cells were assessed by continuance impedance tracings over 48 hours until injury area was completed, and barrier maturation was achieved. Impedance tracings at 16,000Hz were collected and normalized to injury at t=0 and barrier resistance was measured at 4000Hz using peak tracing value.

<u>Microvessel Isolation for RNA Analyses</u>

To confirm the RNAseq results, we performed RT-PCR on RNA isolated from brain microvessels of mice with EAE. To additionally quantify upregulation or downregulation of our inducible tissue-specific knockout, wild type mice were aged to 30 days. adoptive transfer EAE was used, and mice were injected with 20 million cells IP each, returned to their cage, and evaluated using the scoring described above. The mice were then deeply anesthetized and perfused with normal saline transcardially to remove all blood. The brain was processed to extract microvessels containing the BBB-ECs by homogenization with a dounce and centrifugation over a layer of 32% 70 kD Leuconostoc dextran (Sigma-Aldrich 313900) in HBSS. The washed pellet was then placed in RNAzol and qPCR for Dcn and Bgn was performed.

<u>Statistical Analysis</u>

All statistical analyses were performed with Prism (GraphPad Software, Inc.). α was set at 0.05 a *priori* for statistical significance.

Results and Discussion

<u>Dcn and Bgn are upregulated in spinal cord endothelial cells during EAE</u>

As previously discussed, both Dcn and Bgn have been shown to be upregulated in brains of EAE mice and MS patients.[47, 48, 93] Most recently, Munji et al. published a single-cell RNAseq dataset obtained from RNA isolated from spinal cord endothelial cells of mice with EAE.[93] Here, as an additional proof-of-principle, we queried this dataset to compare

all ECM genes during various stages of EAE. As shown in the volcano plot (**Figure 3.1A**) and heat map (**Figure 3.1B**), Dcn and Bgn expression are elevated in microvessels isolated from mice at all four stages of EAE.

Bgn/Dcn$^{BBB/KO}$ alters the clinical course of EAE

By utilizing genetic manipulations and EAE, we were able to test our initial hypothesis whether the CNS endothelial double knockout of SLRPs is associated with faster recovery of EAE-induced angiogenesis and improved clinical paralysis, Bgn, the most closely related SLRP family member to Dcn, has a very limited involvement in tumorigenesis [91] and despite the relatively high degree of homology, it is interesting that Dcn and Bgn do not have more extensive overlapping functions in tumorigenesis. Our findings suggest that this relationship could be harmoniously similar to that in angiogenesis of MS and EAE. Our Bgn/Dcn$^{BBB/KO}$ mice resulted in two unique changes to the clinical course of EAE. During the Onset phase of EAE, the Bgn/Dcn$^{BBB/KO}$ mice had worse scores than their Cre- littermates (**Figure 3.2A**). Conversely, Bgn/Dcn$^{BBB/KO}$ mice had better scores at Peak and during the Recovery phase. Bgn$^{BBB/KO}$ mice, however, significantly increased the onset and severity of EAE when compared to their Cre⁻ littermates (**Figure 3.2B**). Earlier studies examining role of Bgn in tumor angiogenesis in EC, have reported Bgn being strongly expressed in inflammatory and fibrotic tissue,[85, 86] and have shown it been upregulated in tumoragenesis, which exhibit a higher migratory potential than normal EC.[87] These reports and the worsening affect during onset of clinical scores (symptoms) and lack of improved recovery in our Bgn$^{BBB/KO}$ mice suggest that Bgn has an undefined essential role during all phases of EAE, including the recovery phase, that requires further

investigation. These results point in the direction of our newly formed hypothesis that it is primarily the loss of Dcn that had a direct positive effect in the Bgn/Dcn[BBB/KO] mice clinical scores.

Bgn/Dcn[BBB/KO] attenuates aberrant angiogenesis in EAE recovery

In vivo angiogenesis analyses were performed using brain slices from Bgn/Dcn[BBB/KO] mice and their Cre[-] littermates post EAE transfer (**Figure 3.3**). In Bgn/Dcn[BBB/KO mice], results demonstrate a significant decrease not only in the total percentage of microvessel (MV) coverage, but concurrently in the number of branches, junctions, and increase in average branch length (**Figure 3.3A-E**). These results indicate that Bgn/Dcn[BBB/KO] attenuates excessive angiogenesis in EAE recovery. Knowing that in MS disease progression, excess synthesis of pro-angiogenic factors have been reported, which lead to increased endothelial cell proliferation, migration, and tubulogenesis, it is consistent with our results that what we are seeing is EC's ability to remodel vascular microenvironments during angiogenesis. Taken into consideration that high levels of Bgn and Dcn have been observed around angiogenic vessels located within inflamed tissues, including athlerosclerotic plaques,[54] granulomatous tissues,[55] granulomas,[56] and granulation tissues of healing dermal wounds[56], and been identified in the perivascular space of vessels in inflammatory lesions of MS patients,[47] our results further support our hypothesis that Bgn and Dcn together, undoubtfully, have an existing role during the recovery phase of EAE, and the deletion of both SLRPs demonstrates a rescue in aberrant angiogenesis and decreased number of immature vessel formations.

Separately, using angiogenesis tube formation assay *in vitro*, we confirmed Bgn/Dcn knockdown in BMVECs reduces endothelial tube formation in culture, and determined the effect Bgn and Dcn have in angiogenic microvessel formation. To induce knockdown, we utilized a cell-penetrating peptide-based Cre recombinase-delivery system, TAT-Cre, developed by Wadia et al.[94] Excision was performed, and cells were transferred to Matrigel-coated wells. As shown in **Figure 3.4A-C**, Bgn/Dcn knockout decreases tube formation when compared to control. Intriguingly, Bgn knockdown alone in BMVECs does not produce any difference in endothelial tube formation, suggesting that there might be other alternative explanations to why Bgn$^{BBB/KO}$ mice have significantly increased onset and severity of EAE when compared to their Cre$^-$ littermates.

