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The Origin of Multiple Sclerosis Revisited: The Case for a Soluble Toxin

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THE ORIGIN OF MULTIPLE SCLEROSIS REVISITED: THE CASE

FOR A SOLUBLE TOXIN

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After nearly two hundred years of scientific inquiry, the cause of Multiple Sclerosis (MS) remains unknown. Although generally considered an autoimmune disease, recent pathological findings have challenged the long-standing autoimmune view of MS. Indeed, lesions that are just hours past their initial onset display evidence of cellular degeneration in the absence of an inflammatory infiltrate. This seminal finding begs the fundamental question, if immune cells do not participate in early tissue damage in MS, what is the mysterious triggering agent?

In the first half of this thesis, I will propose that a soluble bacterial toxin, Clostridium *perfringens* epsilon toxin (ETX), may be the long sought environmental trigger for MS. ETX is a neurotoxin with a unique tropism for the brain tissues that are specifically damaged during acute MS exacerbations, the blood-brain barrier and the myelin sheath. Using a Western blot seroreactivity assay, I have identified a 10-fold increase in anti-ETX seroreactivity when comparing MS patient samples to that of controls. Furthermore, PCR analysis of bacterial cultures from patient stool samples has yielded the discovery of the first human known to harbor C. *perfringens* type B, an ETX secreting bacterium that is not part of the normal human microbiota. Intriguingly, this individual was found to be in the early stages of developing MS. I have also determined that MS

patients are 2X less likely to harbor the human commensal C. *perfringens* type A, which has been shown to outcompete toxin-producing C. *perfringens* strains, e.g. type B, within a shared ecological niche. Therefore, commensal type A may protect against type B dysbiosis and may protect against developing MS.

Although the ETX/MS hypothesis is obscure at best, ETX's extreme potency has driven interest in identifying its cellular receptor. Indeed, ETX is the third most potent toxin known to man, following only Clostridium *botulinum* and *tetanus* toxins in lethality. For this reason, it is considered a legitimate bioterrorism threat. Despite decades of research, the ETX receptor is still yet to be identified.

In the second half of this thesis, I will propose that the tetraspan proteolipid, Myelin and Lymphocyte protein (MAL) is a bona fide ETX receptor. Recombinant expression of MAL by an ETX resistant Chinese Hamster Ovary (CHO) cell line confers both ETX binding and susceptibility. Additionally, probing tissues harvested from MAL knockout (KO) mice with fluorescently labeled ETX demonstrates a complete abolishment of ETX binding. Finally, MAL KO mice display a remarkable level of ETX resistance even when exposed to 1000X of our experimentally determined LD_{50} for wild type animals. Taken together, these data suggest that MAL is both necessary and sufficient for ETX binding and toxicity.

In summary, I have generated data suggesting that an increased exposure to C. *perfringens* epsilon toxin may exist in the MS population when compared to controls. This may be important, as ETX specifically targets the blood-brain barrier and the myelin sheath, both of which degenerate during acute MS exacerbations. To my knowledge, ETX is unlike any other candidate MS trigger,

as it provides a clear mechanism for how blood-brain barrier disruption and demyelination may occur during an MS relapse. I have also identified Myelin and Lymphocyte protein, as a functional ETX receptor. Identifying the mechanism by which ETX targets cells may help counteract its use as a biological weapon. Furthermore, this discovery may open new avenues for translational research, e.g. novel methods for targeting and bypassing the blood-brain barrier; a major hurdle to drug delivery in the central nervous system.

I dedicate this thesis to two incredibly influential people in my life, my late grandmother, Lorine Pitt and the late Dr. Timothy Murrell. I cannot express in words the gratitude that I have for my grandmother's presence in my life, but I can honestly say that without her love and constant encouragement I would not be on the path that I am on today. Although I never had the pleasure of meeting Dr. Timothy Murrell, the true author of the ETX/MS hypothesis, it is obvious from his writings and communications that he was a brilliant man. My best wishes go out to his family, as I am sure that they miss him dearly.

specifically targets the BBB, as a candidate MS trigger. It didn't take too long for me to realize that this was what I wanted to spend all of my time studying. Indeed, I ended up writing my undergraduate thesis on the idea that C. *perfringens* might be at the heart of MS. By the time I was ready to leave the Barres lab to attend medical school, ETX was found to target myelin once bypassing the BBB. This 2008 finding made it clear to me that there was no turning back from this point on.

I arrived at the Tri-Institutional MD/PhD program and soon sought out people who either currently study MS or had studied MS in the past. One faculty member, Dr. John Zabriskie (Professor Emeritus at the Rockefeller University), gave this strange sheep/MS hypothesis an honest listen. Dr. Zabriskie no longer performed experiments, but introduced me to Dr. Vincent Fischetti, his former student, who currently runs the Laboratory of Bacterial Pathogenesis. Although MS is not his field, Dr. Fischetti kindly provided lab space and reagents and I sincerely thank him for that. During medical school, I tried to experiment when I could, but it was clear that I needed more time and I also needed access to patient samples. This is exactly when Dr. Timothy Vartanian arrived as the new Director of The Judith Jaffe MS Center. Like Dr. Zabriskie, Tim met the sheep/MS hypothesis with an open mind and we quickly formed a collaboration, which has been one of the most enjoyable work experiences imaginable. Tim has taken on this project as his own, and has provided insights and support without which the following work would not be possible.

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CHAPTER 1

The Current View of Multiple Sclerosis

Multiple Sclerosis (MS) is a neurological disease that cripples its victims. Although rarely fatal, it significantly diminishes quality of life. MS is considered a disease of young adults, as the diagnosis is usually made between the ages of 20 and 40. Due to the youth of its target population, MS affects not only the patient, but also the family and the community, as it impairs the productivity of young people in their prime.

One in 500 individuals of European ancestry living in temperate climates suffer from MS (Haines 2004). Additionally, women are 2.3x more likely to acquire the disease than men (Alonso and Hernan 2008). However, African Americans and other minorities are also affected, often suffering from a more aggressive disease course (Cree, Khan et al. 2004).

Interestingly, Multiple Sclerosis comes in two different forms; a Relapsing Remitting form (RRMS), characterized by relative periods of health that are abruptly interrupted by acute episodes of symptomatic exacerbations, and a Primary Progressive form (PPMS), in which the patient suffers from a steady decline of neurologic function. It is important to note that RRMS comprises close to 90% while PPMS comprises about 10% of MS cases (Miller and Leary 2007). Because of this distribution, RRMS will be the main focus of this thesis.

Common MS symptoms include disturbances in vision, balance, motor, sensory, autonomic and cognitive function. Despite the initial waxing and waning pattern of RRMS, many patients will later make the transition to a

secondary progressive phase (Tremlett, Yousefi et al. 2009), leading to a steady decline in neurological function and ultimately resulting in severe disability.

Acute MS lesions are characterized by focal breakdown of the neuroprotective blood-brain barrier (BBB) and by the loss of oligodendrocytes and CNS myelin, the fatty insulation of CNS axons, which facilitates saltatory conduction (Arroyo 2000). Additionally, the typical MS lesion is sharply circumscribed with a well-defined lesion edge. It is also important to note that axons are relatively spared in active MS lesions (Prineas and Parratt 2012).

The BBB defined: The BBB is a protective barrier formed by the blood vessels of the brain. The capillary network of the central nervous system forms a barrier that limits the flow of solutes from the blood to the brain. This barrier is extremely important for maintenance of brain homeostasis and suitable ionic concentrations for controlled neuronal excitability. In addition to preventing the entry of many systemic toxins from entering brain tissue, the BBB also reduces the frequency of brain immune surveillance when compared to other organs.

To perform such specialized functions, brain endothelial cells (ECs), which form the lumen of blood capillaries, possess many unique properties when compared to endothelial cells in the periphery. CNS ECs form high electrical resistance tight junctions between their adjoining cell walls (Rubin and Staddon 1999). Tight junctions prevent virtually all molecules from entering the brain by forming tight seals between adjacent EC membranes, creating fence like structures. This adaptation prevents much of the paracellular transport that occurs in other organs. CNS ECs also display lower rates of transcytosis and lack the fenestrations observed in peripheral ECs (Kaya, Chang et al. 1996),

adaptations that limit the transcellular transport of fluid phase molecules. Fenestrated vessels are no more permeable to plasma proteins than are BBB vessels, but such vessels are much more permeable to water, ions, and small solute molecules (Kaya, Chang et al. 1996). Meanwhile, lipophilic molecules of low molecular weight can enter the brain through passive diffusion. However, the brain possesses transporters such as P-glycoprotein, which generally transports these lipophilic molecules back into the blood (Rubin and Staddon 1999). While these features have evolved to maintain a stable CNS environment and restrict the entry of toxins, active transporters present on the surface of endothelial cells supply the brain with specific nutrients and survival factors.
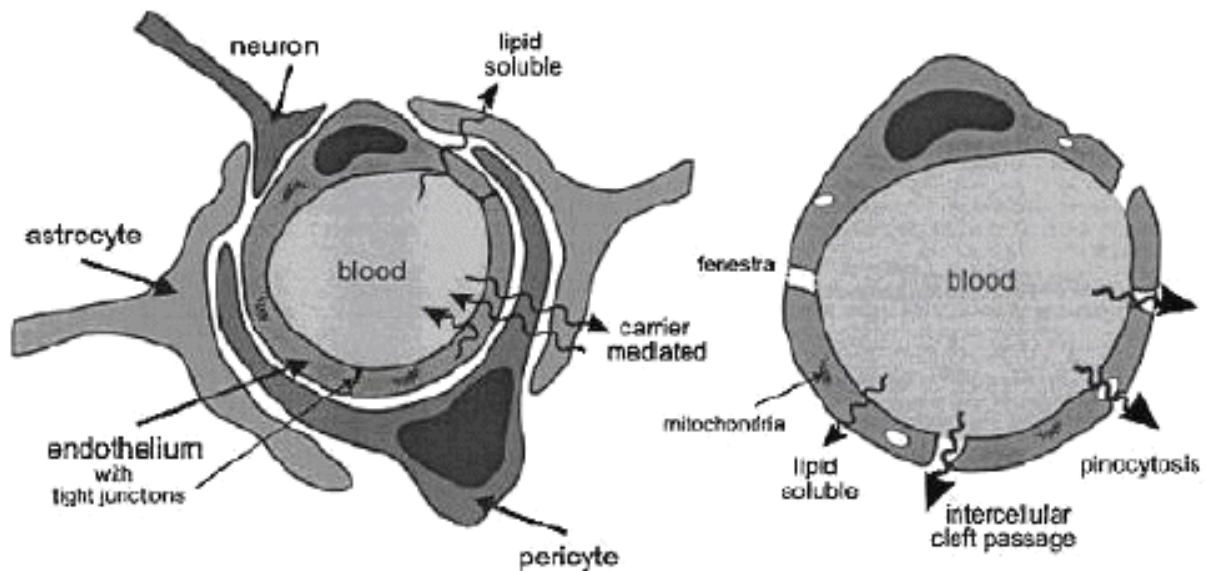
Figure 1. An Illustration of the Blood-Brain Barrier. Schematic comparison between brain (left) and peripheral (right) capillaries. (Misra, Ganesh et al. 2003)

CNS myelin defined: CNS myelin is a multilamellar spiral of specialized membrane, made by oligodendrocytes, that ensheathes axons larger than 1 µm in diameter. Myelinated axons are completely covered by myelin sheaths except at nodes of Ranvier, the small gaps (less than 1 µm in length), directly exposed to the extracellular milieu. By reducing the capacitance and increasing the resistance, myelin reduces current flow across the internodal axonal membrane, thereby facilitating saltatory conduction at nodes (Arroyo 2000).
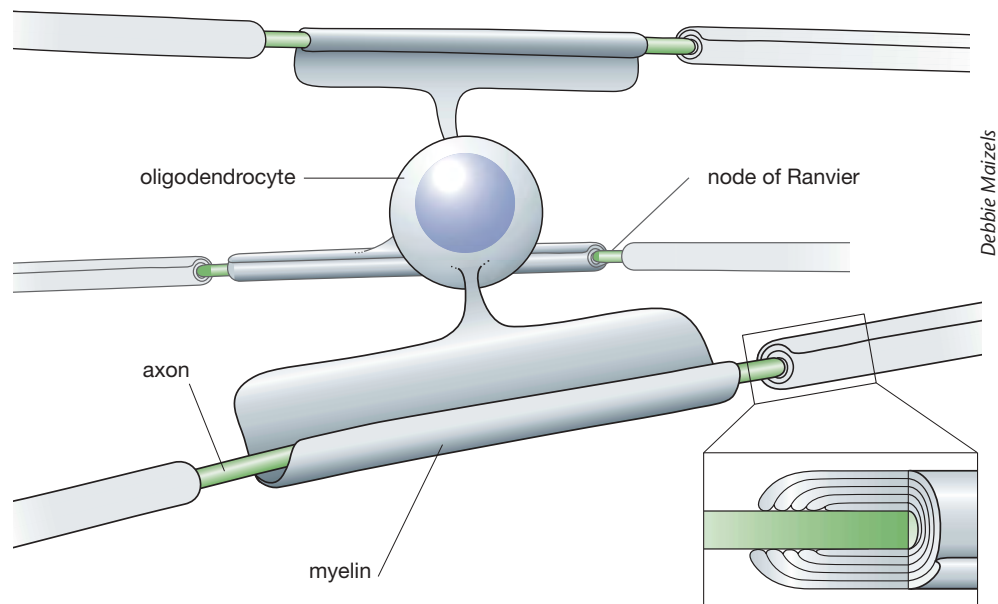
Figure 2. An Illustration of CNS Myelin. The figure schematically depicts an oligodendrocyte simultaneously wrapping multiple axons with a myelin sheath. Also shown are nodes of Ranvier, which are unmyelinated regions where the voltage-gated sodium channels localize. The box shows a longitudinal section through a myelinated axon, illustrating its multilayered structure. (Popko 2003)

A simplified view of the autoimmune MS hypothesis: In addition to BBB damage and loss of CNS myelin, termed *demyelination*, it has been noted that acute MS lesions display a heavy accumulation of immune infiltrates such as activated T cells, B cells and phagocytic macrophages. While it is generally accepted that the histopathological hallmark originates from a breach in the integrity of the BBB, how this initial event occurs remains unknown. As illustrated below in Figure 3, it is suspected that an unknown trigger causes upregulation of adhesion molecules on the endothelium of the brain, optic nerves and spinal cord, allowing leukocytes to home to and traverse vessel walls to enter the normally immunologically privileged central nervous system. If lymphocytes programmed to recognize myelin antigen exist within the cell infiltrate, they may trigger autoimmune demyelination and result in MS.
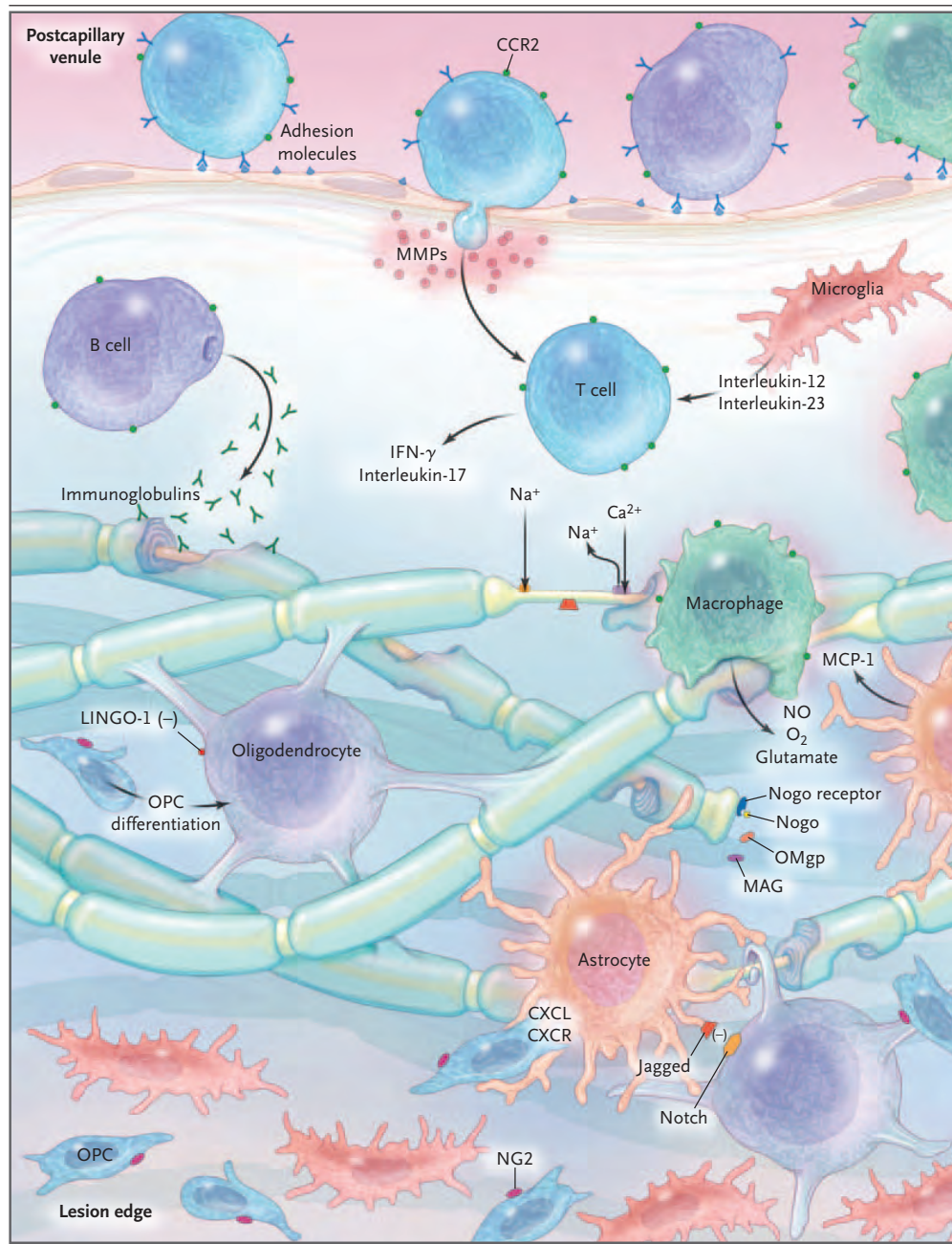
Figure 3. A Model for Autoimmune Demyelination. Leukocyte infiltration and damage of the CNS during an acute MS attack. Blood leukocytes adhere to the luminal surface of the brain endothelium and enter the CNS. T cells secrete pro-inflammatory cytokines. B cells secrete myelin reactive antibodies and macrophages strip myelin off axons. (Frohman, Racke et al. 2006)

Early Descriptions of MS

In the 1824 publication *Maladies de la moelle epiniere*, Charles Prosper Ollivier D'Angers provided the first description of Multiple Sclerosis. In 1808, he encountered a 20-year-old man who developed transient weakness of his right foot. At the age of 17, the young man recalled a period of feeling tired and languid. By 29 years of age, he had weakness of both legs. By age 30, he noted that the hot waters of a spa induced loss of feeling in his right leg and numbness and clumsiness of his hands; an early description of the "hot bath test." He suffered from urinary retention, and a progressive deterioration in his motor function, speech and ability to write. The young man felt cold water as hot and he felt "galvanic shocks" when his paretic right hand touched his thigh. He seemed intellectually intact and retained the "gaiety of his character" despite his advanced disability. Ollivier D'Angers suspected the cause to be a "myelitis" due to an infection, and suggested a treatment regimen of bleeding and blood leeching (Murray 2005).

Robert Carswell, a British pathologist, provided the first pathological description/drawings of MS in an atlas of pathological conditions published in 1838 (Carswell 1838). He noted a brown discoloration of the spinal cord (Figure 4) and postulated that the lesions were caused by a deficiency of blood supply to the affected regions. Meanwhile, Jean Cruveillhier, a French contemporary, contributed similar drawings published sometime after 1841. He was the first to recognize the essential feature that islands of "grey degeneration" were replacing the white matter. Cruveillhier, felt that this condition was caused by a general suppression of sweat (Murray 2005).

Figure 4. An Early Drawing of MS Lesions. An illustration by Robert Carswell depicting brown discoloration of the normally white spinal cord. (The Multiple Sclerosis Society of Ireland)

Despite the prior contributions mentioned above, Jean-Martin Charcot is credited with the "discovery" of MS. In his 1868 lectures, Charcot distinguished MS as a unique disease state and clearly defined its specific symptomatology. He outlined the clinical appearance of intention tremor, nystagmus, and scanning speech as three reliable indicators of an MS diagnosis, which would later be called the Charcot triad. Charcot not only provided the first clear delineation of MS symptoms, but also conducted his own pathological investigation. Like Carswell and Cruveillhier, he drew many illustrations of gross anatomical lesions. In addition, he produced renderings of the microscopic changes. Using a carmine stain, Charcot noted the common presence of a central capillary, with surrounding demyelinated axons. He noted that although the axons were relatively spared, there was axonal thinning. Charcot also depicted "voluminous fatty globules," the result of myelin breakdown, and eventual removal of the fatty myelin sheath (Murray 2005). But most important for Charcot was the proliferation of astroglia and their reticulated fibers (the glial scar). He felt that MS was primarily a disease of glial overgrowth with secondary changes in the blood vessels (Compston 2005). Charcot writes:

> *Undoubtedly, the multiplication of nuclei and the concomitant hyperplasia of the reticulated fibers of the neuroglia constitutes the initial, fundamental fact, and necessary antecedent; the degenerative atrophy of the nerve elements, is consecutive and secondary; it had already begun when the neuroglia gave way to the fibrillary tissue through the wasting, afterwards, proceeded with greater rapidity. The hyperplasia of the vascular parieties plays merely an accessory part.*

Not until the early 1900's did improvement on these early works appear. In 1916, the Scottish pathologist James Walker Dawson published his seminal thesis work *The Histology of Disseminated Sclerosis*, which is widely recognized to be the first systematic study to significantly advance beyond Charcot's

observations. It is perhaps the most important contribution to British neurology in that generation (McAlpine 1946).

Dawson studied nine MS cases, and provided many key pathological insights. He observed the following key features:

- *MS plaques are deposited in relation to the distribution of the veins and to the walls of the ventricles.*

- *Both grey and white matter may be affected, however, lesions more often affect the white matter.*

- *Older patches are firmer than the normal brain substance (sclerotic), but fresh patches are softer (malacotic) than the normal brain.*

- *A striking microscopic feature of lesions is the sharp demarcation of the lesion edge.*

- *An inflamed blood vessel is often present in the center of a lesion.*

After his detailed investigation into the pathologic features of MS, Dawson contemplated the theories of the day regarding a triggering agent. In his thesis, he quotes Byrom Bramwell's theory that "the sclerotic lesions are the result of some irritant which is distributed through the nerve centres by the blood-vessels"(Dawson 1916). Dawson juxtaposes the exogenous irritant theory with the idea championed by Strumpell and Mueller that MS is triggered by an endogenous factor. Dawson summarizes the endogenous theory as he writes, "the disease is due to some developmental or congenital defect of the neuroglial or nervous tissue (perhaps similar to, or analogous to, the gliomatosis in cases of syringomyelia), which renders it more vulnerable or liable to be affected by irritation than the neuroglial or nervous tissue of the normal individual" (Dawson 1916). Charcot also favored the endogenous hypothesis, as he believed that MS was a consequence of astroglial overgrowth.

Although initially unimpressed with the exogenous toxin theory, feeling that the effects of a toxin would be diffuse rather than focal, Dawson later changed his mind and concluded the following:

- *Multiple Sclerosis is likely due to a specific external agent.*

- *The external agent is likely a soluble toxin.*

- *Somehow the agent enters only certain areas due to some local vascular change.*

It should be noted that in the present day study of MS, Dawson's name continues to be linked with the flame-like plaques radiating off the corpus callosum on sagittal sections of the brain on MRI that are pathognomonic for a diagnosis of MS, called Dawson's fingers.
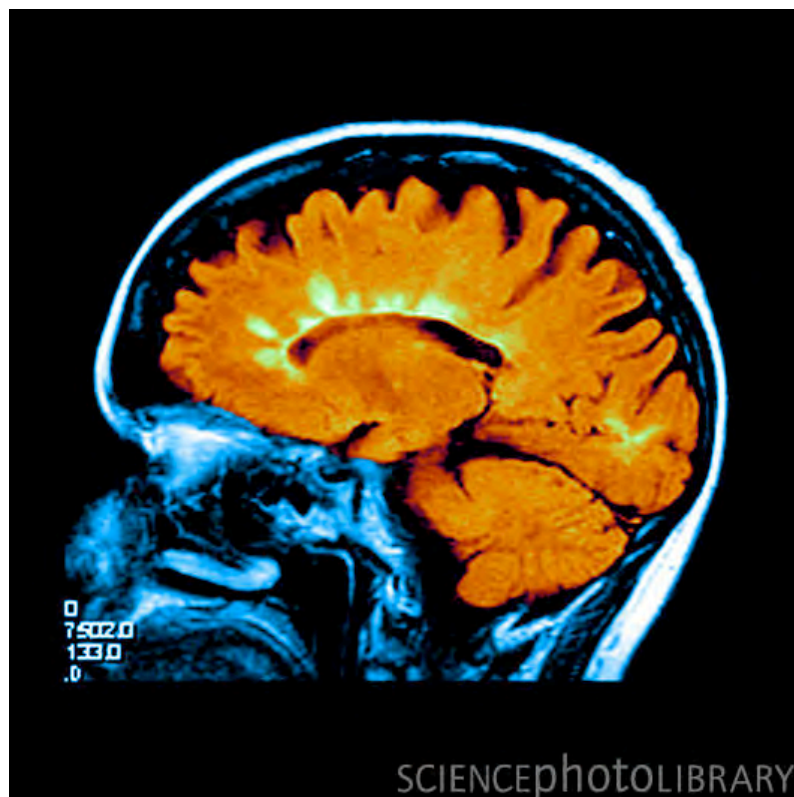
Figure 5. An MRI Image of Dawson's Fingers. A sagittal MRI of an MS patient with flame-like lesions radiating off the corpus callosum (Dawson's fingers).

What Causes MS? Current Ideas

The theories about what causes MS changed as the major advances in medical science changed. In the era of Pasteur and Koch, it seemed to be an infectious disease; in the era of environmental illnesses, it seemed a disease due to some toxin; when epidemiological techniques improved, interest centered on mysterious demographic and environmental factors; as immunology flourished, it became an immunological disease, and in this age of genetics, gene probes, and the human genome, there's great interest in a genetic factor. With advances in virology, slow virus infections, and now prions, it is again being considered an infectious disease, with the virus perhaps acting as a trigger in a genetically predisposed individual. One might ask if the theories of MS just follow the current interest in science, but it is evident that each stage provided important building blocks for the next. (T. Jock Murray 2005)

The cause of MS has eluded researchers for centuries. The longevity of this mystery has led to scientific inquiry encompassing almost every biological discipline imaginable; bacteriology, virology, immunology, genetics, epidemiology, endocrinology and the list continues. Perhaps the most appropriate place to start when summarizing the many theories proposed for the elusive MS trigger would be investigations into the epidemiology of the disease.

Genes and MS

Familial studies have contributed greatly to our current understanding of the genetic factors involved in MS. Sadovnick et al. performed a comprehensive study of the risk of developing MS if a relative had a confirmed MS diagnosis (the proband). As depicted in the table below, there is a marked difference between the relative risk for a monozygotic twin (RR=190), who shares 100% of the proband's genetic material, as compared to an adopted sibling (RR=1), who is completely unrelated. While this clearly shows that genes matter in MS, the fact

that there is only a 38% chance of a healthy monozygotic twin later developing

MS suggests that an environmental trigger likely exists.

Table 1. Comparison of Age-Adjusted Risks By Relationship to The Proband.
Data for a Northern European population living in a temperate climate
(Sadovnick, Dircks et al. 1999).

Relationship to proband	Approximate risk (%)	Relative risk to general population	% Genetic sharing with the proband
General population	0.2	1	0
First-degree relative	3–5	15–25	50
Dizygotic twin	3–5	15–25	50
Monozygotic twin	38	190	100
'Adopted' first-degree relative	0.2	1	0
Half-sib	1.3	6.5	25
Offspring of conjugal MS[a]	29.5	147.5	50[b]

[a] It may be more appropriate to compare crude rates for this group (18).
[b] The child shares 50% of the genetic material with the affected mother and 50% of the genetic material with the affected father.

With the advancement of genetic probing techniques, MS investigators

have identified more and more genetic loci that seem to be associated with MS.

The strongest genetic association was identified relatively early in the modern

era of genetics. In the early 1970's, high density SNP analysis revealed a strong

association with the HLA-DRB1* 1501 haplotype. The International Multiple

Sclerosis Genetics Consortium has determined the odds ratio for HLA-DRB1*

1501 to be 5.8; 95% CI, 3.53 to 9.53 (p= 1.83×10−17) (Hafler, Compston et al. 2007).

The Human Leukocyte Antigen (HLA) system is the name given to major

histocompatibility complex (MHC) in humans. The MHC's function is to present

both self and foreign antigens to T lymphocytes. HLA-DRB1* 1501 is part of the

MHC class II system that is found on the surface of professional antigen

presenting cells (dendritic cells, macrophages and B cells). Antigens that exist

outside of the antigen-presenting cell are phagocytosed, degraded and loaded

onto the MHC II molecule as a short polypeptide for presentation to CD4+ helper
T cells. Additionally, intracellular (self) antigens can also be presented via MHC
II following autophagy (Blum, Wearsch et al. 2013).
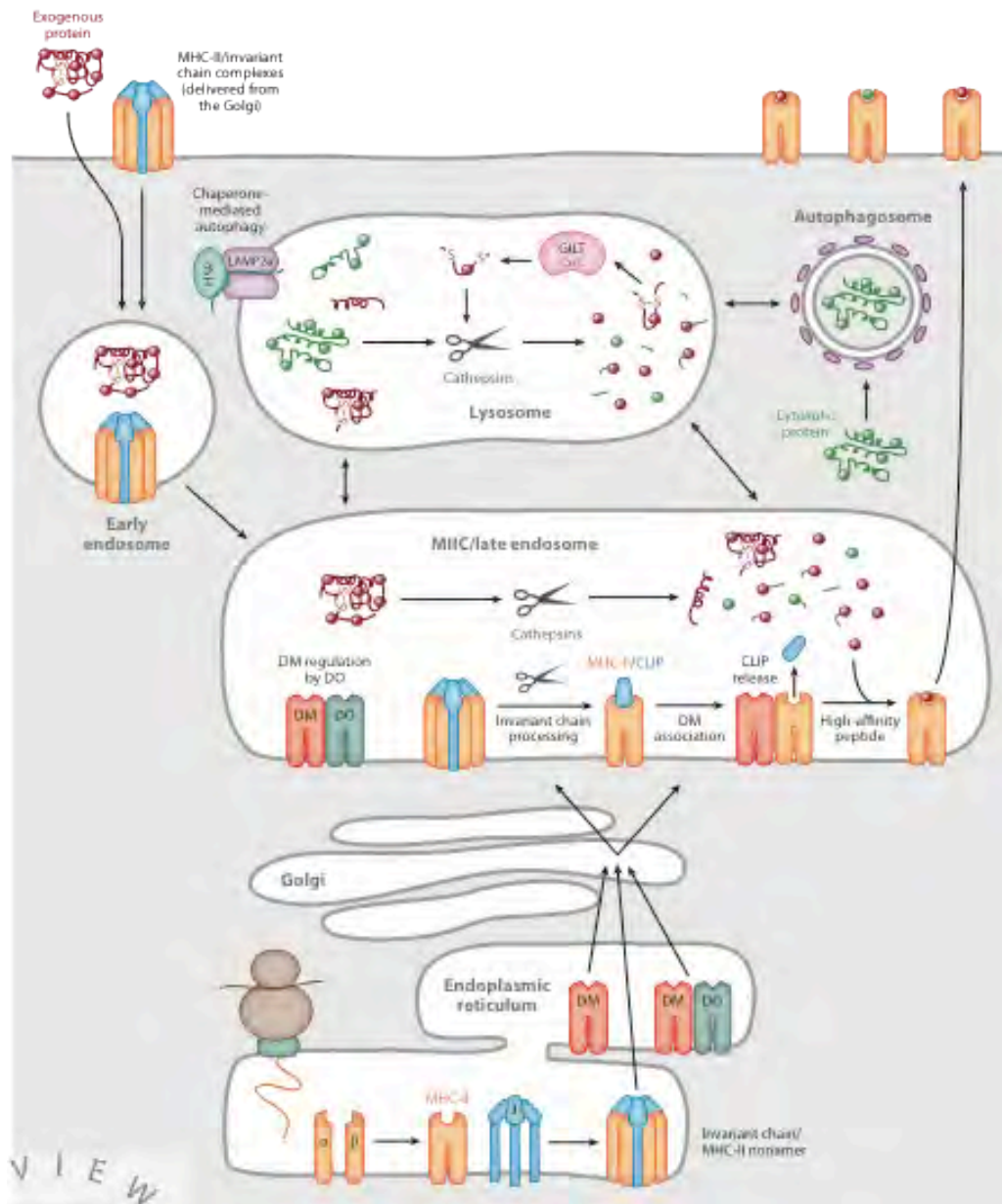
Figure 6. A Schematic of MHC Class II Antigen Processing (Blum, Wearsch et
al. 2013)

On face value, HLA-DRB1* 1501's association with MS seems to support an autoimmune view of MS, as this haplotype is also associated with stereotyped autoimmune diseases such as Goodpasture syndrome (Phelps and Rees 1999), Systemic Lupus Erythematosus (SLE) (Takeuchi, Nakano et al. 2005) and Sjogren's syndrome (Manoussakis, Georgopoulou et al. 2004) for which autoantigens are known. However, it should also be noted that the HLA-DRB1* 1501 haplotype is also associated with diseases of clear infectious origin; cervical cancer (human papilloma virus) (Apple, Erlich et al. 1994). Additionally, infectious triggers for autoimmune disease such SLE are still popular areas of investigation. Therefore, distinguishing a disease as autoimmune or infectious in nature is often times not clear-cut. Finally, although there is a concentration of the HLA-DRB1 *1501 haplotype in the MS patient population, it is not present in all MS patients. Additionally, not all people who carry HLA-DRB1 *1501 have MS; thus HLA DR haplotypes are not necessary or sufficient to produce the disease.

Other strongly associated loci are Interleukin 2 receptor-alpha (IL2RA); 2 synonymous SNPs in intron 1, and Interleukin 7 receptor-alpha (IL7RA); 1 non-synonymous SNP in exon 6 (Hafler, Compston et al. 2007). Functionally, the non-synonymous SNP in exon 6 of IL7RA would lead to a decrease in IL7/IL7RA signaling. The mutation is found in a transmembrane domain and causes increased skipping of the exon, increased production of soluble IL7RA and decreased expression of membrane-bound IL7RA (Online Mendelian Inheritance in Man). The contribution of this SNP appears to be modest, however, with an odds ratio of 1.18; 95% CI 1.11 to1.26 (p = $2.94 \times 10-7$)(Hafler, Compston et al. 2007).

In summary, while we can identify that genes are associated with MS, it is clear that genes are not enough to trigger the disease. It is striking that many of the MS associated loci are involved in immune function, and it is tempting to assume that because of this, MS must be autoimmune in nature. However, it is important to consider that the immune system evolved primarily to fight invading pathogens, such that these MS associated loci may just as likely be involved in host-pathogen interactions as they may be in an autoimmune process.

The Geography of MS

The incidence and prevalence of MS seem to increase with latitude. MS is rare in equatorial regions (< 5 per 100, 000) and common in temperate regions (>30 per 100, 000) (Marrie 2004).