Taken together, these results indicate that the presence of Bgn and Dcn in the recovery phase of observable neurological deficit in EAE-induced animals exhibits BBB dysfunction, and deletion of both SLRPS attenuates aberrant angiogenesis in EAE recovery. In addition, our double knockout demonstrates fewer microvessels density, in conjunction with decreased number of branches, junctions, and an increase in average branch length, further strengthening our hypothesis of Dcn and Bgn dual harmful role by promoting uncontrolled angiogenesis.

Barriergenesis is enhanced in BMVECs deficient in Bgn/Dcn but not Bgn alone

Only a handful of early studies have considered how Dcn and Bgn may influence endothelial function.[95] Worsening the problem, there are conflicting reports of both overlapping and divergent roles that these two SLRPs play in endothelial function, with very few reports of their roles in CNS endothelium. Given the results from our angiogenesis

assays, we narrowed our search to evaluate two important endothelial angiogenic functions in the context of transgenic deletion of Bgn or Bgn/Dcn. Specifically, to assess real-time changes in BMVEC migration and barriergenesis, we employed Electric Cell-Substrate Impedance Sensing (ECIS) and measured resistance over time following a voltage-based wound to the subsection of the monolayer where endothelial cells are over the sensors. This allows for monitoring the migration of peripheral cells as they re-cover the sensors while continuing to monitor the barrier maturation following the reformation of the BMVEC monolayer. With this method, Bgn and Dcn gene excision was performed in primary mouse BMVECs isolated from $Bgn^{fl/0}$ or $Bgn^{fl/0}/Dcn^{fl/fl}$ mice (**Figure 3.5**). Surprisingly, neither Bgn or Bgn/Dcn excision resulted in reduced or enhanced migration, suggesting their potential role in aberrant angiogenesis does not involve cell migration (**Figure 3.5A,C**). However, Bgn/Dcn excised BMVECs display increased barrier formation and resistance when compared to non-excised monolayers (**Figure 3.5A,B**), and consistent with the pattern observed in other results, no changes were found in the Bgn excised monolayers (**Figure 3.5C,D**). ECIS was able to give us a better insight into if barrier function was reduced or enhanced due to expression modifications. Excitingly, data suggest that double knockout can increase barrier integrity and endothelial cell resistance.

<u>All signs point to Dcn</u>

After determining that the effects of Dcn/Bgn transgenic double knockouts are divergent from $Bgn^{BBB/KO}$ mice, we decided to take a closer look at Dcn. To confirm the RNAseq results, we performed RT-PCR on RNA isolated from brain microvessels of wild-type mice with EAE, and surprisingly, our data indicated that only Dcn was upregulated,

whereas Bgn remained unchanged (**Fig. 3.6A-B**). One potential reason for this could be the difference between spinal cord-specific endothelial cells used in the RNAseq work,[93] and isolated brain-specific endothelial cells used in ours.

Taken together, our results indicate that loss of endothelial Dcn and Bgn expression together can have a positive outcome in the recovery phase of EAE. In contrast, the loss of Bgn alone did not mirror the bi-transgenic knockout results which suggests that it is primarily the loss of Dcn that has a direct positive effect on endothelial cell migration, barrier maturation, and angiogenesis. Given these factors and our recently generated Dcn[BBB/KO] mice, I evaluated the impact of Dcn single knockout in clinical progression of EAE. As expected, loss of Dcn has a significant positive effect on the clinical scores in EAE recovery (**Figure 3.7**).

Thus far, studies involving Dcn/Bgn deposition into the vascular basement membrane (BM) during MS or EAE have not considered the recovery stages of disease. Dcn and Bgn in MS is rarely studied, and their function is poorly understood. It has, however, been shown that Dcn inhibits TGF-β in the EAE model, which may have benefits in the treatment of the acute phase.[95] On the contrary, it has also been shown to be involved in the formation of perivascular fibrosis in chronic lesions and can limit the recruitment of immune cells.[47, 96]

Our data is consistent with these studies, showing that Dcn is involved in inflammation, angiogenesis, and other biological processes of brain-specific endothelium. The role of Dcn, however, in angiogenesis remains controversial, with studies indicating a pro-angiogenic role such as enhanced endothelial cell migration via increased integrin interaction with collagen type I.[97] Our results suggest that Decorin plays a role in

progressing autoimmune and inflammatory diseases such as MS and therefore, it is critical to continue exploring the role of Dcn in disease development and treatment. More evidence is needed to support whether it can be used as a therapeutic target, which is why we investigated whether silencing of Dcn could be beneficial in the recovery of MS.