Worldwide prevalence of multiple sclerosis per 100 000 population.
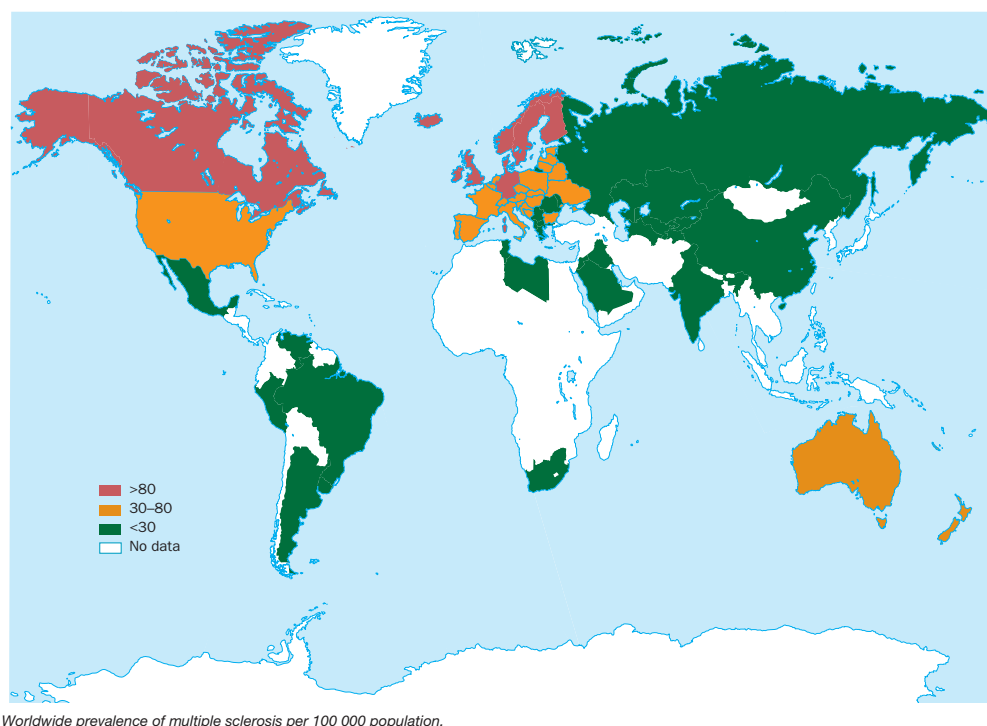
Figure 7. The Global Distribution of MS (Marrie 2004)

The exact reason for this pattern of global distribution remains unknown, but a few explanations have been offered. 1) The global distribution mirrors the concentration of Northern European settlements; people of this racial background being more at risk for developing MS (Bulman 1992).

2) The distribution may reflect the prevalence of a particular pathogen that triggers MS (Kurtzke 1993). 3) Decreased vitamin D due to reduced sunlight exposure in temperate regions may influence MS susceptibility (Ramagopalan, Dobson et al. 2010). 4) Increased hygiene in developed countries (temperate zones) may alter host-pathogen interactions and may cause an increased risk of developing MS (Ascherio and Munger 2007). However, it should be noted that the latitude gradient is weakening (Ascherio and Munger 2007). Each of these hypotheses is explored in further detail below.

1) Latitude, Northern European Settlements and MS

Upon closer inspection of the global distribution map, it is striking that the latitude gradient applies to the Western Hemisphere, and Western Europe, but it breaks down as one approaches Asia. The simplest explanation for this phenomenon would be the difference in racial backgrounds in these regions. It is possible that a European background and a latitude-based modifying factor act synergistically to trigger MS. To determine if this is the case, we must observe regions that are genetically homogenous, but differ significantly in latitude. Unfortunately, there are regions of relatively homogenous populations that obey the latitude rule e.g. (Australia/Tasmania/New Zealand and the U.K) and regions that fail to obey this rule e.g. (Norway and Italy/Sardinia) (Rosati 2001).

Therefore, it is difficult to identify an unequivocal role for latitude in modulating the risk of developing MS, even when genetic background is controlled.

2) Latitude and a triggering pathogen

John F. Kurtzke has proposed that a pathogen responsible for triggering MS may have arisen in Southern Norway and spread from this focus throughout Europe and to North America thus explaining the latitude gradient (Kurtzke 1974). It is conceivable that European settlers may have transported such a pathogen to the Southern Hemisphere (Australia, Tasmania and New Zealand). This dependence on European settlements for the spread of said pathogen makes it difficult to distinguish the existence of bona fide MS pathogen from confounding variables such as a purely genetic MS risk or shared cultural practices that may lead to some other environmental exposure.

3) Latitude and Vitamin D Deficiency

The influence of vitamin D on MS susceptibility is currently the subject of intense investigation. In humans, the main source of vitamin D comes from the conversion of 7-dehydrocholesterol in skin to active vitamin D_3 by an ultraviolet B (UVB) dependent process. This UVB light comes from sunlight exposure and since this is reduced with increasing latitude, investigators have postulated that vitamin D deficiency may play a role in triggering MS (Berlanga-Taylor, Disanto et al. 2011). Furthermore, there are reports that MS patients are often vitamin D deficient (Faridar, Eskandari et al. 2012). George Ebers et al. have proposed a mechanism for how vitamin D may be involved in MS. Active vitamin D binds to its nuclear receptor and acts as a transcription factor via the

vitamin D responsive element. This molecular complex modulates the expression HLA-DRB1* 15, an MHC II molecule, which has shown a strong association with developing MS. Paradoxically, vitamin D seems to increase the expression of HLA-DRB1* 15. It should also be noted that vitamin D has been found to modulate up to 5% of gene expression in treated cells, so HLA- DRB1* 15 cannot be considered an exclusive vitamin D target (Berlanga-Taylor, Disanto et al. 2011). Despite this paradoxical vitamin D mediated increase in HLA-DRB1* 15 expression, we must also consider that vitamin D has been shown to be protective in experimental autoimmune encephalomyelitis (EAE), the current animal model for MS (Ascherio and Munger 2007).
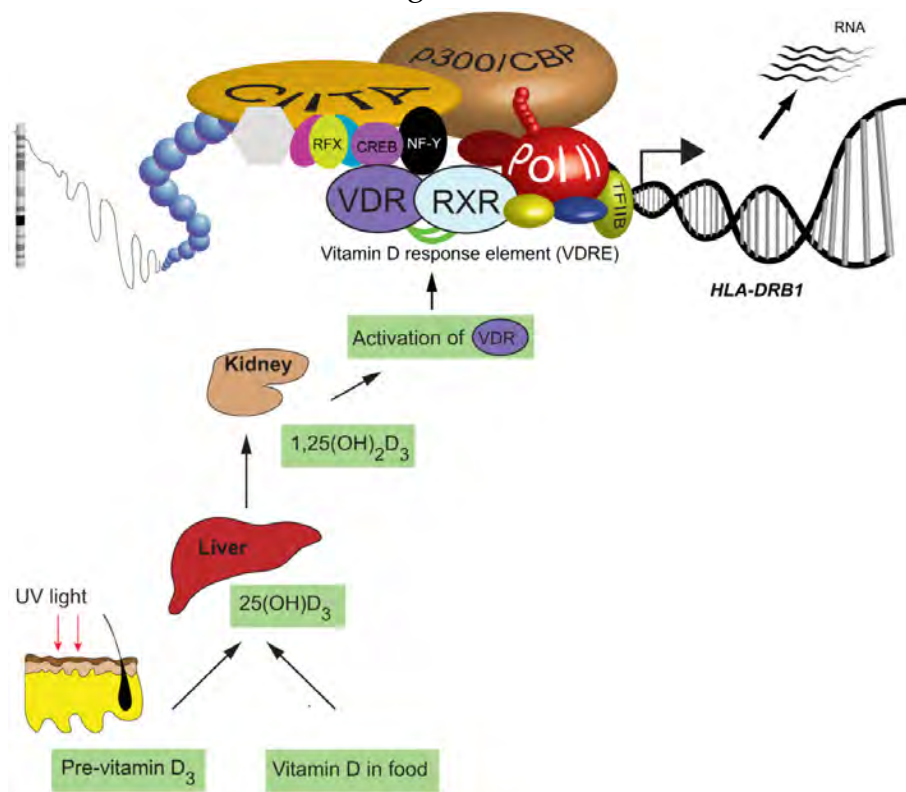
Figure 8. A Schematic of Vitamin D Regulating HLA-DRB1. A conserved VDRE confers responsiveness to vitamin D to HLA-DRB1. (Handunnetthi, Ramagopalan et al. 2010)

4) Increased hygiene in developed countries of temperate zones

The hygiene hypothesis suggests that increased sanitation causes a delay in the timing of common childhood infections. This delay causes a shift from early childhood infections, when the disease process may be benign, to infections that arise during adolescence or young adulthood. At this later exposure, the disease may manifest itself in a more malignant form e.g. polio and potentially MS (Ascherio and Munger 2007). Poorer sanitation in the developing world, i.e. the tropics, may be protective against developing MS by exposing the population to the triggering pathogen earlier in life.

Migration and MS

Migration studies have given risen to some provocative concepts in MS. Despite their many caveats, MS migration studies have consistently shown that one can reduce risk by moving from a high-risk region to a low-risk region. However, the reverse statement is not true. Moving from a low-risk region to a high-risk region does not increase one's risk, providing that the migration is made after the age of 15. This suggests that there is a critical period during the first two decades of life when one acquires a protective factor(s), which remains stable throughout one's lifetime (Marrie 2004).

MS Clusters and Outbreaks

MS outbreaks provide a valuable opportunity to potentially identify a triggering agent. There have been multiple reports of unusually high MS incidences and identifying a common theme between these clusters may be the key to identifying said trigger.

MS outbreak in swayback researchers: In 1947, four of seven researchers developed signs and symptoms of MS. They were studying swayback, which is neurological disease of lambs caused by copper deficiency. Another researcher joined the group later in the study, making a final group of eight men. The chance of four or more out of eight men developing MS is about one in a billion. Of interest, MS has not occurred in workers studying swayback disease elsewhere (Dean, McDougall et al. 1985).

MS outbreak in Key West: In 1984, Dr. William Sheremata, a neurologist from The University of Miami drew attention to the paradoxically high incidence of MS in Key West, the southern most tip of the United States of America. He determined that of the 26, 000 Key West residents, 37 of them had MS (140 per 100, 000), which does not fit the expected low prevalence rate for its respective latitude. Dr. Sheremata speculated that the exposure may be related to the fact that Key West did not have an adequate sewage treatment plant and often endured exposure to unpotable water because of sewage contamination (Sheremata, Poskanzer et al. 1985). Interestingly, nine of the 37 patients were nurses, who had at some time in their careers worked in the same community hospital.

MS outbreak in Mansfield, MA: Fourteen MS cases were identified in the small town of Mansfield, Massachusetts (population 10, 000). Remarkably, eight of these patients all lived within the same block between 1932 and 1936. All eight of these patients lived on the town's water supply, which was heavily contaminated at the time. In August 1932, the Department of Public Health

urged Mansfield to consider a sewerage system and disposal plan to eradicate excessive sewage bacteria found in all waterways in the center of the community. A pond located in the thickly settled area of town where the patients lived was highly contaminated. Eastman writes, "One can only assume that multiple episodes of exposure to a common contaminated water supply occurred during the four-year period." (Eastman, Sheridan et al. 1973)

Multiple Sclerosis outbreak on the Faroe Islands: This outbreak, identified by John F. Kurtzke, is perhaps the most well known and the most carefully investigated. The Faroe Islands are a group of 18 Danish islands in the North Atlantic Ocean situated between Norway and Iceland. According to Kurtzke, there were no native Faroese suffering from MS in the 20th century before July 1943, when the first of 21 cases occurred. From April 1940 until September 1945, British troops occupied the Faroe Islands during World War II. Because the presence of British forces coincided with the MS cases not only in time, but also in space, Kurtzke postulated that they brought to the Faroes an infectious agent responsible for triggering MS. Kurtzke goes on to identify a coincident increase in acute gastrointestinal diseases during the British occupation and proposes that the MS agent may be an enteric pathogen spread by fecal-oral transmission (Wallin, Heltberg et al. 2009).

Infections Implicated in MS

Pierre Marie (1853-1940) was the first to champion the idea that an infection causes MS. He firmly believed that one day "the vaccine of Pasteur or lymph of Koch" would be sufficient to eradicate the disease (Murray 2005).

Despite Marie's optimism, we still struggle with MS in 21st century due to the fact that such an infection is yet to be identified. Many infections have been proposed, but viruses have received the most attention. This is partly because demyelination is a prominent feature in brain lesions caused by known human viruses e.g. measles virus, which causes SSPE, and JC virus, which causes PML (Lipton, Liang et al. 2007). Furthermore, viruses can cause demyelinating lesions in other species, as shown below in Table 2.

Table 2. Experimental Animal Models of Chronic Virus Induced Demyelination. (Lipton, Liang et al. 2007)

Virus	RNA Family	Host	Viral Persistence[a]/Reservoir[b]
Canine distemper virus[83,84]	Paramyxovirus	Dog	+/astrocyte
Mouse hepatitis virus[85,86]	Coronavirus	Mouse	+/astrocyte
Theiler's virus[87,88]	Picornavirus	Mouse	+++/macrophage
Visna virus[89,90]	Retrovirus	Icelandic sheep	++/monocyte/macrophage
[a]Comparative level of persistence: + = viral antigen only; ++ = viral antigen + limited cell-associated infectious virus; +++ = viral antigen + infectious virus. [b]Principal cell harboring virus during persistent infection.			

Human herpesviruses represent the most studied in MS. Among them HHV-3 (varicella zoster, VZV), HHV-4 (Epstein Barr, EBV) and HHV-6 have all been implicated.

HHV-3: Varicella-zoster virus, VZV, causes chickenpox and is acquired by 95% of adults in the developed world. VZV establishes latency in the dorsal ganglia of most healthy people and viral reactivation affects approximately 1% of the population. Conflicting data have been reported regarding the presence of VZV DNA and visualization of VZV virions in MS tissues (Kakalacheva, Munz et al. 2011).

HHV-4: Epstein-Barr virus, EBV, mainly infects B-lymphocytes and can establish latency. Exposure to this virus in early childhood is usually asymptomatic, but infection in adolescence or young adulthood can lead to Infectious Mononucleosis (IM). Seroreactivity against EBV antigens is found in 99% of MS patients when compared to 89-95% in controls. In addition, IM occurs in the same geographic regions as MS (more common farther away from the equator) and Operskalski et al. found that IM was strongly associated with eventually developing MS (Operskalski, Visscher et al. 1989). For these reasons EBV has been the favored MS associated virus (Tselis 2011).

HHV-6: This lymphotrophic virus exhibits a tropism for CD4+ T cells but has a tendency to infect neural cells. HHV-6 DNA has been detected in brain tissue, CSF and PBMCs from some MS patients (Challoner, Smith et al. 1995; Kim, Lee et al. 2000; Alvarez-Lafuente, Garcia-Montojo et al. 2008). Furthermore, a mechanism of molecular mimicry has been proposed based on the virally encoded U24 protein's amino acid homology with myelin basic protein (MBP) (Tejada-Simon, Zang et al. 2003). Despite these positive findings, an association with MS remains tenuous due to studies with contradictory results (Martin, Enbom et al. 1997; Mirandola, Stefan et al. 1999).

Other infections have also been implicated in MS; canine-distemper virus (Rohowsky-Kochan, Dowling et al. 1995), human endogenous retroviruses (Nexo, Christensen et al. 2011) and most recently a bacterium, Chlamydia

pneumonia (Sriram, Stratton et al. 1999). However none of these studies have yielded conclusive results.

The Immunological Hypothesis

The production of clonally restricted antibodies in the CSF, which are absent in the serum (oligoclonal bands)(Davenport and Keren 1988), a presence of immune cells in MS plaques (Lassmann 2011) and changes in the number of helper and suppressor cells in peripheral blood depending on disease activity (Waksman 1981) have prompted investigation into a possible immune abnormality that may be responsible for triggering MS. Similarities between MS and an immunization-mediated encephalomyelitis (EAE) have also contributed to the immunological hypothesis.

Although provocative and the prevailing present day hypothesis, the immunological theory has some striking inconsistencies. To elucidate these inconsistencies, we must first review the current immunological theory of MS.

Cellular autoimmunity: Thomas Rivers and Francis Schwentker of The Rockefeller University developed EAE in 1935 (Rivers and Schwentker 1935). In their initial experiments, they intramuscularly injected eight monkeys with heterologous brain tissue from rabbits and observed myelin destruction in 7/8 injected animals. Affected animals suffered from ptosis of the eyelids, mask-like expression of the face, facial paralysis, abnormal position of the head (held to the right or to the left), blindness and ataxia. Little or no paralysis of the extremities was detected (Rivers and Schwentker 1935). This was the first evidence that a

demyelinating disease could be triggered by vaccination with brain tissue and thus excited the MS community.

In modern EAE protocols, whole brain homogenates are no longer used. Rather, specific immunogenic myelin peptides are injected with Freund's adjuvant (immunostimulatory, inactivated mycobacteria) along with pertussis toxin which weakens the BBB (Stromnes and Goverman 2006). This vaccination model represents the Active Induction EAE model, and is characterized by an ascending flaccid paralysis with CD4+ T cell mediated inflammation targeting the spinal cord. Along side Active Induction there is also a Passive Induction model in which myelin reactive CD4+ cells are adoptively transferred from a vaccinated animal into a naïve animal, again leading to ascending flaccid paralysis with a predilection for the spinal cord (Stromnes and Goverman 2006).

One striking inconsistency between EAE and MS is that EAE animals suffer from a flaccid paralysis while MS patients suffer from spastic paralysis (Stromnes and Goverman 2006). Additionally, clonally restricted CD8+ T cells dominate MS lesions while both active and passive EAE result from CD4+ T cell mediated disease (Sriram and Steiner 2005). However, efforts are being made to develop CD8+ T cell mediated EAE models by engineering recombinant viruses that express myelin proteins (Stromnes and Goverman 2006). In addition to the differences in paralysis (flaccid vs. spastic) and effector T cells present in EAE vs. MS lesions (CD4 vs. CD8), Table 3 lists many of the other differences between EAE and MS.