Conclusion

Here we demonstrate for the first time that altering expression of two CNS endothelial-restricted SLRP genes, *Dcn* and *Bgn*, can lead to significant alterations in the degree of EAE-induced paralysis. Specifically, Bgn/Dcn$^{BBB/KO}$ and Bgn$^{BBB/KO}$ mice had worsened paralysis in the early stages of EAE, whereas, Bgn/Dcn$^{BBB/KO}$ and Dcn$^{BBB/KO}$ had improved recovery during late stage EAE. Collectively, implying that CNS endothelial cell-derived decorin plays a significant role in the pathogenesis of chronic EAE. Excising SLRPs in our mice have shown to help with the recovery phase and in turn slow down angiogenesis in EAE. Similarly, studies performed on endothelial cells *in vitro* suggest that genetic deletion of SLRPs decreases angiogenesis and enhances barrier function. Together, this data strongly suggests that alterations of decorin in EAE-induced angiogenesis can provide a basis for anti-angiogenic therapy in EAE, such as siRNA-mediated knockdown. There is a colossal need to develop a multi-target drug able to act simultaneously on different pathogenic factors which could improve the outcome of the disease and patient's quality of life. The link between chronic inflammation and angiogenesis, which are both involved in the pathogenesis of MS, makes gene-editing and synthetic inhibitors of Type I SLRPs a potential therapeutic target that would block the angiogenic processes with anti-inflammatory as well as anti-angiogenic properties.
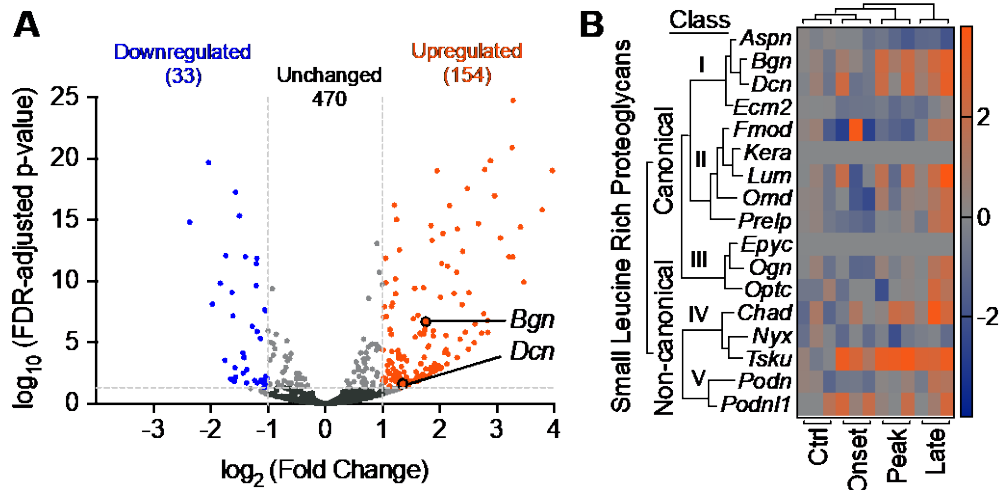
Figure 3.1   Expression levels of SLRPs are altered during EAE progression.
In silico analysis of ECM genes in spinal cord endothelial cells identified upregulation of SLRPs at all stages of EAE.[93] A) Volcano plot of identified genes at the peak stage of disease shows upregulation of Bgn and Dcn (arrows). B) Heat map of the SLRP family over the course of EAE shows strong enrichment for Bgn, Dcn, and other SLRPs at the peak and late stages of disease.
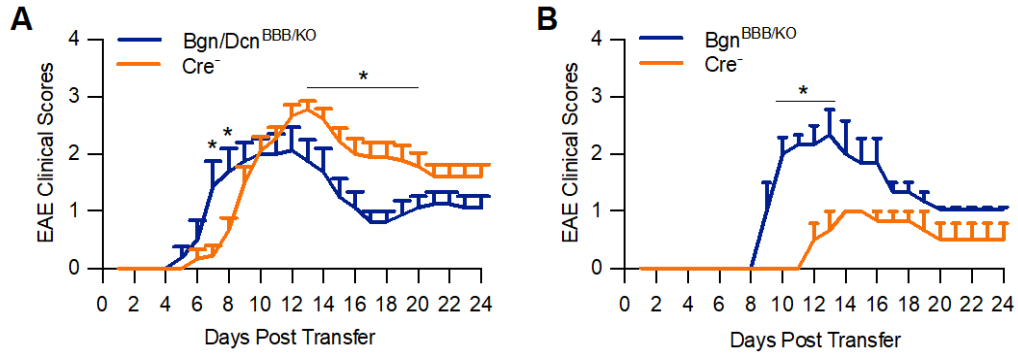
Figure 3.2 **Bgn^BBB/KO worsens while Bgn/Dcn^BBB/KO improves EAE outcomes.**
Both *Slco1c1-iCreER^T2 / Bgn^fl/0* mice and the bitransgenic *Slco1c1-iCreER^T2 / Bgn^fl/0/Dcn^fl/fl* mice were aged to 10 weeks and injected with tamoxifen to produce BBB-specific knockout of these SLRPs, i.e., Bgn^BBB/KO and Bgn/Dcn^BBB/KO mice. A,B) Following tamoxifen-induced excision (3 weeks), cells from C57BL/6 mice with EAE were transferred to transgenic mice and clinical scoring was conducted for 24 days post transfer. A) Bgn^BBB/KO mice significantly increased the onset and severity of EAE compared to their Cre^- littermates (*n=3*). B) Bgn/Dcn^BBB/KO resulted in two unique changes to the clinical course of EAE. During the Onset phase of EAE, the Bgn/Dcn^BBB/KO mice had worse scores than their Cre- littermates. Conversely, Bgn/Dcn^BBB/KO mice had better scores at Peak and during the Recovery phase (n=8-9). Data represents mean scores $\pm$ S.E.M. * Indicates $p < 0.05$.