Table 3. Immunopathology and Response to Therapy in EAE and MS. (Sriram and Steiner 2005)

	EAE	MS
Pathology		
Location of demyelination	Predominantly, perivenous sleeves of myelin loss in spinal cord and brain	Demyelination not restricted to perivenous regions of white matter; extensive demyelination of cerebral cortex in the absence of inflammation is common
Location of lesions	Dependent on the autoantigen used for induction: inflammation dominates in lumbar regions in MBP and PLP EAE and brainstem in MOG EAE	Periventricular areas, cortical mantle, brainstem, optic nerves, and upper cervical cord; lesions are uncommon in thoracic and lumbar regions
Phenotype of cellular infiltrate	$CD4^+$ T cells (MBP and PLP EAE) activated macrophages and few $CD8^+$ T cells	Activated macrophages and $CD8^+$ T cells of a restricted clonotype
Cytokine predominance	TH1 bias in MBP and PLP EAE; TH2 bias worsens MOG EAE	Variable; no clear cytokine preponderance
CSF immunology	Antibodies to myelin antigens present in CSF	Antibodies to myelin antigens are infrequent in CSF and do not constitute the antigen specificity of oligoclonal bands
Effect of immunotherapies		
γ interferon	Depends on route of administration and can either worsen on ameliorate EAE	Worsening of inflammatory lesions unproven
β interferon	Variable; can worsen EAE if given after immunization	Decreases relapse rate: effect on progression modest
Anti–TNF antibody	Reverses EAE	Worsens MS
Anti–VLA-4 antibody	Reverses EAE	Decreases relapses; effect on progression not known
Anti–CD4 antibodies	Cures EAE	No evidence of clinical efficacy on relapses or progression
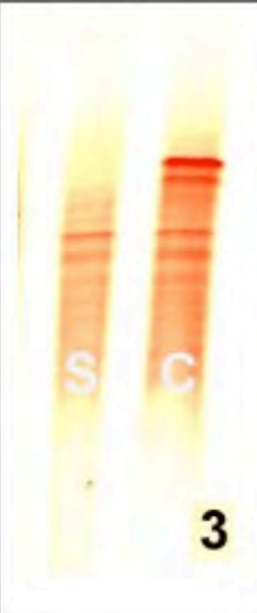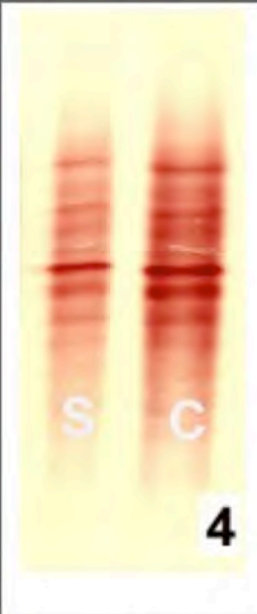
EAE = experimental allergic encephalomyelitis; MS = multiple sclerosis; MBP = myelin basic protein; PLP = proteolipoprotein; MOG = myelin oligodendrocyte glycoprotein; CSF = cerebrospinal fluid.

Despite these differences, EAE begs the question; Are there major differences in the T cell repertoires in MS patients vs. healthy controls? The short answer to this questions is no. Myelin reactive T cells can be found in most human beings (approx. 70%) and MS patients fail to show a substantial difference in the prevalence of such cells (approx. 80%) (Martin, Jaraquemada et al. 1990).

Humoral autoimmunity: Antibody abnormalities are well established in MS and are an important part of the determining a diagnosis. The most striking abnormality is oligoclonal banding. In 1964, E.C. Lature used gel electrophoresis

to show that in MS cerebrospinal fluid there was intrathecal synthesis of clonally restricted IgG (Lature 1964). These unique CSF antibodies gave a different electrophoretic pattern when compared to serum IgG from the same patient.

Oligoclonal bands are not unique to MS, but are present in other neurologic diseases, typically of an infections or inflammatory origin. In the case of an identifiable infection, the oligoclonal antibodies usually react with pathogen-derived antigens that are either found in the brain tissue or are part of an antigen specific serological assay e.g. an ELISA. Below is a summary of different gamma globulin banding patterns:

Conditions	Pattern 2	Pattern 3	Pattern 4
	2	3	4
Infection	Yes (CNS)	Yes	Yes (systemic)
Inflammation	Yes (CNS)	Yes	Yes (systemic)
Paraneoplastic	Yes	Yes	Yes
Neoplastic	Rare	Rare	Rare
GBS	No	No	No
Other neuropathies	No	No	Yes
Multiple Sclerosis	Yes	Yes	No
Vascular disease	No	No	Rare
Degenerative disease	No	No	Rare

GBS = Guillain-Barré syndrome, S = serum, C = CSF

Figure 9. Gamma Globulin Banding Patterns and Associated Conditions.
Pattern 1 (not shown) is an absence of banding in both the serum and CSF and reveals an absence of both systemic and intrathecal IgG synthesis. **Pattern 2** shows oligoclonal banding in the CSF only and can be seen in MS. **Pattern 3** (a more than pattern) shows oligoclonal banding both in the serum and CSF, however, there are additional bands in the CSF. Pattern 3 can also be seen in MS and indicates both systemic and intrathecal IgG synthesis. **Pattern 4** (a mirror pattern) suggests systemic IgG synthesis with an absence of intrathecal synthesis. This pattern is not usually seen in MS. (University of Birmingham 2013)

Because the oligoclonal CSF antibodies fail to react against myelin proteins, as they do viral antigens e.g. SSPE caused by the measles virus, investigators have wondered if MS CSF antibodies are "nonsense" antibodies that have been produced as part of a non-specific inflammatory process. However, Gregory P. Owens et al. have shown that the intrathecal plasma cell clones that secrete these antibodies have undergone somatic hypermutation, suggesting a response to a particular antigen (Owens, Kraus et al. 1998). Furthermore, they have shown binding of human IgG to an unknown antigen at the edge of MS lesions (Owens, Gilden et al. 2011). Conflicting data exist regarding the binding of human IgG in MS lesions, as Barnett et al. conclude that IgG staining of disrupted myelin in MS lesions is a non-specific feature found in neurologic diseases other than MS. (Barnett, Parratt et al. 2009).

One provocative study has proposed a bona fide MS autoantigen. In 2012, Srivastava et al. screened serum IgG from MS patients for antibodies capable of binding to brain tissue. They identified that 47% of MS patients possessed serum IgG reactive against the first extracellular loop of the KIR4.1 potassium channel compared to 1% in patients with other neurologic disease and 0% in healthy controls ($p<0.001$). Injection of affinity purified MS IgG (using KIR4.1 as bait) led to changes in the astrocyte cytoskeleton and complement deposition. Although oligodendrocytes also express KIR4.1, no evidence of damage to these cells was reported (Srivastava, Aslam et al. 2012). Since the anti-astrocyte IgG (anti-aquaporin 4) is proving to be the primary pathophysiologic mechanism for Neuromyelitis Optica (NMO) (Roemer, Parisi et al. 2007), another demyelinating disease, one could argue that anti-KIR 4.1 may be of importance in the demyelination that occurs in MS. However, NMO and MS differ significantly in

their pathological features. NMO lesions are characterized by astrocyte loss, neutrophil and eosinophil infiltration, significant axonal injury and profound inflammation, where as MS lesions display oligodendrocyte apoptosis, mild inflammation and minimal axonal injury (Lassmann 2011).

Back to the Basics: Modern Day MS Pathology

Despite important contributions from many disciplines, the cause of MS still evades us. However, modern advances in MS pathology may hold the key to unlocking this centuries old mystery. Detailing the cellular changes that occur soon after the MS lesion begins to unfold gives us the best chance of revealing key features of the elusive trigger. Two research groups, Lucchinetti/Lassmann and Barnett/Prineas, have dominated the field for the last 15 years. Unfortunately, these groups are not in agreement regarding the nature of MS lesions.

The standard view of MS lesions: MS plaques vary in size and in number but are most conspicuous in areas with myelin enriched axon tracts, termed *white matter*. These areas include the periventricular white matter, the visual system, deep white matter, brainstem, and spinal cord. Active MS lesions are usually localized to white matter regions and are characterized by a mixture of lipid-laden macrophages, large reactive astrocytes, accompanied by varying perivascular inflammation. Axons are relatively well preserved, although where damage is most severe, axons are also lost or fragmented and display irregular

tortuous and clubbed profiles. Besides focal demyelinated plaques, global diffuse injury of the so-called "normal"- appearing white matter (NAWM) is found in the brains of MS patients. The pathology of NAWM in MS is characterized by diffuse, mainly CD8+, T cell infiltrates, gliosis, microglial activation, diffuse axonal injury and nerve fiber degeneration. Chronic inactive MS lesions are hypocellular with no evidence of active myelin breakdown. Astrocyte proliferation and hypertrophy (reactive gliosis) is prominent and, although axonal injury is considered mild when compared to the axonal loss observed in an infarct, some degree of axonal damage can be appreciated. Mature oligodendrocytes are markedly diminished or absent in chronic inactive lesions and inflammation, especially in perivascular regions, often remains (Lucchinetti, Parisi et al. 2005).

MS according to Claudia Lucchinetti/Hans Lassmann: In an analysis of 51 biopsies and 32 autopsy specimens, Lucchinetti/Lassmann concluded that a profound heterogeneity exists between patients with respect to the inflammatory response, oligodendrocyte survival and patterns of demyelination. However, they observed a striking homogeneity among lesions within a given patient. In their opinion, MS may represent a common name for different pathologic entities that unify on the special vulnerability of CNS myelin to various immune and toxic mediators (Lucchinetti, Bruck et al. 2000). The heterogeneous phenotypes observed in MS patients can be classified into four distinctions:

 Pattern I: Macrophage-associated demyelination

 Pattern II: Antibody/complement-associated demyelination

 Pattern III: Distal dying-back oligodendrogliopathy

Pattern IV: Primary oligodendrocyte degeneration

Although each pattern involves an inflammatory response composed mainly of T lymphocytes and macrophages, differences in plaque geography, extent and pattern of oligodendrocyte pathology, immunoglobulin deposition, complement activation, and myelin protein loss were observed.

In patterns I and II, macrophages and T cells predominate in well-demarcated plaques that surround veins and venules; only pattern II lesions demonstrate local precipitation of immunoglobulin and activated complement in regions of active myelin breakdown. The expression of all myelin proteins is similarly reduced. Pattern III lesions also contain an inflammatory infiltrate, composed of macrophages, activated microglia and T cells; however, the lesions are ill defined and seldom surround vessels. There is no evidence of immunoglobulin deposition or complement activation, and Myelin Associated Protein (MAG) is selectively lost compared to other myelin proteins. MAG is a myelin protein localized to the most distal extension of the oligodendrocyte cell body (the periaxonal region), and early loss of this protein is believed to reflect a dying-back phenomenon in the oligodendrocyte, which precedes the apoptosis found to be prevalent in pattern III. Pattern IV lesions also contain T cells and macrophages but no preferential MAG loss, immunoglobulin deposition or complement activation is observed. Instead, there is evidence of non-apoptotic oligodendrocyte death, the mechanisms of which are unclear. In summary, inter-individual rather than intra-individual differences in lesion heterogeneity with respect to demyelination is a characteristic feature of MS (Lucchinetti, Parisi et al. 2005).

A rebuttal to the Lucchinetti/Lassmann model: Although a provocative concept, the heterogeneity of MS lesions as described by Lucchinetti and Lassmann has come under question. Breij et al. analyzed 131 lesions from 39 patients with long-standing MS. The aim of the study was to determine if inter-individual differences persist in patients who are temporally farther away from their first symptoms. They found a homogenous pattern of demyelination in active lesions in patients with long-standing MS. The authors suggest that the immunopathological appearance of active demyelinating lesions in established MS is uniform. Initial heterogeneity of demyelinating lesions in the earliest phase of MS lesion formation may disappear over time as different pathways converge in one general mechanism of demyelination (Breij, Brink et al. 2008).

MS according to Michael Barnett/John Prineas: In an analysis of 10 lesions collected hours-days after the deaths of 7 patients with fatal MS lesions (the earliest lesion being 17 hours old), Barnett/Prineas concluded that newly forming lesions arise in the absence of an inflammatory infiltrate. Instead, there was evidence of primary oligodendrocyte degeneration/apoptosis, BBB breakdown and early microglial activation. In their opinion, some local change to which oligodendrocytes are uniquely susceptible is responsible for lesion formation (Barnett and Prineas 2004). They offer the following timeline: Within hours of lesion initiation, oligodendrocytes throughout the affected tissue appear apoptotic (as shown in Figure 10), myelin sheaths stain positively for activated complement while immunoreactivity for CNP and MAG is diminished, and ramified microglia with thickened processes appear in increased numbers. T

34

cells, early-activated macrophages and myelin phagocytes are rare or absent in the apoptotic zone but are present elsewhere in the lesion. After 1 or 2 days, oligodendrocytes disappear, most presumably phagocytosed by the now amoeboid microglia present in the tissue. The tissue appears vacuolated because of the presence of widespread intramyelinic edema, which is the usual accompaniment of oligodendrocyte loss. The third and most protracted stage involves fragmentation and uptake of vacuolated and smudged (vesiculated) myelin sheaths by macrophages in the presence of infiltrating T cells and macrophages (Barnett and Prineas 2004).

Surprisingly, Barnett and Prineas were not the first to describe apoptotic oligodendrocytes and early microglial activation in the absence of an inflammatory infiltrate. In 1952, Adams and Kubik described a lesion, which they estimated was 48 hours old. In this newly forming lesion, they observed unstained or poorly stained, but still intact myelin, and pyknotic nuclei, which they attributed to degenerating oligodendrocytes. They went on to postulate that the process that damages the myelin, at the same time destroys oligodendrocytes and causes a microglial reaction. Most importantly, there was no perivascular infiltration in this early lesion (Adams RD 1952).
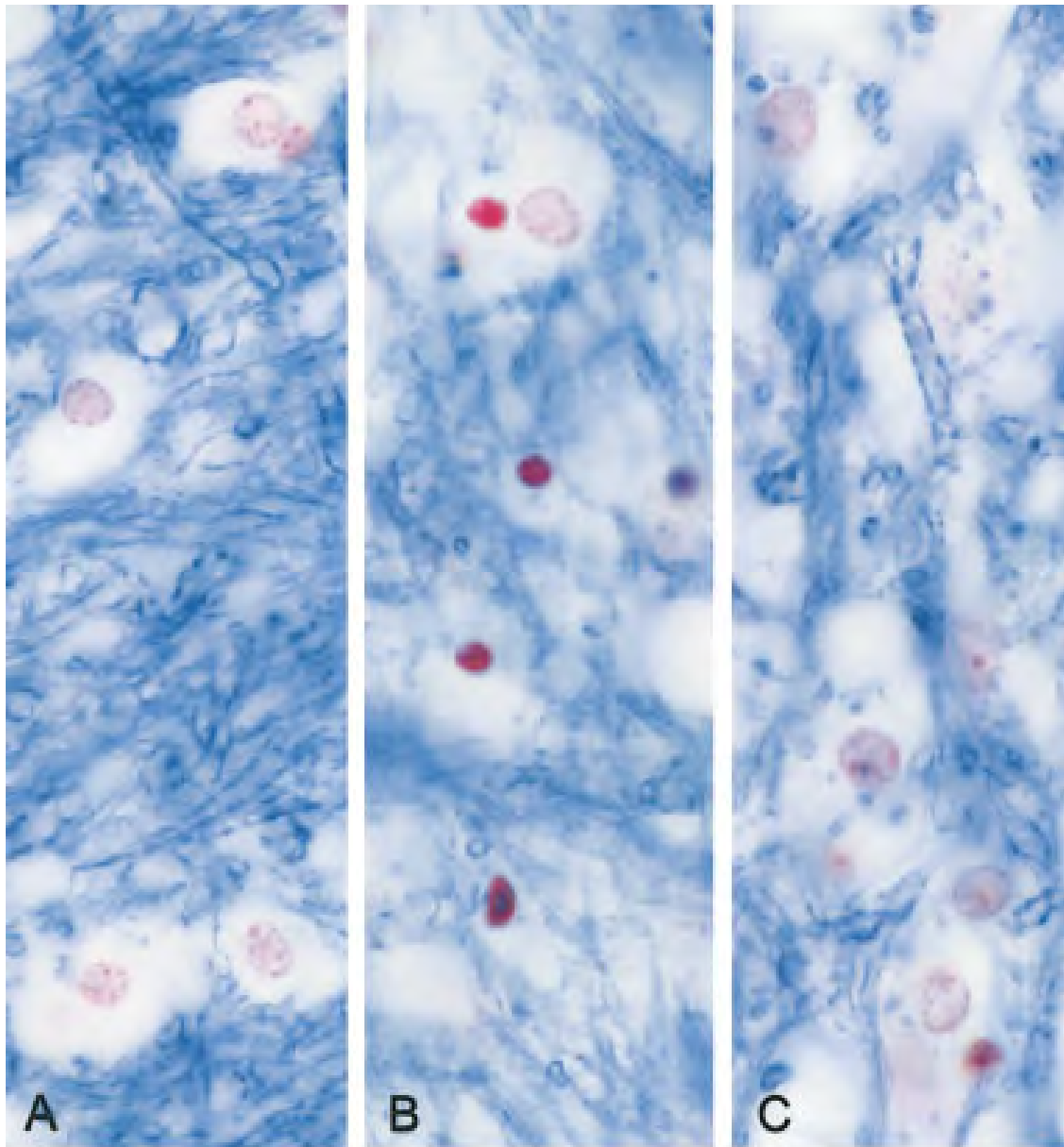
Figure 10. Histology of Nascent MS Lesions. (A) Unaffected white matter. A stain the colors nuclear chromatin red has been used to identify nuclei in tissue stained blue for myelin. (B) Apoptotic oligodendrocytes with shrunken nuclei and densely compacted nuclear chromatin. (C) Commencing phagocytosis of vacuolated myelin. Apoptotic nuclei and normal appearing oligodendrocytes were almost entirely absent in this region of the plaque. Luxol fast blue periodic acid-Schiff with nuclear fast red counterstain. Mag. 630X. (Barnett and Prineas 2004)

In contrast to Lucchinetti/Lassmann, Barnett/Prineas conclude that these "prephagocytic" oligodendrocyte changes represent the initial process for all MS lesions and that inter-individual lesion heterogeneity does not exist. Furthermore, they argue that the Lucchinetti/Lassmann findings represent different temporal stages in lesion evolution. While Barnett and Prineas admit that their study provides no direct evidence for what might be the cause of oligodendrocyte apoptosis, they point out that one unexplained finding that may relate to the genesis of a new lesion is the occasional occurrence of perivascular cuffs of mononuclear cells adjacent to periventricular white matter, at the corticomedullary junction and close to the pia, all areas where new MS lesions tend to form (Barnett and Prineas 2004). Furthermore, Barnett and Prineas report serum protein leakage and the accumulation of perivenular monocytes in the absence of oligodendrocyte apoptosis or demyelination. These data suggest that insult of the endothelium and a subsequent innate immune response may be the earliest of changes in acute MS lesions. (Prineas and Parratt 2012)

<u>Resurrecting The Vascular Theory of MS</u>

Although these newly forming lesions lacked an inflammatory infiltrate within the CNS proper, the fact that immune cells were found to congregate around CNS vasculature in normally appearing white matter may be telling. There has been long-standing interest in a vascular hypothesis for the genesis of the MS lesion dating back to the mid-1800s when Eduard Rindfleisch (1836-1908) noted, in 1863, the consistent location of a blood vessel in the center of MS plaques. Rindfleisch, later followed by Prineas and others, observed cellular

infiltrates that remained confined to the perivascular space. He postulated that MS was caused by a primary insult to CNS blood vessels and their ramifications, and that the perivascular infiltrates were secondary to this primary insult. Rindfleisch writes:

> *If one looks carefully at freshly altered parts of the white matter…one perceives already with the naked eye a red point or line in the middle of each individual focus…the lumen of a small vessel engorged with blood…All this leads us to search for the primary cause of the disease in an alteration of individual vessels and their ramifications; all vessels running inside the foci, but also those which transverse the immediately surrounding but still intact parenchyma are in a state characteristic of chronic inflammation.* (Eduard Rindfleisch, 1863)

In accordance with this vascular theme, Dawson's seminal 1916 thesis on the pathology of MS ultimately concluded that MS is likely caused by a soluble, hematogenous toxin that enters the CNS due to an unknown vascular abnormality (Dawson 1916). Here Dawson summons both the vascular theory, originated by Eduard Rindfleisch and the soluble toxin theory, originated by Byrom Bramwell.

Published data support a vaso-centric interpretation of MS with MS-associated retinal phlebitis perhaps being the most intriguing example. Retinal phlebitis is the inflammation of the venules and veins of the retina. The retina resembles the brain and spinal cord in that it consists of neurons, astroglia (muller cells) and vasculature that restricts the free flow of solutes from the blood into its parenchyma, the blood-retinal barrier (BRB). Most importantly, the retina lacks oligodendrocytes, the myelin forming cells of the CNS, and thus lacks myelin. Therefore, inflammation of the retinal vasculature cannot be secondary to a demyelinating process. Despite this lack of myelin, many researchers have observed inflammatory scarring of the retinal microvasculature

(Kerrison, Flynn et al. 1994). This phenomenon remains unexplained.

Ter Braal and Herwaarden first reported MS associated retinal phlebitis in 1933 (Kerrison, Flynn et al. 1994). Since their initial observation, two types of inflammatory scar or "sheathing" have been described, active and inactive. Active venous sheathing consists of infiltrates that disappear over a period of months to as long as two years. Inactive venous sheathing consists of sharp, well-defined, permanent lines along veins; a presumed sequelae of chronic, active phlebitis. Inactive sheathing causes the venular walls to become thick and laminated with collagen. This occurrence has been the subject of a number of clinical studies, and the frequency of venous sheathing has been estimated to be between 9% and 36%. Furthermore, a multi-study analysis of the frequency and duration of venous sheathing predicts a frequency of 11.5% and an average of 3.6 episodes during the course of the disease (Kerrison, Flynn et al. 1994).

In addition to venous sheathing of the retinal veins, ocular coherence tomography (OCT) studies have identified retinal abnormalities in MS patients even though the retina lacks myelin. Investigators have identified an increased thickness of the inner nuclear layer (INL) of the retina, which correlates with increased disease activity and contrast enhancing MRI lesions. In the same study, they also found evidence of microcystic macular oedema (MMO) suggesting a break down of the blood-retinal barrier (BRB), which is analogous to the blood-brain barrier in the brain (Runkle and Antonetti 2011). The authors mention that BRB breakdown occurs in approximately 20% of MS patients and occurs concurrently with BBB breakdown during active disease (Saidha, Sotirchos et al. 2012).

Blood-retinal barrier dysfunction is not the only provocative vascular abnormality that has been observed in MS. Other investigators have described early evidence of microvascular injury in acute MS lesions. Despite these vascular lesions, the surrounding myelin remains preserved and the reactive changes in the cerebral parenchyma are absent (Adams, Poston et al. 1985; Wakefield, More et al. 1994). This early damage to the CNS microvasculature has been described as focal endothelial cell associated fibrin deposition, which can be found in many thin-walled vessels, including veins and capillaries. Investigators of this phenomenon suggest that activation of the cerebral endothelium is a primary event in MS (Adams, Poston et al. 1985; Wakefield, More et al. 1994).

Vascular inflammation in the absence of demyelination, and even in the absence of myelin itself, as observed in retinal phlebitis, raises three important questions:

1. What causes the vascular injury observed in MS patients?

2. Why is the CNS vasculature targeted while the peripheral vasculature is spared?

3. Is the demyelination that typifies the MS lesion secondary to the vascular insult or is it a separate process?

Because the primary role of blood vessels is to transport molecules and cells throughout the body, the idea that a soluble, blood-borne, noxious agent may trigger MS quickly followed Rindfleisch's mid-19th century vascular theory. Two of the most influential MS researchers of the early 20th century, Dawson and Marburg, supported the soluble toxin theory.

Both Dawson and Marburg were struck by the reproducibly close proximity of MS plaques to the ventricular system and the equally reproducible presence of a vein or venule at the center of a lesion. Dawson also commented on the remarkable symmetry of periventricular plaques in MS. He explained this distribution on a vascular basis and concluded that MS was due to a specific morbid agent, probably a soluble toxin, which is conveyed to the nervous system by the blood channel (Dawson 1916; McAlpine 1946). Similarly, Marburg speculated that the causative agent might be an enzyme or immuno-agent that diffuses from the blood or CSF into the brain (Adams, Poston et al. 1985).

Although the soluble toxin theory's popularity peaked in the early 20th century, current data has re-invigorated this past theory. The advent of more advanced myelin stains and MRI imaging techniques has made clear that MS lesions are not confined to the subcortical white matter. Indeed, modern research has shown that cortical demyelination exists and this demyelination occurs in a very interesting pattern. Cortical lesions typically emanate from the subpial surface (the area of the brain that is in direct contact with the CSF). Additionally, these lesions typically occur as long strips (Figure 11), suggestive of a diffuse process, in contrast to the focal characteristics seen in subcortical white matter lesions with a venular or capillary focus (Rudick and Trapp 2009). Dr. Bruce Trapp maintains that a soluble and diffusible agent may be at play (personal communication), an inference that revisits the conjecture of his 20th century predecessors, Bramwell, Dawson and Marburg. In support of this view, Lassmann et al. found that cortical lesions predominantly occur in deep indentations of the cortical ribbon and in cortical sulci, and this is consistent with a soluble mediator as cerebrospinal fluid flow is more restricted in these areas

than at the outer cortical surface (Lassmann 2011). It should be noted, that Trapp and Lassmann believe this soluble mediator to be derived from inflammatory cells residing in the meninges (Rudick and Trapp 2009; Lassmann 2011).
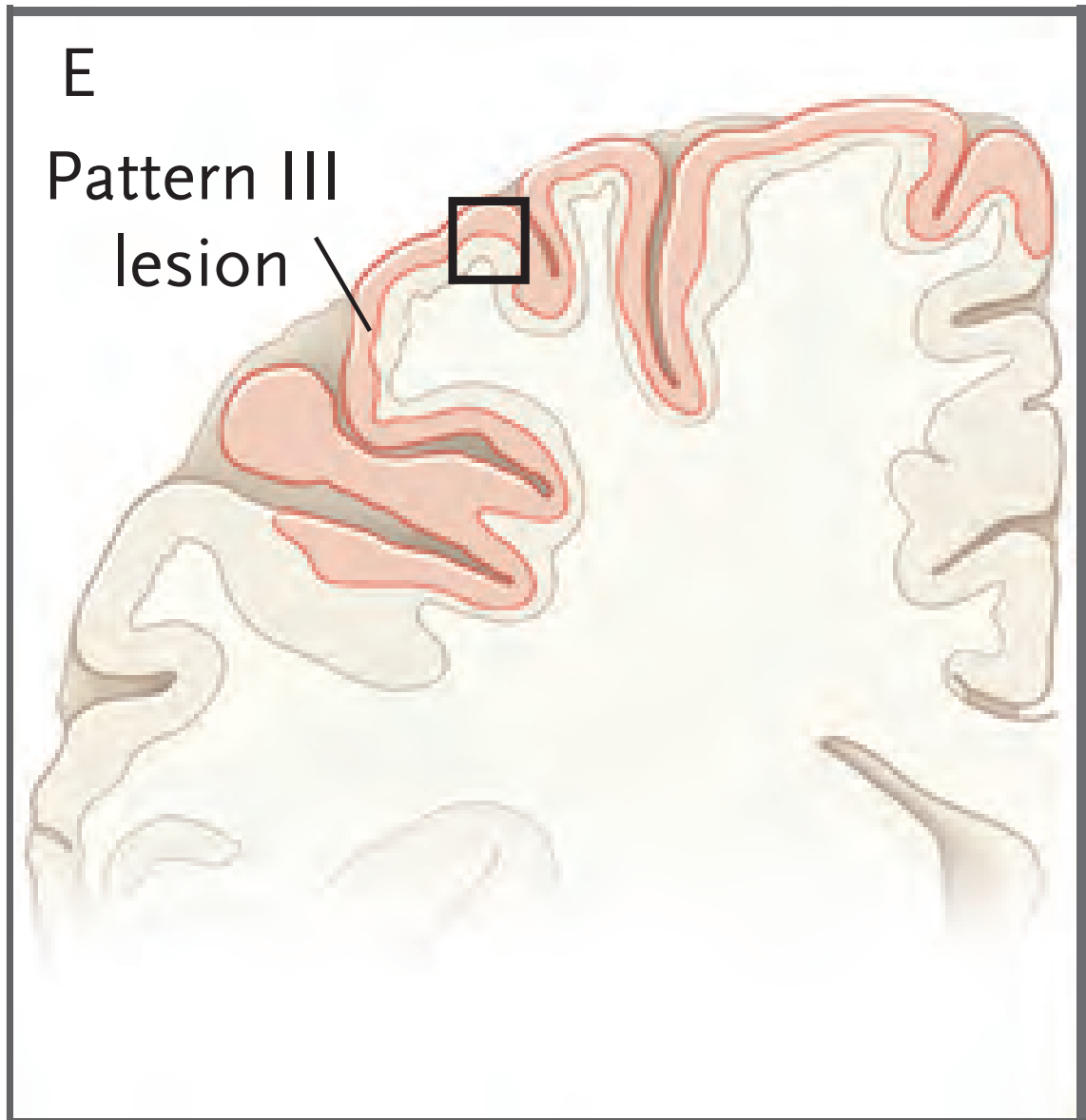
Figure 11. A Ribbon-Like, Sub-Pial Gray Matter MS Lesion. (Rudick and Trapp 2009)

CHAPTER 2

Could Clostridium *perfringens* Epsilon Toxin Be a Soluble MS Trigger?

To expand the vascular/soluble toxin theory, I wish to return to questions raised in the previous chapter. If we accept that vascular inflammation occurs in the absence of demyelination (perivascular cuffs in normal appearing white matter) and in the absence of myelin itself (retinal phlebitis and microcystic macular edema) then the below questions naturally follow:

1) What causes the vascular injury observed in MS patients?

2) Why is the CNS vasculature targeted while the peripheral vasculature is spared?

3) Is the demyelination that typifies the MS lesion secondary to the vascular insult or is it a separate process?

In this chapter, I will suggest that a soluble bacterial toxin, epsilon toxin (ETX) derived from Clostridium *perfringens* types B and D, adequately addresses each of these fundamental questions.

C. perfringens is a gram positive, spore forming, anaerobic rod that is sub-categorized into five toxinotyopes based on combinatorial carriage of α (plc), β (cpb1), ε (etx) and ι (iap/ibp) toxins. Clostridium *perfringens* enterotoxin (cpe) and β2 (cpb2) are considered major Clostridial toxins but do not factor into the toxinotype classification. Unlike other Clostridial species, C. *perfringens* is non-motile and does not invade host tissue. C. *perfringens* is wide spread in the environment and can be readily found in soil and sewage (McDonel 1980). It is

also commonly found in the intestines of animals, including humans where it can be pathogenic under certain conditions (Rood and Cole 1991).

In humans, C. *perfringens* causes gas gangrene, gastrointestinal disease e.g. food poisoning (cpe toxin) and necrotic enteritis (β toxin), whereas in other animals, enterotoxaemic diseases occur more frequently. While C. *perfringens* does not invade tissues, it produces a variety of toxins and enzymes responsible for the associated lesions and symptoms. The toxins produced depend on the particular C. *perfringens* toxinotype involved and each toxin produces a specific syndrome (Table 4) (Petit, Gibert et al. 1999).

Table 4. Diversity of Clostridium *perfringens* Toxinotypes, Genotypes and Associated Diseases. (Petit, Gibert et al. 1999)

Toxinotype	Major toxin[a]				Genotype	Associated pathology[b]	
	α	β	ε	ι		Humans	Others animals
A	++	−	−	−	plc plc, cpe plc, cpb2[d] plc, cpb2, cpe	Gangrene Gastrointestinal diseases[c] (food poisoning, antibiotic-associated diarrhea, sporadic diarrhea, some cases of sudden infant death syndrome)	Diarrhea[c] (foals, pigs…) Necrotic enteritis in fowl
B	+	+	+	−	plc, cpb1, etx plc, cpb1, etx, cpe		Dysentery in newborn lambs Hemorrhagic enteritis in neonatal calves and foals Enterotoxemia in sheep
C	+	+	−	−	plc, cpb1 plc, cpb2 plc, cpb1, cpe plc, cpb2, cpe plc, cpb1, cpb2 plc, cpb1, cpb2, cpe	Necrotic enteritis (Pigbel, Darmbrand)[e]	Necrotic enteritis in piglets, lambs, calves and foals Enterotoxemia in sheep
D	+	−	+	−	plc, etx plc, etx, cpe		Enterotoxemia in lambs, sheep, calves and goats
E	+	−	−	+	plc, iap, ibp plc, iap, ibp, (cpe)[f] plc, iap		Enterotoxemia in calves

[a]Toxinotypes based on mouse lethality tests.
[b]Data taken from Refs 2–4,7.
[c]Caused by enterotoxigenic strains.
[d]Some strains containing the *cpb2* gene are classified as type A by the mouse lethality test.
[e]*C. perfringens* type C necrotic enteritis has been observed in New Guinea populations (Pigbel) and in Germany after World War II (Darmbrand).
[f]Gene in brackets is not expressed.

C. *perfringens* type A is ubiquitous, is the most common of the toxinotypes

and is commensal to the human gut. Approximately 50% of healthy North

Americans harbor intestinal C. *perfringens* type A (Carman, Sayeed et al. 2008).

However, the distribution of the other toxinotypes is more restricted. Types B

and D are predominantly found in ruminants; type C in pigs and type E in

calves. Host specificity probably depends on particular host factors and specific

bacterial virulence factors e.g. adhesins that promote digestive tract colonization.

Although adhesins have not been identified for C. *perfringens*, it has been

reported that toxinotype C adheres to intestinal villi (Walker, Murrell et al. 1980).

Additionally, bacterial enzymes involved in utilizing certain substrates could

promote rapid growth in the digestive tracts of specific animals e.g. in

Bacteroides sp. the locus containing α-galactosidase and a regulatory gene for

polysaccharide utilization are critical for the colonization of the mouse digestive

tract (Petit, Gibert et al. 1999).

Interestingly, type A is the dominant toxinotype in soil where it is

thought to outcompete the minor toxinotypes for resources. It appears that the

minor toxinotypes (B, C, D and E) depend on a mammalian host for survival

(Itodo, Adesiyun et al. 1986), however, the host-pathogen interactions that

dictate toxinotype/species coupling are poorly understood. Even when the

minor toxinotypes (of intestinal origin) are inoculated directly into soil, they die

out within a few months as they cannot compete with the better adapted normal

soil inhabitants (McDonel 1980).

Of the seventeen toxins that C. *perfringens* secretes, ETX is the most potent,

trailing only Clostridium *botulinum* and *tetanus* toxins in lethality (Popoff 2011).