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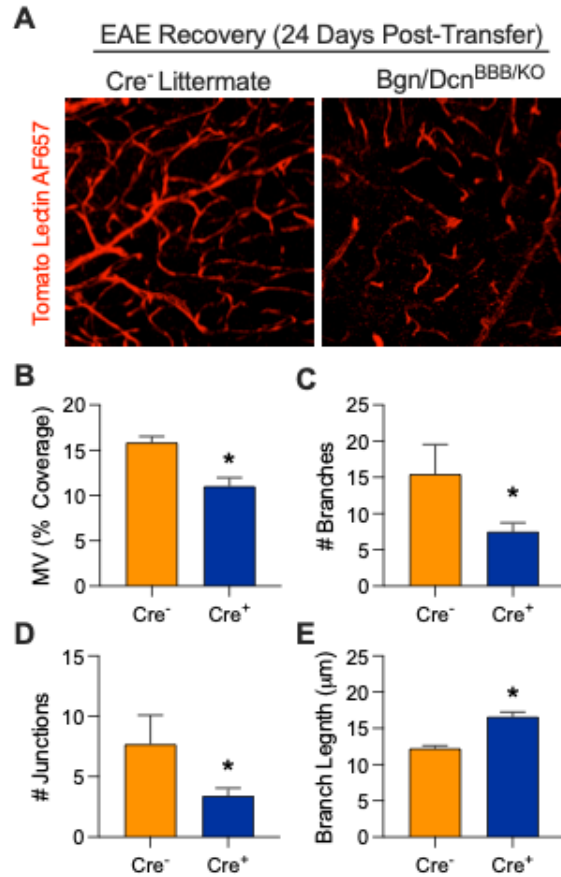
Figure 3.3   Bgn/DcnBBB/KO attenuates excessive angiogenesis in EAE recovery.
In vivo angiogenesis analyses were performed in Bgn/DcnBBB/KO mice and their Cre-littermates. 24 days post transfer, harvested and fixed brain slices were stained with tomato lectin AF647 to fluorescently label all blood vessels. A) Representative confocal micrographs. B-E) Morphometric analyses of confocal micrographs measuring (B) the total percentage of microvessel (MV) coverage, C) number of branches, D) number of junctions, and E) the average branch length. Data represents mean scores ± S.E.M (*n=4*). * Indicates p < 0.05.
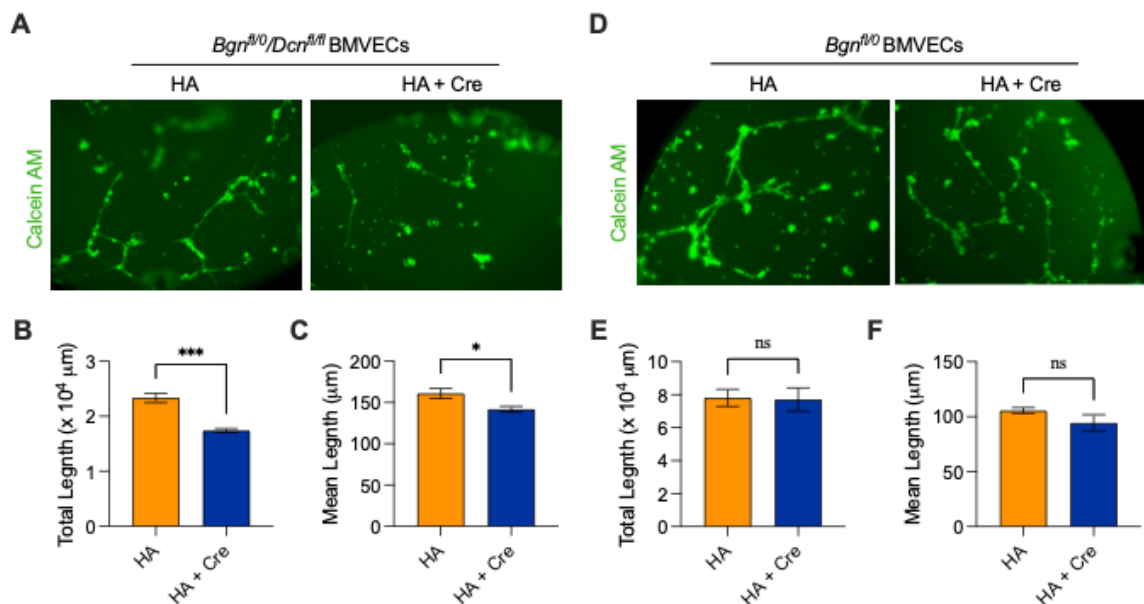
Figure 3.4   Bgn/Dcn knockdown in BMVECs reduces endothelial tube formation in culture.

A-F) Angiogenesis analyses were performed using isolated BMVECs from transgenic (Bgn) and bitransgenic (Bgn/Dcn) floxed mice. In vitro Cre-mediated excision was achieved by pre-treating BMVECs with TAT-HA (HA) and TAT-Cre recombinase (Cre). Following knockdown, cells were passaged into Matrigel-coated cultureware and incubated for 24 hours. Tube formation was measured by fluorescent imaging of the calcein AM loaded cells (A,D) and quantified as total tube length (B,E) or mean tube length (C,F) using NIH ImageJ software. Data represents mean scores ± S.E.M ($n=4$). * Indicates $p < 0.05$.
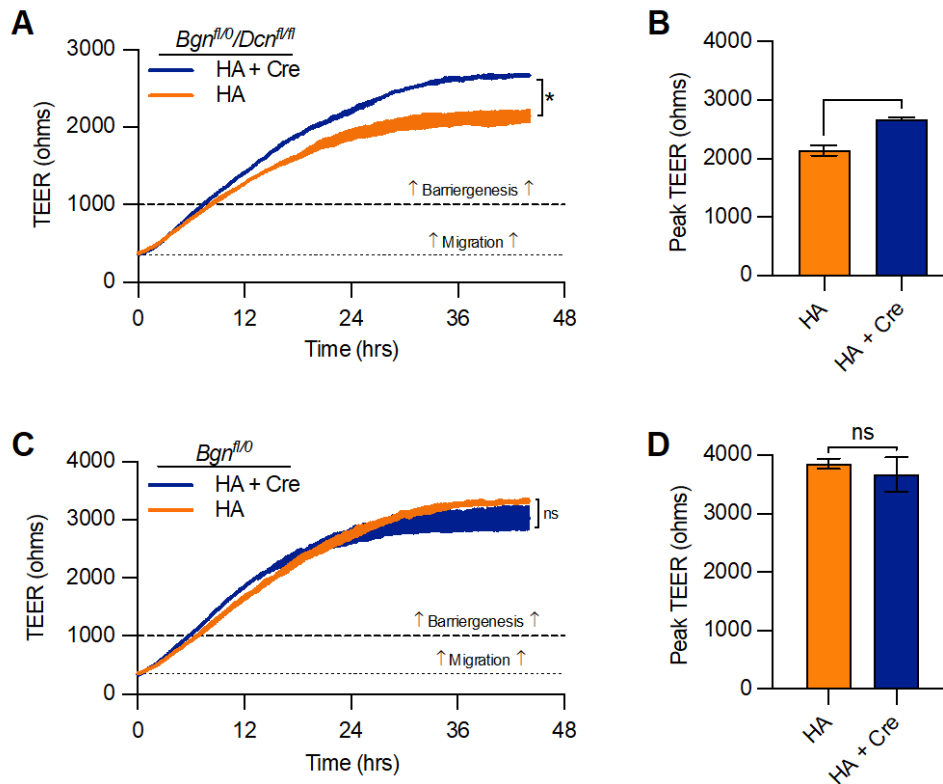
Figure 3.5 Bgn/Dcn knockdown in BMVECs increases endothelial barriergenesis in culture.

Primary BMVECs were assessed using ECIS. A-B) A significant difference in resistance is shown between *Bgn*^{fl/0}/*Dcn*^{fl/fl} cells treated with recombinant TAT-Cre (HA+ Cre) and control (HA), demonstrating higher barriergenesis in BMVECs lacking Bgn/Dcn. C-D) No difference in resistance was found between *Bgn*^{fl/0} cells treated with Cre and control (HA), suggesting limited Bgn involvement in barriergenesis. *Indicates $p < 0.05$.
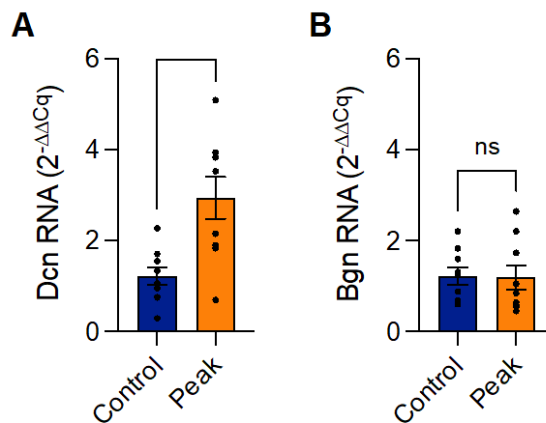
Figure 3.6   Decorin is elevated in brain microvessels at peak EAE.
RT-PCR results on RNA isolated from brain microvessels of wild-type mice scored at peak EAE, indicating that only Dcn was upregulated (A), whereas Bgn remained unchanged (B). ** indicates $P < 0.01$, ns = $P > 0.05$.
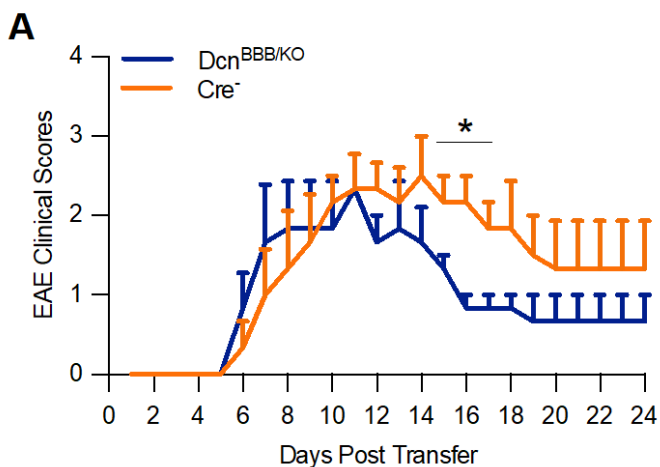
Figure 3.7   DcnBBB/KO mice have significantly improved EAE recovery.
Following tamoxifen-induced excision, cells from C57BL/6 mice with EAE were transferred to transgenic mice and clinical scoring was conducted for 24 days post transfer. A) Dcn$^{BBB/KO}$ mice had a significantly increased recovery from EAE compared to their Cre$^-$ littermates (*n=3*). * indicates $P < 0.05$.

CHAPTER FOUR: OVERCOMING THE CHALLENGES OF PRECLINICAL MURINE HEMAPHERESIS WITH STATE OF THE-ART MICROFLUIDICS

Introduction

Autoimmune and inflammatory diseases are major health problems affecting over 200 million people worldwide. Chronic inflammatory diseases have been recognized as one of the major causes of death in the world today, with more than half attributed to inflammation-related diseases such as ischemic heart disease, stroke, cancer, and autoimmune and neurodegenerative conditions.[98, 99] The search for new therapeutic approaches is not only to help understand the process of disease occurrence and development but also to alleviate patients' symptoms and reduce the economic burden on society.