C. *perfringens* types B and D, which typically colonize the G.I. tract of sheep, goats and cattle but not man, carry two unique extrachromosomal ETX plasmids (Havard, Hunter et al. 1992) and secrete ETX upon exponential bacterial growth. Secreted as a 33kDa inactive precursor within the ruminant intestine, ETX is cleaved by trypsin, chymotrypsin and/or an additional clostridial exotoxin, lamda toxin. Proteolysis removes 11-13 N-terminal amino acids and 29 C-terminal amino acids yielding a 31kDa cleavage product that is 1000X more active. Activated ETX permeablizes the gut epithelium, enters the blood stream and binds to an unknown receptor on the luminal surface of brain endothelial cells (Figure 12). Once bound to brain microvascular, ETX breaks down the BBB resulting in cerebral edema (Popoff 2011). For this reason, ETX has been classified a category B select agent and a bioterrorism threat (Bokori-Brown, Savva et al. 2011).
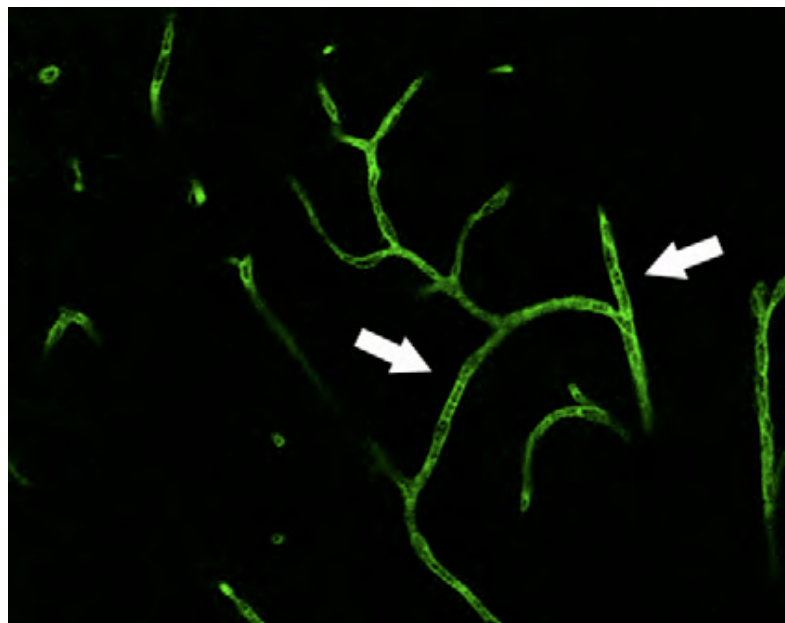
Figure 12. ETX Binds to BBB Vasculature. A sheep brain section incubated with GFP-ETX fusion protein. White arrows indicate ETX binding to brain vasculature in the brain slice. (Dorca-Arévalo, Soler-Jover et al. 2008)

In addition to its known effects on BBB vasculature, ETX has been found to specifically bind to myelin when incubated with mammalian brain slices (Figure 13) (Dorca-Arévalo, Soler-Jover et al. 2008). We have also confirmed this finding in the mouse brain (Figure 14). Furthermore, we find that incubating mixed glial cultures with active ETX causes selective damage to oligodendrocytes (Figure 15). Interestingly, cultured oligodendrocytes that make physical contact with microglial cells display increased sensitivity to ETX (picomolar range), displaying membrane blebbing and extensive morphological changes. However, cultured oligodendrocytes that have no contact with microglial cells display ETX sensitivity in the nanomolar range and cell death is typified by detachment of the cell body from the substrate, leaving the processes attached to the dish (data not shown). In summary, this unique ability to interact specifically with the tissues that are damaged in MS, the BBB and the CNS myelin-oligodendrocyte unit, makes ETX a promising candidate as an environmental MS trigger.
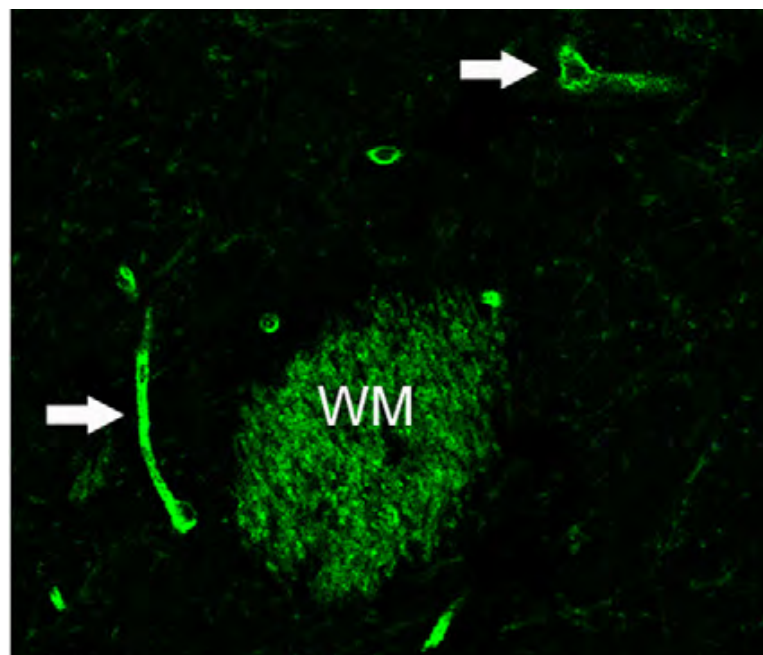
Figure 13. ETX Binds to White Matter. A sheep brain section incubated with GFP-ETX fusion protein. White arrows indicate ETX binding to brain vasculature in the brain slice. (WM) indicates ETX binding to a myelinated white matter region of the sheep brain. (Dorca-Arévalo, Soler-Jover et al. 2008)
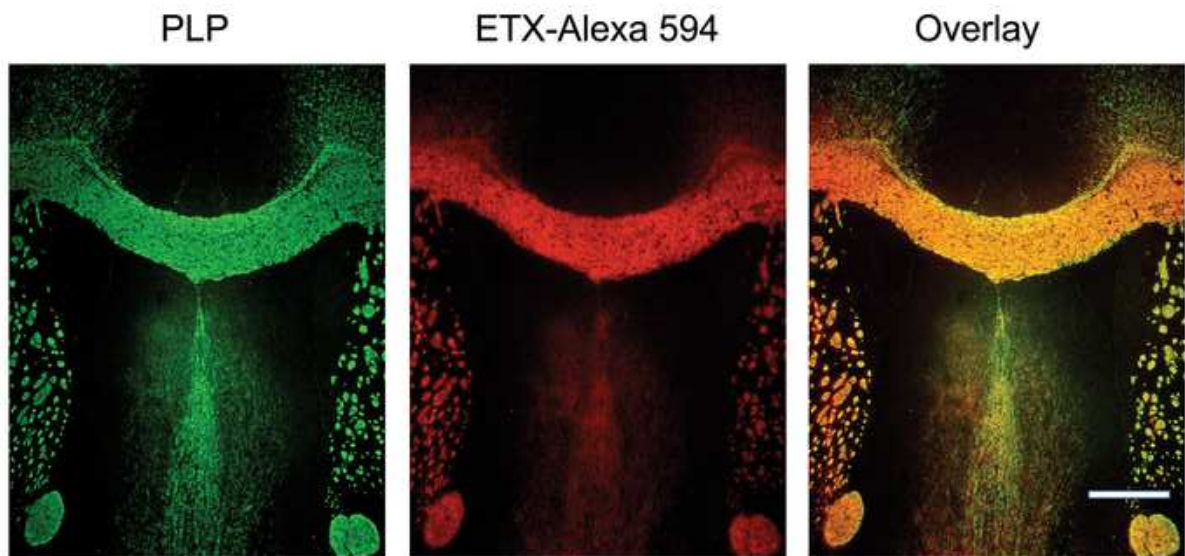
Figure 14. ETX Binds Specifically to Myelin. Fixed frozen coronal sections from adult mouse brain through the corpus callosum were stained for proteolipid protein (PLP, green), and Alexa 594-ETX (red). Intense staining with ETX is observed in all PLP-positive white matter tracts. Merged PLP and ETX images reveal essentially complete overlapping fluorescent signal. Bar = 200 um.

A.
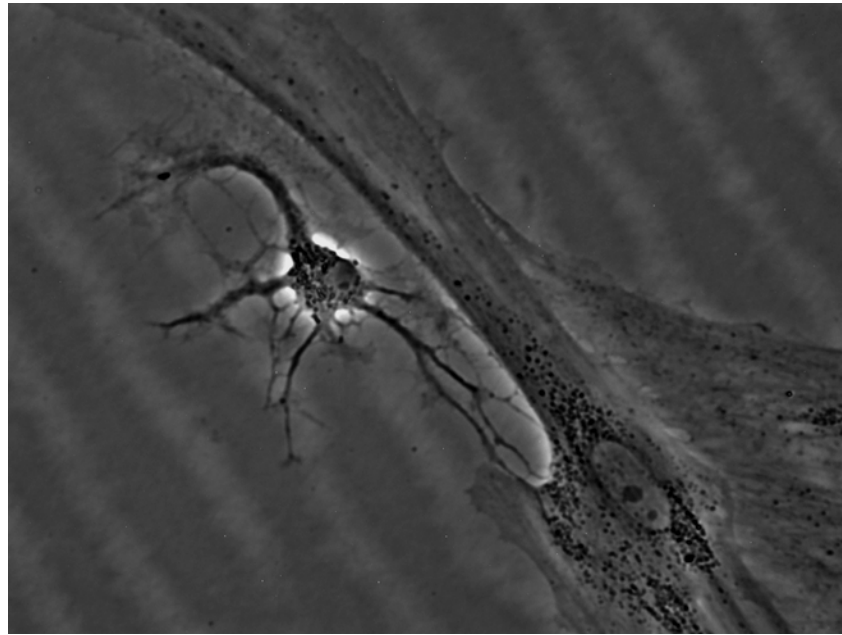
B.
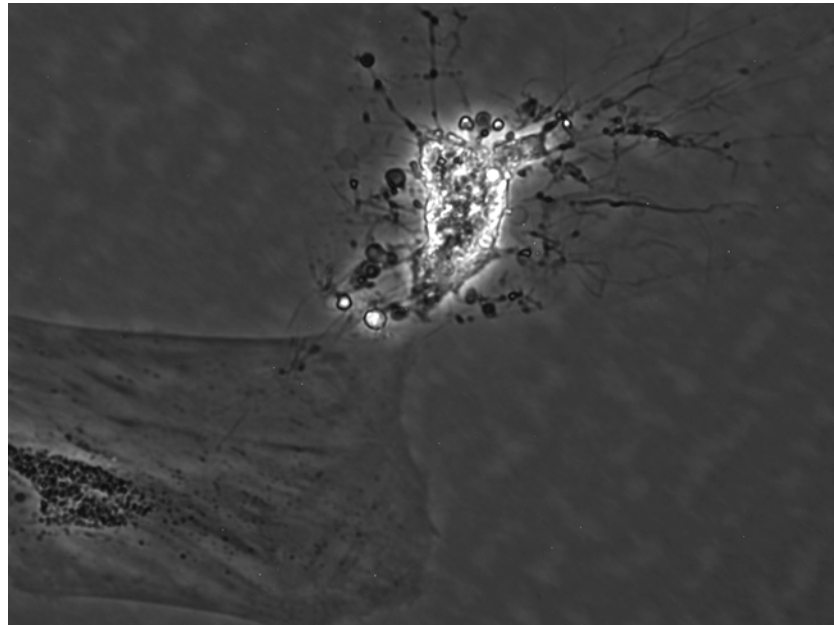
Figure 15. ETX Kills Cultured Oligodendrocytes. A) Untreated mixed glial culture from rat brain, oligodendrocyte (left) and microglial cell (right). B) Mixed glial culture from rat brain treated with 600pM ETX overnight, oligodendrocyte (top), microglial cell (bottom). Note the swelling of the oligodendrocyte cell body, blebbing of the membrane and damaged processes. The microglial cell below shows no signs of injury.

In fact, ETX has been proposed as an MS trigger in the past. Reviewing the idea that MS is associated with the concentration of global sheep populations, Dr. Timothy Murrell identified historically aberrant MS outbreaks and proposed that these epidemics stemmed from human contact with sheep (Murrell, O'Donoghue et al. 1986). He went on to delineate pathogens that may be transmitted from sheep to humans. Intriguingly, he offered *C. perfringens* type D as a possible infectious agent, as sheep are the natural reservoir for this bacterium. Importantly, humans typically carry C. *perfringens* type A, which does not carry an ETX encoding plasmid (Carman, Sayeed et al. 2008).

Murrell notes that ETX causes MS like symptoms upon entering the animal's blood stream such as blindness, ataxia, opisthotonos (a form of spastic paralysis) and central nervous system derangement due to malacia in brain areas with a distribution similar to MS; periventricular lesions perhaps being the most provocative, as they are commonly observed in both ETX intoxicated animals and MS patients (Murrell, O'Donoghue et al. 1986). Murrell concludes by suggesting that ETX mediated BBB breakdown may open the human CNS to a demyelinating neurotropic virus resulting in MS. However, the 2008 finding that ETX specifically binds to myelin once it gains access to the CNS may obviate the need for a neurotropic virus to cause demyelination.

There are many other properties of ETX that make it an attractive causative agent for MS. ETX mediated veterinary disease bears the name focal symmetrical encephalomalacia (FSE), and if one examines the meaning of this clinical term, similarities between FSE and MS start to emerge. The characteristic subcortical white matter MS lesion is indeed focal in nature, matching the pathologic description of FSE. MS lesions are often symmetrical, especially

lesions that form close to the lateral ventricles (McAlpine 1973). Finally, encephalomalacia literally means "brain softening," while sclerosis means "scar" or "hardening." Although these two terms seem to oppose each other, sclerosis is indeed a misnomer when it comes to MS. One must consider that MS lesions harden over time. In reality, fresh MS lesions that are in the processes of forming during the active disease state are soft. The earliest of MS investigators Carswell, Cruveillhier and Dawson noted that fresh lesions were softer than the normal brain substance. Therefore, in terms of active disease, "encephalomalacia" more accurately describes MS than "sclerosis"(Murray 2005).

The experimental injection of rodents with ETX also shows many provocative parallels to the MS disease state. ETX not only binds specifically to brain microvessels, but also has a penchant for vasculature residing in the myelinated regions of the brain (Finnie 1984). Additionally, intoxicated mice often develop periventricular lesions, similar to the lesion distribution seen in MS (Figure 16) and display a form of spastic paralysis evinced by neck retroflexion (Morgan and Kelly 1974). Similarly, MS patients often suffer from spastic paralysis, typically of the lower limbs. In this way, ETX intoxication may better characterize MS than the current EAE model. EAE mice develop a flaccid rather than the spastic paralysis that occurs in MS (Stromnes and Goverman 2006; Stromnes and Goverman 2006).

Figure 16. ETX Causes Periventricular Lesions. A coronal brain section of a mouse injected with ETX shows bilateral periventricular lesions and a lesion in the corpus callosum. (Morgan and Kelly 1974)

Strikingly, intraperitoneal injections of rats with the ETX precursor protein, which is 1000X less active, results in the formation of focal ovoid lesions within the corpus callosum, in which the long axis of the ovoid is oriented perpendicular to the surface of the lateral ventricle (Zhu, Ghabriel et al. 2001). These ETX induced lesions resemble the flame-like lesions that radiate from the lateral ventricles in MS, termed Dawson's fingers (Figure 17). Dawson first described this specific lesion morphology and the radiographic equivalent is all but pathognomonic for clinically definite relapsing remitting multiple sclerosis (Dawson 1916). To date, no MS animal model reproduces this highly specific lesion morphology.
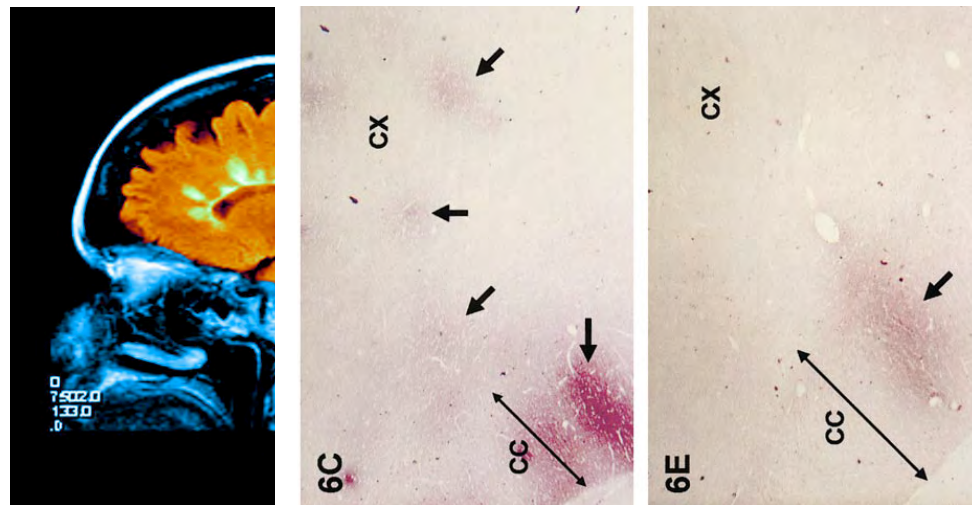
Figure 17. ETX Recapitulates Dawson's Fingers. A comparison of MS Dawson's fingers shown on sagittal MRI, left panel (SCIENCEphotoLIBRARY) and ovoid lesions in ETX precursor injected rats, middle and right panels (Zhu, Ghabriel et al. 2001). Sagittal sections of the rat brain (middle and right) show that BBB breakdown occurs with ovoid lesions appearing throughout the corpus callosum (CC). Focal lesions are also seen in the cortex (CX).

The fact that ETX can disrupt the BBB, bind to myelin, damage cultured oligodendrocytes, and recapitulate Dawson's finger lesion morphology and the spastic paralysis seen in MS are all provocative findings. However, if we look more closely at the mechanism by which ETX damages cells and compare this to what Barnett and Prineas observed in newly forming MS lesions more similarities become clear.

ETX forms heptameric pores in the cell membrane and allows the free flow of ions, water and hydrophilic solutes (up to 1kDa) across the cell membrane. This osmotic imbalance causes cellular swelling, membrane blebbing and cellular damage. ETX toxicity also leads to ATP depletion, AMP-activated protein kinase stimulation, mitochondrial membrane permeabilization and mitochondrial-nuclear translocation of apoptosis-inducing factor, which is a potent caspase 3-independent cell death mechanism characterized by a marked reduction in nuclear size and nuclear pyknosis (Popoff 2011). Barnett and Prineas, reported swelling of the oligodendrocyte cell body and the myelin sheath. More interestingly, they found a reduction nuclear size and nuclear pyknosis (Figure 10) and caspase 3-independent cell death (Barnett and Prineas 2004), all of which are reminiscent of the type of cell death described in ETX mediated cytotoxicity.

As previously mentioned, the retina contains no myelin yet retinal vasculitis, microcystic macular edema and thickening of the retinal inner nuclear layer (INL) mysteriously occur in MS, all pointing to a primary insult of the BRB. Because the BRB is analogous to the BBB, which is broken down with every new MS lesion, it is tempting to postulate that primary damage to CNS vessels occurs

in MS. Although ETX is known to bind to and break down the BBB, its effects on the BRB have not been studied. We find that ETX stains retinal vessels when incubated with retinal cryosections (Figure 18), further supporting a potential role for ETX as an MS trigger.
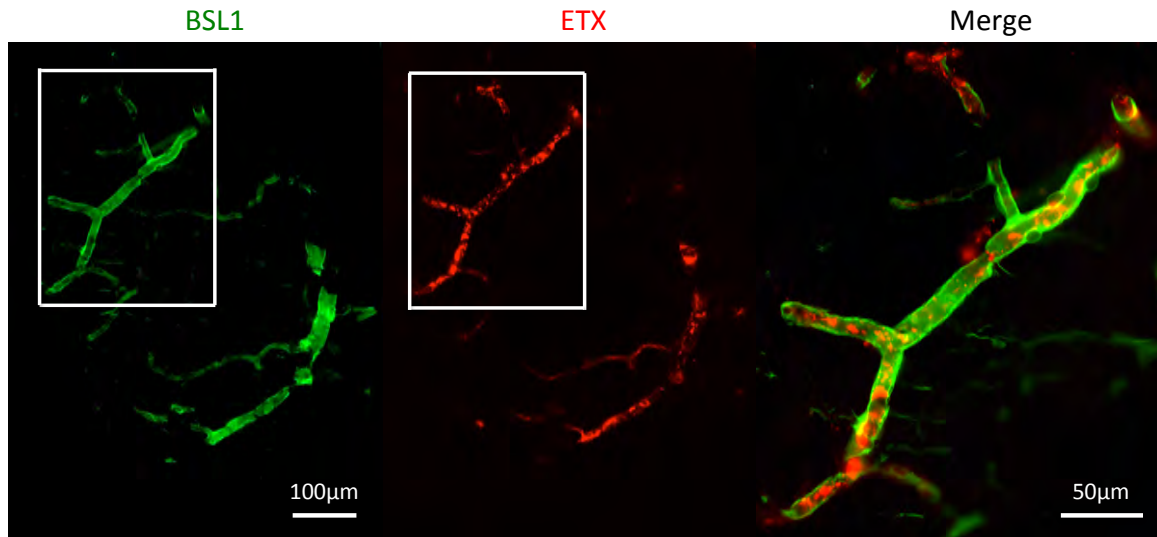
Figure 18. ETX Binds to Retinal Vessels. (Left panel) FITC labeled BSL1 (green), a pan-vessel marker, staining retinal vessels. (Middle panel) Alexa 594 labeled ETX (red) staining retinal vessels. (Right panel) a merge and enlargement of staining in the white boxes of the left and middle panels. BSL1 and ETX co-label a retinal vessel.