The standard treatment of relapsing multiple sclerosis (MS) consists of medications for disease symptoms, including treatment for acute exacerbations. Harmoniously, Therapeutic Plasma Exchange (TPE) is a well-established method of treatment for steroid-refractory relapses in MS[100], although, several studies corroborated its efficacy in only some patients undergoing TPE[100-102]. Moreover, TPE should only be carried out in conditions where there is reliable evidence of its effectiveness as the side-effects alone include disturbances of coagulation, vasovagal episodes, fluid overload, and allergic reactions due to plasma infusion.[103] Relapses in MS are often associated with significant

disability impairment, which also results from poor response to corticosteroids.[102] Currently, there is no therapy that alters the progression of physical disability associated with this disease. Therapies developed on the basis of this theory decrease the relapse rate by approximately one-third but do not fully prevent the occurrence of exacerbations and are largely ineffective against progressive forms of multiple sclerosis.[104] The prevention of inflammatory lesions during the onset early in the disease may delay the development of progressive multiple sclerosis, highlighting the importance of developing more effective therapies for relapsing–remitting multiple sclerosis.[105]

As discussed previously, inflammation is a principal defense mechanism that is vital to our health. It is the immune system's response to pathogens, and damaged cells, and acts by initiating the healing process. The result of inflammation is a known protection from the spread of infection, followed by restoring affected tissues to their normal structural and functional state.[106] During acute inflammatory responses, cellular and molecular events and interactions efficiently lessen threatening injury or infection.[107] This mitigation process contributes to the repair of tissue homeostasis and resolution of the acute inflammation. However, uncontrolled acute inflammation may become chronic, contributing to various chronic inflammatory diseases. Recurring inflammation contributes significantly to the pathogenesis of atherosclerosis, cancer, chronic obstructive pulmonary disease, inflammatory bowel disease, neurodegenerative disease, rheumatoid arthritis, or MS.

The blood brain barrier (BBB) is dysfunctional during the early phase of MS, due to the local recruitment of pathogenic T cells. Differential expression of pro-inflammatory cytokines, chemokine receptors, and integrins by infiltrating lymphocytes mediates

disruption of the BBB in MS.[37] Upon recognizing the myelin antigen, CD4+ T cells release a host of proinflammatory cytokines recruiting other immune cells such as neutrophils that release neurotoxin chemicals and cytokines, propagating proinflammatory conditions.

Due to an already weakened blood-brain barrier and lack of tight junctions between endothelial cells, neutrophils are able to exit blood vessels unrestricted. The intensity of neutrophil infiltration controls the number of T cells recruited into antigen sites.[108] In addition, mast cells regulate the cytokine microenvironment of the contact hypersensitivity response.[109] Given the multitude of cell types involved in chronic inflammation, it is not surprising that this response is orchestrated by a complex network of cytokines and their receptors. For example, the autoimmune response inflammatory reaction depends on the activity of cytokines. Cytokines are small-secreted proteins that aid in communication between the cells, and when dysregulated[110], are responsible for systemic inflammation contributing towards the etiopathogenesis of MS. The imbalance between inflammatory and anti-inflammatory cytokines, can have deleterious effects. During neuroinflammation specifically, a host of factors lead to disruption of the endothelial barriers within the brain and spinal cord, worsening the inflammatory injury.

The proinflammatory cytokines have a crucial role in the MS pathogenesis and regulate lymphocyte infiltration across the BBB.[111] Pro-inflammatory cytokines such as GM-CSF, IL-17, Il-6, IL-1β, IL-22, INF-γ, OSM are accountable for the initiating and the MS progression. Further uncontrolled production of cytokines leads to further Th cell upregulation, production of proteinases, and destruction.

In addition to cytokines, it has been reported that there are increased plasma levels of mitochondrial DNA and pro-inflammatory cytokines in patients with progressive

multiple sclerosis[112] as well as other pro-inflammatory factors such as miRna and Histones. A recent study of whole-blood samples from patients with relapsing-remitting MS found that 165 miRNAs were differentially expressed compared with healthy controls[113, 114], investigating miRNA profiles as a potential diagnostic and prognostic biomarker for multiple sclerosis. Histone citrullination was increased in animal models of demyelination and in patients with MS.[113, 115]

Chronic inflammation is a significant driver of MS. Continuation of inflammation is a risk in itself because inflammation damages tissue, and necrosis can provoke further inflammation. Multiple built-in mechanisms typically ensure resolution. Chronic inflammation may result from deficits or abnormalities in mechanisms that typically ensure resolution. This greatly complicates the development of anti-inflammatory therapies. Anti-inflammatories that are clinically useful and acceptably safe are a fraction of those whose development was undertaken.[106]

Furthermore, several studies indicate that Oncostatin M (OSM), a cytokine from interleukin 6 family plays a major role in cellular processes to include formation of T cells outside thymus, immune tune up of brain's endothelial cells, and deterioration of the nervous tissue.[116-118] OSM has been found both in lesions and in increased concentrations in serum of MS patients,[119, 120] and when found with increased concentrations of hepatocyte growth factor, may be a strong indicator of MS.[119] Finally, Oncostatin M (OSM) has also been found in the blood plasma of patients with relapsing-remitting MS and it has been identified that during the effector phase of EAE there are increased levels of circulating cytokine OSM concurrent with a pathogenic increase in OSM+ neutrophils.

OSM's structure and its detailed role in plasma, makes it a perfect target of interest for patients with MS.

Circulating cytokines are a hallmark of inflammatory diseases. Serum from mice with experimental autoimmune encephalomyelitis (EAE) causes barrier dysfunction. In humans, one potential treatment is hemapheresis. This, unfortunately, hasn't worked with rodents. After data is collected from this study, I aimed to use this procedure to selectively reduce circulating cytokines in mice with (EAE) and to establish the MSM micropump as a highly selective blood-filtering device capable of eliminating specific cytokines in mice. Overall, this would offer the possibility of providing a "bench-to-bedside" screening tool for testing new hemapheresis modalities.

Methods

Animal Use

Wild type C57BL/6 mice were maintained under a 12-hour light-dark schedule with food, water, and libitum. All experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at or Boise State University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Pump

Collected blood from mice was circulated through magnetic MSM pump. Blood was run at 300rpm for duration of 47 min. The total circulating volume was approximately 700ml, however, goal to be closer to 150μl to reduce the dead volume, which is the 10% of circulating blood volume of a mouse.

<u>IgG removal</u>

Protein G Dynabeads were captured in μ-Slide VI 0.4, prior to circulating mouse blood through the MSM pump. Blood was circulated through silicone tubing connected to a μ-Slide that contained 50μl of Dynabeads and placed on the magnet. Once Dynabeads were spread across, circulation of the mouse blood began throughout the MSM pump at 300 rpm.

<u>Blood smear</u>

Blood was collected from wild type mice by placing under 3% isoflurane anesthesia. Gem –Wright stain was complete on whole blood.

<u>Microscopy</u>

The morphology and presence of red blood cells and cytokines was visualized using a Leica DM 1000 LED transmitted light microscope.

<u>Western blotting</u>

Western blotting and analysis were performed using standard blotting techniques and the protein bands were imaged on Odyssey® CLx (LI-COR Biosciences USA, Lincoln, NB).

Results / Discussion

Using mouse brain microvascular endothelial cells (BMVEC) and sera from mice with systemic inflammatory response syndrome (SIRS), a condition caused by bacterial infection where cytokines are released at alarming amounts,[2,3] we have found that barrier function is significantly reduced (**Figure 4.1A**). Similarly, serum from mice with experimental autoimmune encephalomyelitis (EAE) causes barrier dysfunction (**Figure**

4.2A). It is our overarching hypothesis that this barrier dysfunction is central to many diseases.

In humans, one potential treatment is hemapheresis- the selective manipulation of blood components to reduce inflammation and disease.[4] This unfortunately hasn't worked with rodents, as their reduced blood volume and vasculature is incompatible with nearly all devices on the market. Through a collaboration with Dr. Peter Müllner of the Boise State Department of Materials Science and Engineering, we have access to a magnetic shape memory (MSM) micropump that overcomes previous limitations: with a very low dead volume and the ability to pump at reasonable speeds for mouse vasculature **(Figure 4.4).** This pilot study, then, will be to establish the MSM micropump as a highly selective blood-filtering device, capable of eliminating specific cytokines in mice, enabling a "bench-to-bedside" screening tool for testing new hemapheresis modalities.

Preliminary characterization of the pump with *ex vivo* blood has been promising. The general setup of the pump system (**Figure 4.2A**) utilizes magnetic particles conjugated to antibodies against specific molecules (**Figure 4.2B**) to clear them from blood. In this assay, the light chain of immunoglobulin G (IgG Lc) was selected against. After running through the pump, the concentration of IgG Lc was significantly decreased (**Figure 4.2C**).

Initial tests show that using anti-mouse IgG capture beads causes a slight decrease in the number of circulating granulocytes (**Figure 4.3A**). Running whole blood through the pump causes few changes to cellular morphology (**Figure 4.3B**), indicating the pump has potential use as a safe hemapheresis device.

Treatment of inflammatory diseases today is mainly based on interrupting the action of mediators that drive the host's response to injury.[60] Non-steroidal anti-

inflammatories, steroids, and antihistamines, for instance, are developed and provided the main treatment for inflammatory diseases. We offer an alternative approach to the development of novel therapeutics based on the endogenous mediators and mechanisms that switch off acute inflammation and bring about its resolution. It is thought that this strategy will open up new avenues for the future management of inflammation-based diseases.

Future Direction

The purpose of this pilot study was multifold: to establish the MSM pump as a safe device to use in murine hemapheresis, quantify the ability of the MSM pump and antibody combination to effectively remove circulating molecules, and use the procedure described below to selectively reduce circulating cytokines in mice with EAE. Due to time constraints, and events that took place that were out of our control, we were unable to test our hypothesis fully we propose the following procedure for future animal studies:

Mice to be used to evaluate the safety and efficacy of removing circulating molecules. Mice to be selected of either sex, then allowed to age until $60 - 80$ days, the age that EAE is usually induced. 30 animals in total to be chosen: 15 for initial testing, and 15 for recovery testing. For the initial terminal testing mice to be euthanized after the hemapheresis procedure. These will be used to verify that the procedure is viable and that there are no immediate complications. Only then to continue to recovery testing, to confirm that there are no long-term side effects from the procedure, allowing to use the pump as an intervention in mice with EAE. Blood would be pumped from one leg, across antibodies to remove GFP, and returned to the mouse. In total, 30 mice would be used; 15 would