Although a promising hypothesis, several caveats must be addressed when considering ETX as a possible MS trigger. First, in addition to binding CNS myelin, ETX has been shown to bind to peripheral nerve myelin when incubated with sciatic nerve tissue slices (Dorca-Arévalo, Soler-Jover et al. 2008), however, MS is a disease that is restricted to the CNS. Radiolabeled ETX injected into mice only targets the CNS, and not the PNS (Tamai, Ishida et al. 2003). We propose that ETX fails to bind to PNS endothelial cells that comprise the blood-nerve

barrier; therefore PNS myelin is not accessible to the toxin in vivo.

Second, one might expect GI discomfort and intestinal lesions in MS if caused by C. *perfringens* overgrowth and toxin production in the gut lumen. However, histological abnormalities are minor, inconsistent and often completely undetectable in intoxicated sheep. Diarrhea is also very uncommon in sheep enterotoxaemia. The same is not true for intoxicated goats that often suffer from hemorrhagic enterocolitis and diarrhea (Blackwell, Butler et al. 1991; Fernandez Miyakawa and Uzal 2003). Therefore, even between ruminants, there is great species variation in the phenotype of ETX enterotoxaemia.

Interestingly, subtle small bowel changes have been observed in MS patients (Lange and Shiner 1976). Lange and Shiner reported varying degrees of villus atrophy, inflammatory cell infiltration and thickening of connective tissue in an analysis of jejunal biopsies from 12 randomly chosen MS patients.

Third, enterotoxaemia in sheep, goats and less frequently cattle often results in severe, often fatal disease. MS attacks, while debilitating, are rarely fatal. Why this difference in disease severity? Attempts to develop a small rodent oral inoculation model for C. *perfringens* enterotoxaemia may help address this question. When inoculated with toxinogenic C. *perfringens* type D, mice remain unaffected unless the anus is sealed to halt intestinal transit (Fernandez-Miyakawa, Sayeed et al. 2007). The authors propose that there may be more stasis in the ruminant gut, which allows for increased toxin accumulation and systemic absorption. Additionally, they propose that the ruminant animal has a larger gut absorptive surface area/body weight ratio than the mouse, thus making enterotoxaemia more likely in the ruminant (Fernandez-Miyakawa, Sayeed et al. 2007).

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Furthermore, when toxinogenic C. *perfringens* strains were orally administered to germ free guinea pigs, all animals showed signs of intoxication, however, when this experiment was repeated in conventional guinea pigs with normal gut flora, none of the animals showed symptoms of intoxication (Horton, Madden et al. 1970). Therefore, a normal gut flora is protective against enterotoxaemia. This may be of importance in animals (humans and small rodents) for which C. *perfringens* types B and D are not natural symbionts. It should be noted that germ free animals inoculated with C. *perfringens* type A, which is commensal to humans, remained unharmed but toxinogenic types B, C, D and E all resulted in a fatality rate of 100% (Horton, Madden et al. 1970).

Finally, ETX intoxication often bears the name pulpy kidney disease suggesting damage to the kidneys. There is no evidence of kidney damage in MS; however, pulpy kidney disease is a misnomer. The pathological changes in the kidneys of intoxicated sheep have been shown to be a post-mortem change characterized by rapid autolysis of the renal tubules. Kidneys that are inspected shortly after death show minimal, if any signs of damage (Popoff 2011).

In contrast to what the name "pulpy kidney" disease may suggest, the kidneys have been shown to be critical to ETX detoxification. Nephrectomy of ETX intoxicated mice greatly increases morbidity, mortality and decreases the LD_{50}. Therefore, the kidneys are actually key players in ETX host defense (Tamai, Ishida et al. 2003).

Have MS patients been exposed to ETX?

Dr. Timothy G.W. Murrell, a general practitioner who considered himself a "human ecologist," was the first to suggest a role for C. *perfringens* epsilon toxin in MS. Murrell suspected that there was a connection between the prevalence of MS and global sheep populations. He explored different neurotropic pathogens that could be transmitted from sheep to humans and he identified C. *perfringens* type D/ETX as potential zoonotic MS trigger.

To test this hypothesis of ETX exposure, Murrell harvested blood samples from MS patients, spouses and age matched control patients with other neurological disorders. He sent the sera to the Commonwealth Serum Laboratories (Melbourne, Australia) to be analyzed for circulating levels of anti-ETX antibodies by a method described in Appendix XIV B8 of the British Veterinary Codes (Murrell, O'Donoghue et al. 1986). No anti-ETX immunoreactivities were found by this method.

There are many reasons why this pilot study may have failed. One reason may be that humoral immunity to epsilon toxin in mammals is transient and incomplete. For example, when vaccinated at $t = 0$ and $t = 6$ weeks with epsilon toxoid, only 50% of goats have protective anti-toxin titers at week nine. By week 30, at the time of the 3rd vaccination only 2% of the goats maintain protective titers. At week 32 (2 weeks after the 3rd vaccination), 100% have protective titers, but by week 56 only 11% show protective titers (Figure 19). Additionally mean anti-ETX titers follow this same trend of seroreversion (Figure 20). Thus, in mammals exposed to epsilon toxin, seronegativity and seroreversion are

common even when the toxin is administered with an adjuvant (Blackwell, Butler et al. 1983).
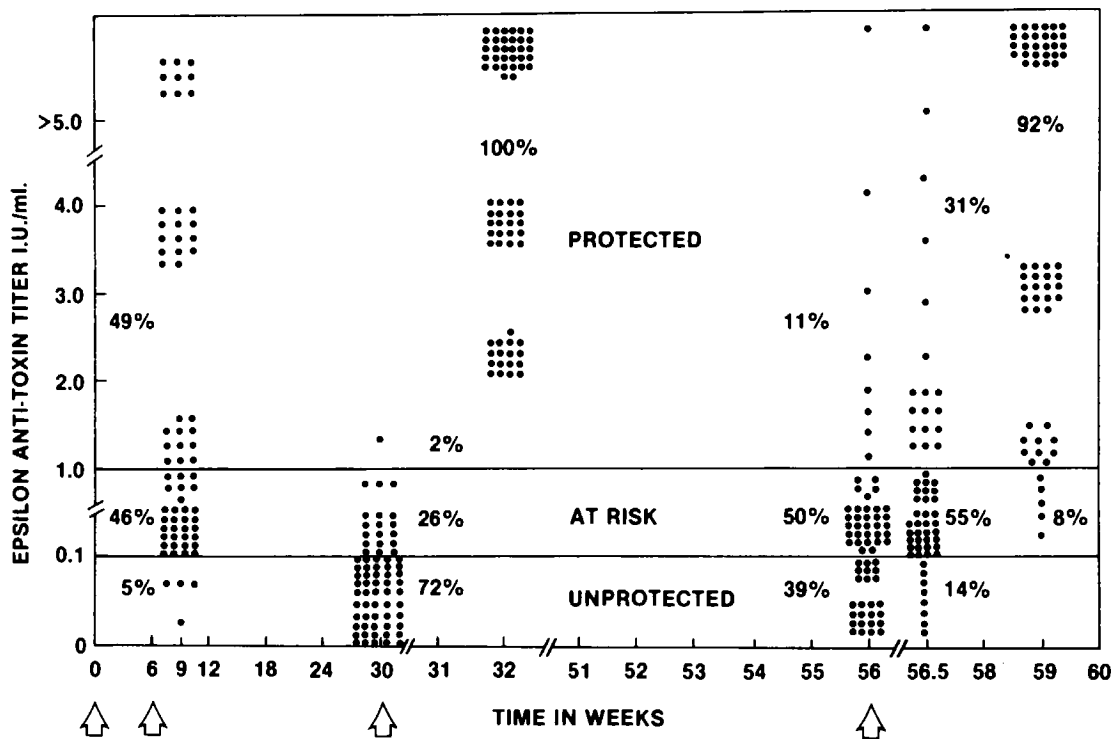
Figure 19. Humoral ETX Immunity is Variable and Short Lived. Response of mature goats following vaccination at time 0, 6, 30 and 56 weeks (white arrows) for the prevention of enterotoxemia. Each dot approximates the epsilon anti-toxin titer in IU/mL for one goat. The goats were vaccinated with either Clostroid C-D (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) or Covexin-8 (Burroughs Wellcome Ltd., Beckenham, Kent, England). (Blackwell, Butler et al. 1983)
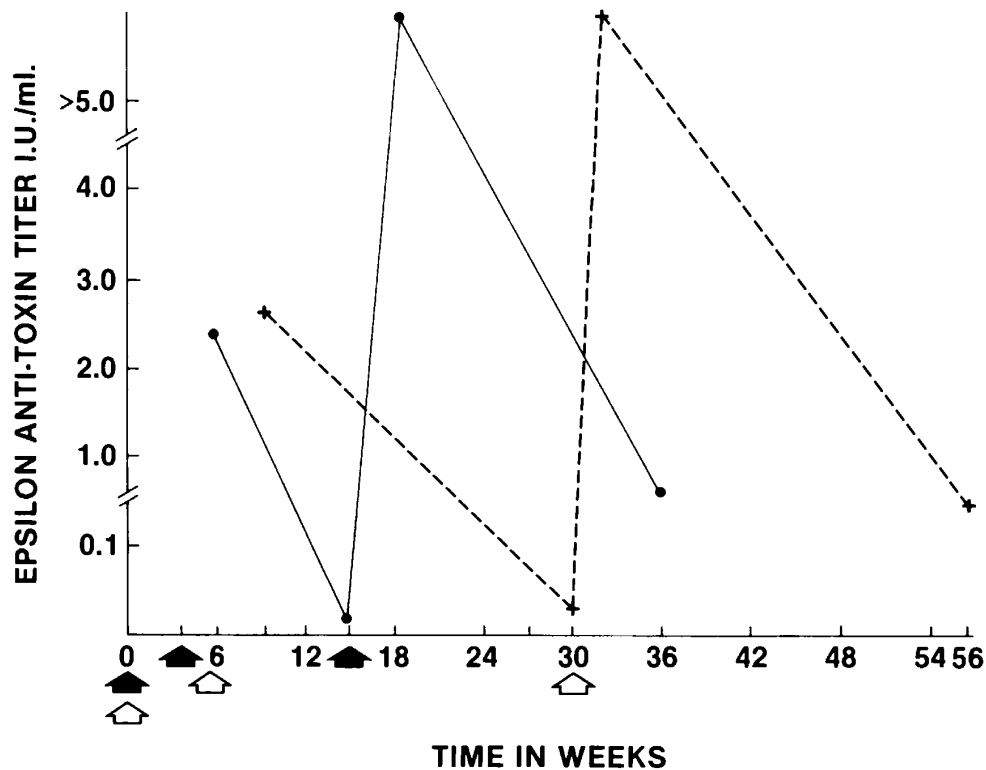
Figure 20. Humoral ETX Immunity is Short Lived. Mean epsilon anti-toxin titers in goats (----) versus sheep (——) following three vaccinations for the prevention of enterotoxemia. (White arrows) vaccine administered to goats, (black arrows) vaccine administered to sheep. (Blackwell, Butler et al. 1983)

In the following study, we continue Murrell's work by performing an immunoreactivity experiment using a Western blot approach. Furthermore, we determine the prevalence of C. *perfringens* toxinotypes in MS patients and healthy controls.

Expt 1. Analysis of ETX Immunoreactivity in MS patients vs. Controls

RESULTS

Increased anti-ETX immunoreactivity in MS vs. controls: We screened sera and CSF from a cohort of MS patients, healthy controls and other diseases for

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immunoreactivity to ETX by Western blot using the proETX protein. We developed a Western blot assay that rigorously excluded the likelihood of false positives. A sample was scored positive if there was clear immunoreactivity for ETX in conjunction with no immunoreactivity to four control toxins. Three of the controls were chosen to represent known gut derived toxins: Cholera toxin beta, Shiga toxin 1 beta and Shiga toxin 2 beta; no cross-reactions ever occurred with these control toxins. A fourth control, protective antigen 63 (PA63) from Bacillus *anthracis* was chosen because like ETX, PA63 is a pore-forming toxin with a hydrophobicity map similar to epsilon toxin (Knapp, Maier et al. 2009). PA63 was also chosen because most humans should be seronegative. Seroreactivity to PA63 would occur only in instances of vaccination or exposure to Anthrax. Most humans are not vaccinated against Anthrax, and in our study, none of the patients or controls were vaccinated. Seroreactivity to PA63 could also be observed in people who have been infected with Bacillus *anthracis* and survived. Since pulmonary and gastrointestinal Anthrax is usually fatal or debilitating, and since cutaneous Anthrax results in a characteristic black eschar, it is unlikely that prior Anthrax would be missed on a directed health questionnaire. Thus, positive immune reactivity to PA63 would strongly suggest non-specific interaction of host antibodies with PA63 or prior exposure to an antigen with a shared epitope. We thus excluded samples that showed immunoreactivity against ETX and PA63 since these indicated equivocal results. In SLE, where there is heightened humoral immunity, cross-reactions were common (Figure 21). Since hydrophobic proteins are more likely to show non-specific interactions with antibodies, we favor the idea that immunoreactivity to PA63 is nonspecific in nature.

We found that 10% of MS patients and 1% of healthy controls, in a cross-sectional analysis, possessed ETX specific antibodies χ^2 p = 0.0044 (Figure 21). Based on the known low rates of seropositivity following immunization, and the common seroreversion rates, this 10% positivity presumably underestimates the true value of ETX exposure.
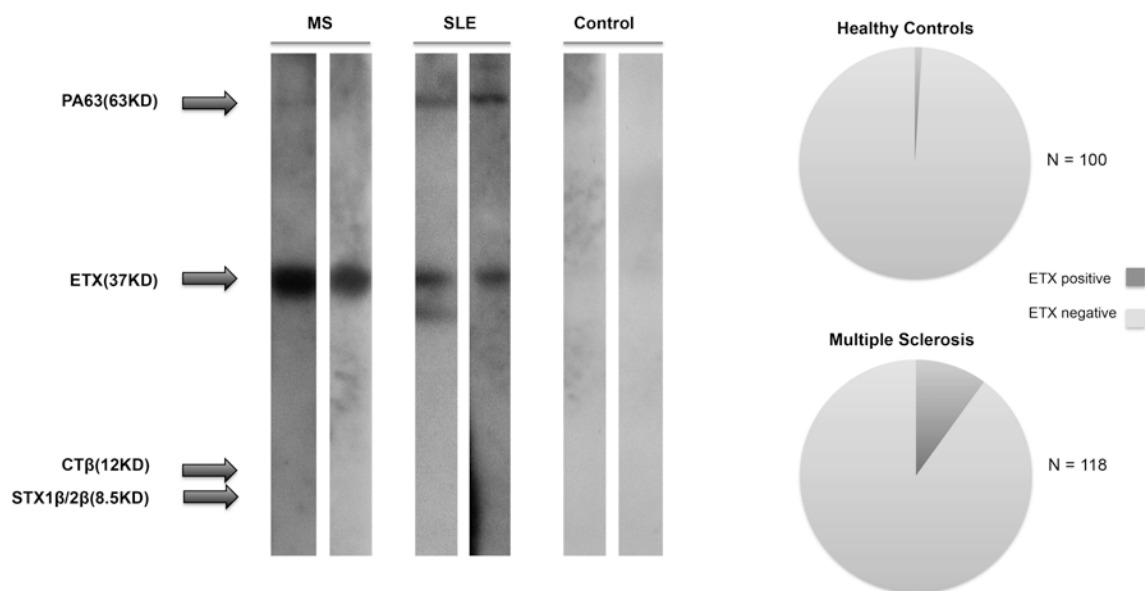
Figure 21. Immunoreactivity to ETX in MS, SLE and Healthy Controls. Left panel shows Western blots. The two MS blots shown are characteristic for true positives: immunoreactivity to the *C. perfringens* proETX protein at 37 kD but not to the other toxins present on the blot including PA63 at 63kD. The two blots probed with SLE sera are characteristic of false positives in that immunoreactivity is also present for PA63. Controls shown are true negatives with no immunoreactivity to any of proteins present on the blot. Note that the proETX gene encodes a protein with a predicted MW of 33kD, which runs on SDS-PAGE with an apparent MW of 37kD. The right panel shows prevalence of immunoreactivity to ETX in serum and/or CSF of people with MS and healthy controls.