undergo terminal testing, allowing for analysis of circulating blood after hemapheresis. The remaining 15 mice would be monitored for 24 hours after hemapheresis to evaluate for side effects. Measured outcomes to include morbidity, blood morphology, pump efficacy, and finally hemapheresis efficacy.
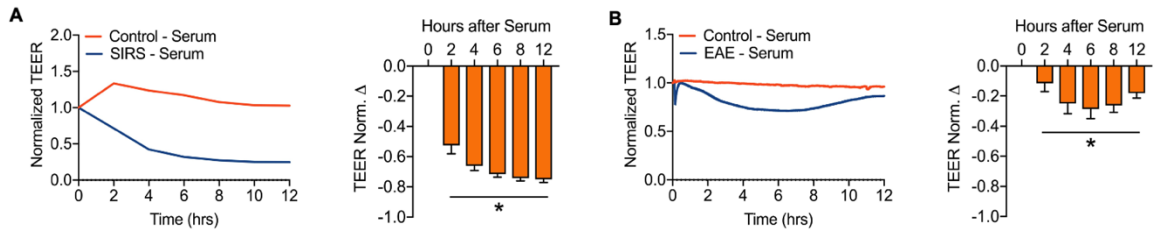
Figure 4.1.   Decreased barrier integrity due to inflammation.
(A) SIRS serum causes barrier dysfunction during systemic inflammatory response to burn injury. (B) ECIS tracings show barrier dysfunction using serum from EAE mice.
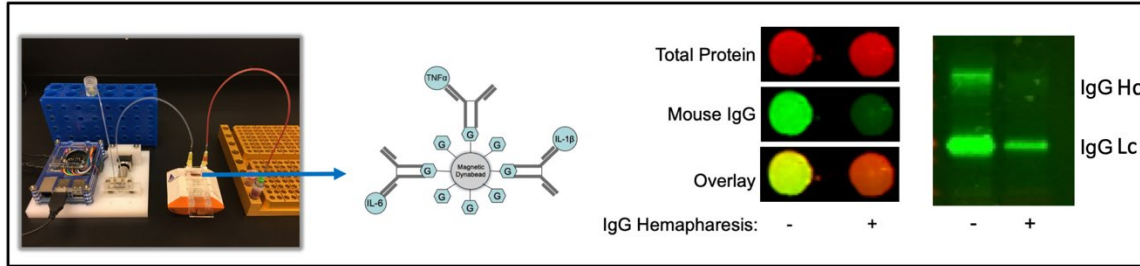
Figure 4.2. **Schematic of the experimental setup for selective elimination of cytokines by IgG antibody from mouse serum.**
(A) Use of MSM pump to isolate antibodies from blood. (B) Dynabeads™ Protein G binding to cytokines. (C) Immunoblotting demonstrating decrease of Mouse IgG protein in serum after hemapheresis in presence of Dynabeads treated with antibodies.
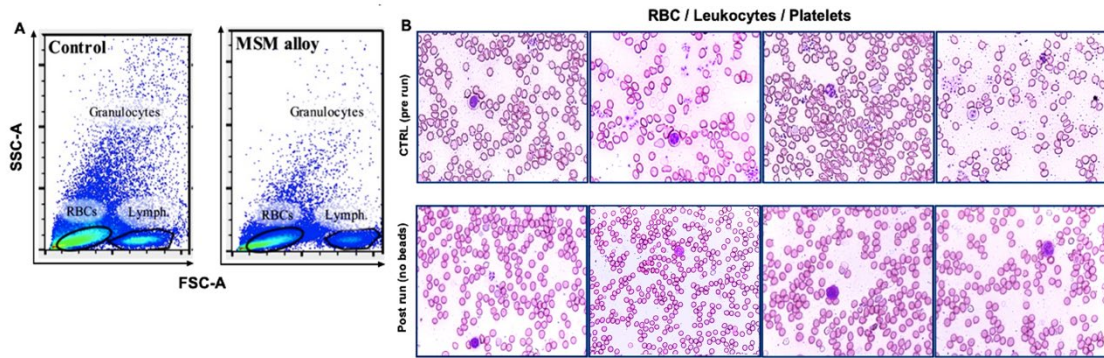
Figure 4.3.   Morphological analysis of blood before and after MSM pump.
(A) Flow cytometry results demonstrate slight decrease in granulocytes after initial testing of the MSM pump. (B) Blood smear showing red blood cells, platelets and circulating leukocytes pre-run (control), post-run without beads demonstrates only slight morphological changes.
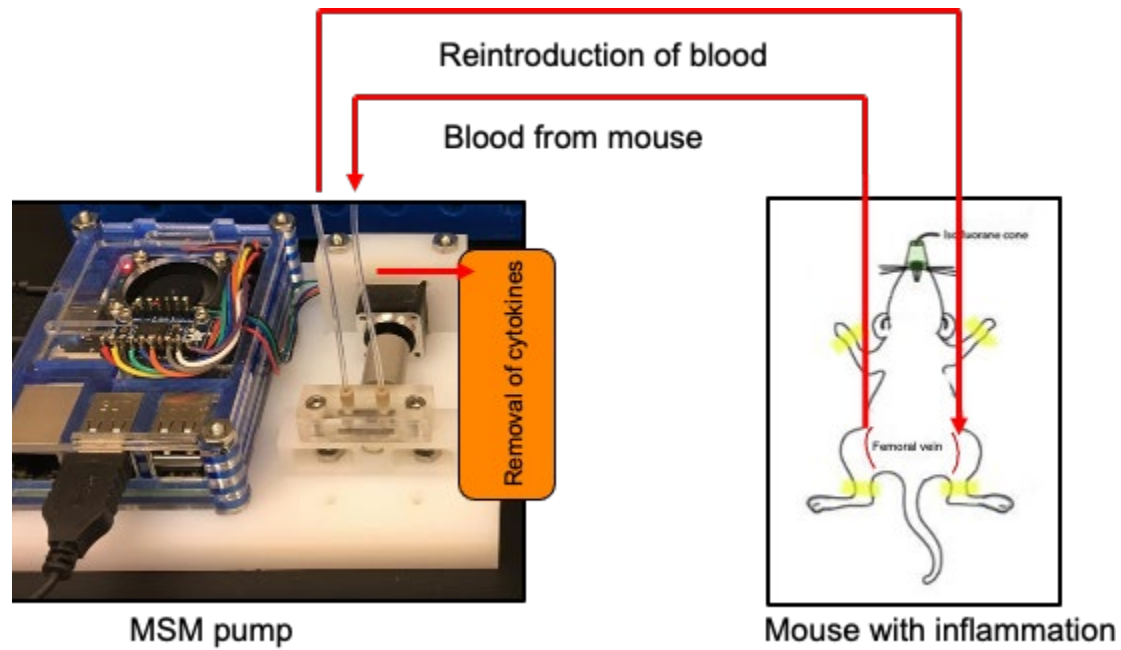
Figure 4.4 **General schematic of device and setup. Magnetic shape memory micropump with biocompatible coating enables autologous mouse hemapheresis.**

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