Expt 2. Analysis of the prevalence of *C. perfringens* toxinotypes in MS patients compared to healthy controls

RESULTS

Reduced prevalence of *C. perfringens* type A in MS compared to healthy controls: Soil studies have identified that the presence of *C. perfringens* type A is coincident with the absence of other toxinotypes, suggesting that toxinotype A may compete with other *C. perfringens* toxinotypes for resources (McDonel 1980; Itodo, Adesiyun et al. 1986). For these reasons, we assessed the prevalence of type A, a human commensal, in MS and healthy controls. We cultured feces from 30 individuals with MS and 31 healthy controls. Bacteria were lysed, DNA isolated and toxinotypes determined by PCR analysis (Meer and Songer 1997). Prior published studies have demonstrated that type A is present in approximately 50% of healthy humans (Carman, Sayeed et al. 2008). Consistent with this, we found that 52% of the healthy controls (n=31) carried detectable type A (Figure 22). However, we found only 23% *C. perfringens* type A carriage in individuals with MS (n=30), χ^2p=0.0227 (Figure 22). This finding supports the concept that MS susceptibility may, in part, be due to host-microbiome influences and that the commensal *C. perfringens* type A may be protective

Healthy Controls

Type A negative
48%

Type A positive
52%

N=31

Multiple Sclerosis

Type A positive
23%

Type A negative
77%

N=30
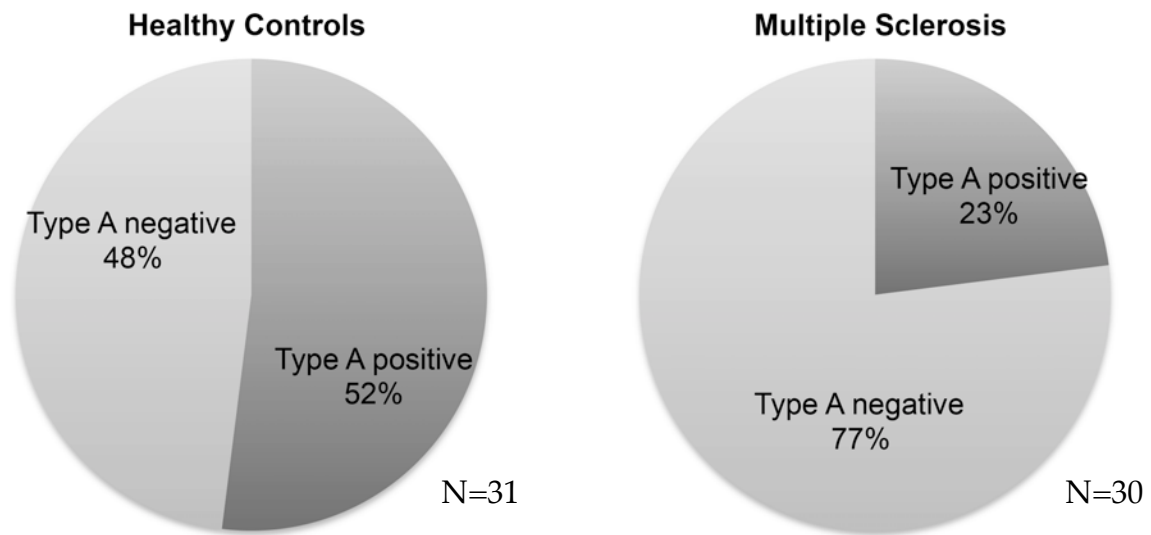
Figure 22. C. *perfringens* **type A prevalence in MS and Healthy Controls.**
Culture of stool in *C. perfringens* compatible growth medium revealed that 52% of
health controls harbor *C. perfringens* type A in the gastrointestinal tract, whereas
only 23% of people with MS harbor type A.

Identification of Clostridium *perfringens* **type B in a woman with Multiple**

Sclerosis: A 21-year old woman (patient 73F) developed left lower extremity

dyscoordination, and imbalance that evolved to its maximum deficit over three

days. Two weeks after onset she was referred to a neurologist due to persistent

symptoms and neuroimaging of the brain revealed multiple foci of increased

T2/flair signal in the deep and subcortical white matter, with several ovoid

lesions within the corpus callosum characteristic of MS. Following

administration of IV gadolinium, several lesions enhanced. CSF analysis revealed

five IgG bands on isoelectric focusing that were not present in the corresponding

serum sample. She met revised criteria for clinically definite relapsing remitting MS at the earliest clinical presentation termed a clinically isolated syndrome (CIS). She received five days of IV methylprednisolone, 1 gram per day, and her symptoms resolved to normal neurological function within three weeks. She was referred to the Weill Cornell MS Center for confirmation of diagnosis and treatment planning. Repeat neuroimaging at Weill Cornell revealed lesions characteristic in morphology and location for Multiple Sclerosis (Figure 23). Approximately three months after onset of symptoms, she was enrolled in the HITMS (**H**arboring the **I**nitial **T**rigger of **M**ultiple **S**clerosis) study, IRB protocol no. 1003010940, and a self-collected stool sample was obtained. Disease modifying treatment was initiated. Eight months after initiation of treatment she remained asymptomatic and her first treatment assessment MRI was performed which revealed several new contrast enhancing lesions (Figure 23). Three months after onset of her first symptoms, patient 73F was found to harbor *C. perfringens* type B in her GI tract. PCR analysis revealed carriage of genes encoding α, β, and ε toxins (Figure 24A). This represents the first human known to carry type B and the first MS patient found to carry an ETX producing *C. perfringens.* To exclude a possible laboratory-derived contaminant, we performed a lysogenic bacteriophage footprint analysis of the laboratory (ATCC 3626) and patient-derived *C. perfringens* strains. Three lysogenic bacteriophage insertions were identified in the laboratory strain, which matched the known whole genome sequence (Figure 24B). The patient's strain contained just two lysogenic bacteriophage insertions, thus confirming that the patient-derived ETX amplicon was not a laboratory contaminant (Figure 24B). Since a combination of

toxinotypes C and D would also result in identification of α, β, and ε toxin genes, we sequenced the patient-derived ETX gene confirming that it was derived from a type B ETX plasmid (Havard, Hunter et al. 1992). Interestingly, eight months after detection of C. *perfringens* type B in patient 73F, repeat analysis showed her to be negative, highlighting the dynamic nature of *C. perfringens* type B colonization.
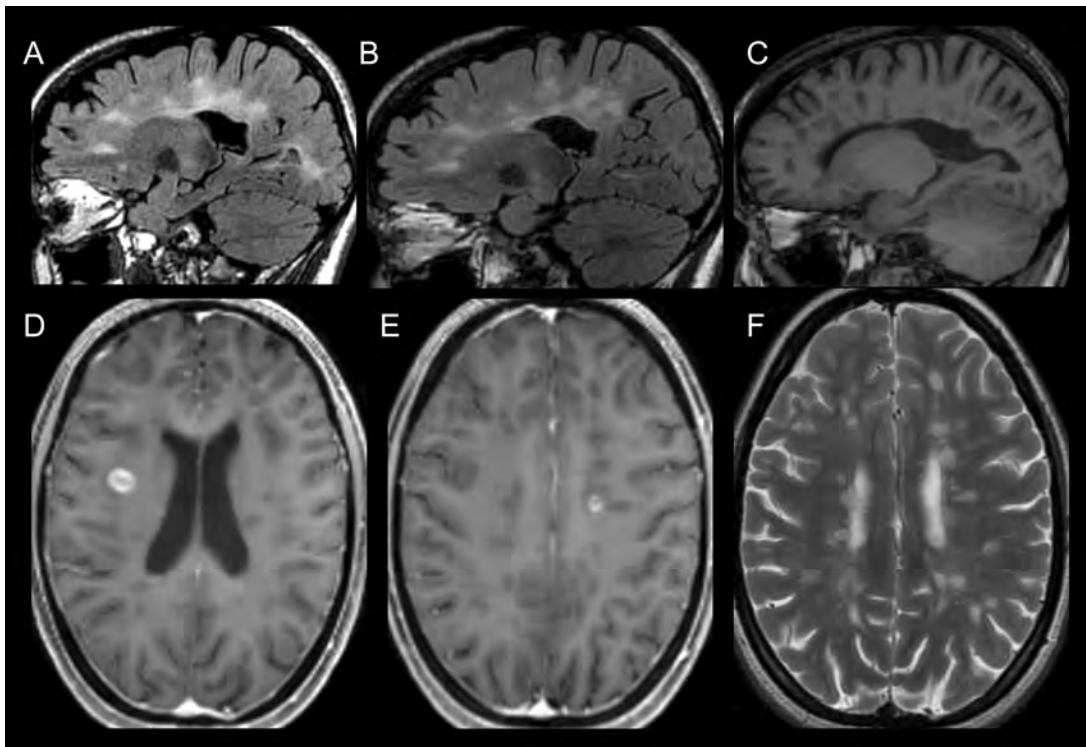
Figure 23. Brain MRI of Patient 73F. (A) FLAIR image from September 2011 showing characteristic lesions in a parasagittal plane. (B-F) MRI from May 2012 revealing characteristic lesions on FLAIR imaging as before (B), T1 hypointensities (C), characteristic contrast enhancing lesions post IV Gadolinium (D, E), and characteristic lesions on T2 weighted axial image (F).
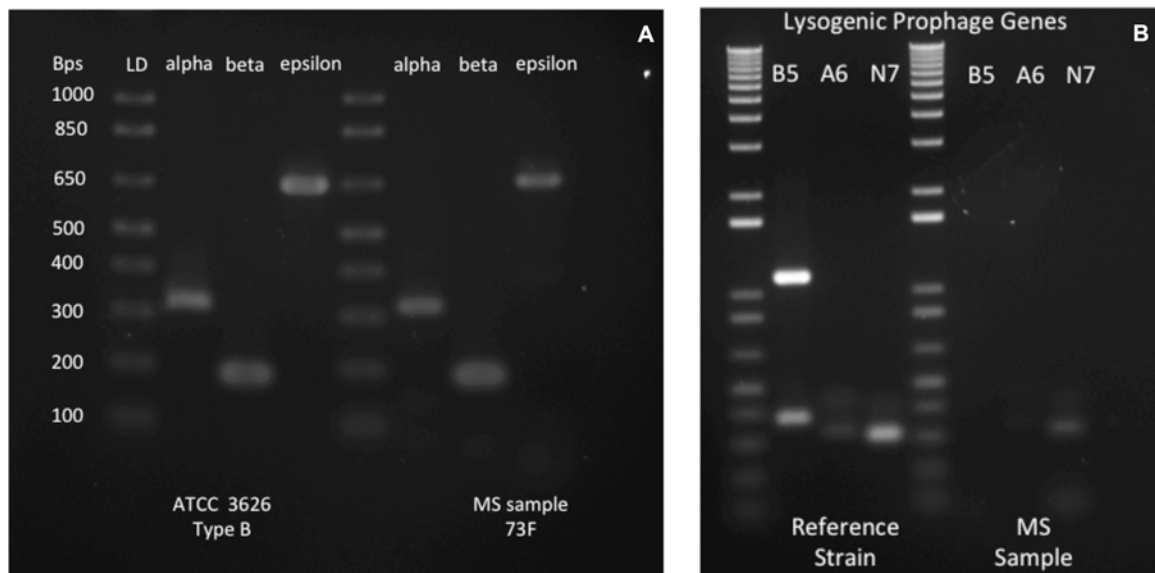
Figure 24. C. *perfringens* type B in Patient 73F. (A) Left panel shows PCR based genotyping of ATCC 3626 type B strain and from patient 73F. PCR products for α, β, and ε toxin are identified in both. (B) To exclude the possibility that the type B strain identified in the stool of patient 73F was a contaminant, the profile of lysogenic prophage genes was determined in the laboratory strain and in the patient isolate (right panel). ATCC 3626 reference strain possesses all three prophage insertions, whereas the patient's strain possesses only the A6 (weakly) and N7 prophage insertions. Phage genes and PCR product size: B1RBB5, 1000bps; B1RAA6, 300bps; Q8SBN7, 300bps.

SUMMARY

We propose that *C. perfringens* ETX fulfills the relevant criteria for an MS disease initiator. ETX binds to BBB and BRB endothelium (Figures 12 and 18 respectively), disrupts barrier function, binds to white matter (Figure 14) and damages cultured oligodendrocytes (Figure 15). ETX intoxication also mimics MS in that it causes Dawson's finger-like ovoid lesions in the rodent corpus

callosum (Figure 17) and causes spastic paralysis in affected animals, similar to the type of paralysis observed in MS. We have found evidence of increased ETX exposure in MS patients vs. controls. We have identified immunoreactivity to ETX in about 10% of people with MS vs. 1% in healthy controls, a 10 fold difference. The low value of immunoreactivity to ETX in MS may be explained by the difficulty mammals have maintaining humoral immunity to ETX (Blackwell, Butler et al. 1983). We also find that people with MS are less likely to harbor *C. perfringens* type A, a toxinotype thought to outcompete *C. perfringens* types B and D for resources in an ecological niche (McDonel 1980; Itodo, Adesiyun et al. 1986). We have identified one case in which a newly diagnosed patient harbored *C. perfringens* type B. This is the first time that a type B toxinotype has been isolated from a human being and this individual suffers from MS. Interestingly, eight months after testing positive for *C. perfringens* type B, she reverted to negative for all *C. perfringens* toxinotypes, an example of the transient nature of bacillus growth and/or detection limits. We expect that identification of *C. perfringens* types B or D in humans will be difficult, as *C. perfringens* forms endospores that are resistant to standard DNA extraction methods. Additionally, the organism is likely to exist in low abundance in the upper GI tract, only rarely entering growth phases that render it detectable.

MATERIALS AND METHODS

Ethics Statement - Research protocol #1003010940 for the collection of samples from individuals with MS and healthy controls was reviewed and approved by the WCMC institutional review board. All participants in the study gave written informed consent.

Serum and CSF collection - CSF and sera from people with MS and healthy

controls were collected at the Weill Cornell MS Center (IRB protocol no.

1003010940). Additional MS and Stroke CSF and sera were obtained from the

Brain Research Institute, UCLA. SLE sera were purchased from Vital Products

Inc.

ETX immunoreactivity - Western blots were performed using human sera/CSF

as primary antibody (Figure 25). SDS page electrophoresis was run and each

well was loaded with a mixture of 100ng of His tagged proETX (BEI Resources)

and molar equivalents of protective antigen 63 (PA63) from Bacillus anthracis

(EMD Millipore) 190ng, Cholera toxin beta FITC (Sigma Aldrich) 36ng, His

tagged Shiga toxin 1 beta (BEI Resources) 26ng and His tagged Shiga toxin 2 beta

(BEI Resources) 26ng. Proteins were transferred to an Immobilon P membrane

(Millipore) and probed with diluted sera/CSF. All serum and CSF samples were

diluted 10,000 fold and 27 fold respectively, while SLE sera were diluted 100, 000

fold to normalize background. HRP conjugated Donkey anti-human IgG 1:10,000

(Jackson Immunoresearch) was used to visualize human antibody binding.
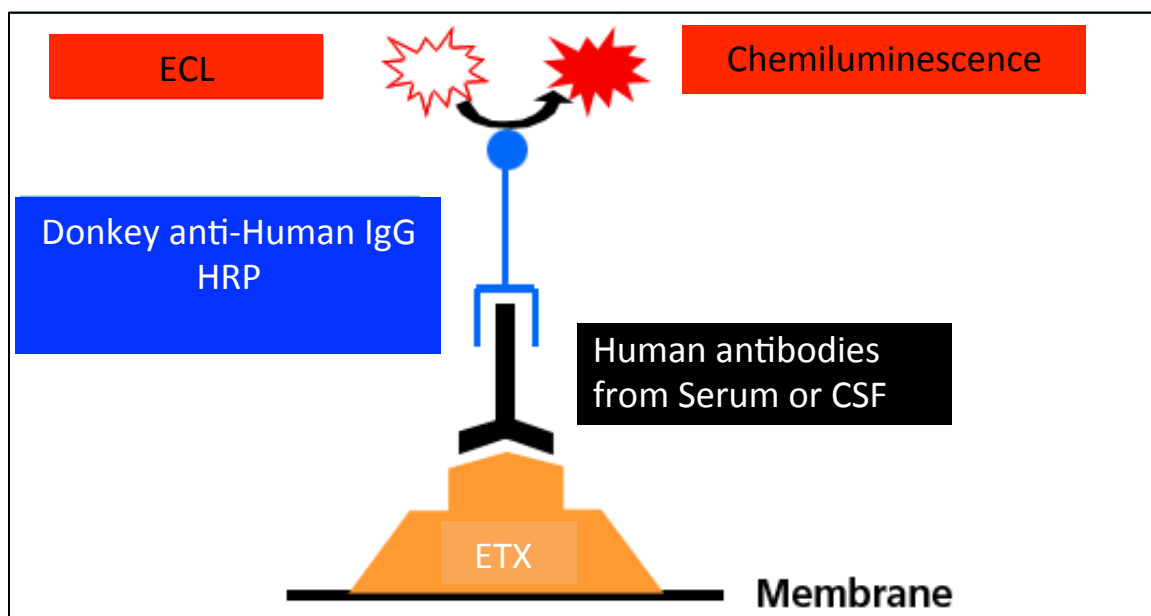
Figure 25. A Schematic of The Western Blot Assay. Protein toxins are run on SDS page and transferred onto an immobilon P membrane. Membranes are incubated with diluted human antibodies, washed and then incubated in HRP-conjugated Donkey anti-human IgG secondary. ECL solution is added and immunoreactivity is detected and captured on hypersensitive film. (Schematic adopted from Sigma Aldrich)

Sample collection/Fecal culture/PCR analysis - Stool specimens were self-collected by patients and healthy controls in a clean single use vessel and stored at -20 degrees C until returned to the MS Center. Approximately one gram of stool was collected and stored in a fecal collection tube (Sarstedt) containing 9ml of buffered glycerin-salt solution (10% glycerin, 71.2mM K2HPO4, 29.4mM KH2PO4, 71.9mM NaCl made in distilled water, adjusted to pH 7.2 and autoclaved) under IRB protocol no. 1003010940. Upon receipt, samples were resuspended in 40ml of modified rapid perfringens media21; D-cycloserine (400mg/L) was substituted for neomycin/polymyxin Band litmus milk was omitted to improve DNA extraction. The resuspended samples were cultured in 50ml falcon tubes with tightly closed caps at 47 degrees C ON.

DNA was extracted was from 1ml of culture supernatant using a Qiagen blood

and tissue kit. Isolated DNA was used as template for the following PCR reactions; the following primers were used:

1) 16S rRNA (positive control) fwd primer: AGAGTTTGATCCTGGCTCA, reverse primer: GGTTACCTTGTTACGACTT

2) Alpha toxin (pan *C. perfringens* marker) fwd primer: GCTAATGTTACTGCCGTTGA reverse primer: CCTCTGATACATCGTGTAAG (Meer and Songer 1997)

3)   Beta toxin fwd primer: GCGAATATGCTGAATCATCTA, reverse primer: GCAGGAACATTAGTATATCTTC (Meer and Songer 1997)

4)   Epsilon toxin   fwd primer: GCGGTGATATCCATCTATTC, reverse primer: CCACTTACTTGTCCTACTAAC (Meer and Songer 1997)

5) B1RB**B5** phage gene fwd primer: AAATGGACAAGAGGGATAAGGAT**,** reverse primer: TTTTCATCACAAATACCAGCCTC

6) B1RA**A6** phage gene fwd primer: TTACAATAAAACCACATGAGCTT**,** reverse primer: TTTTATTTAACATACTCCGTTTT

7) Q8SB**N7** phage gene fwd primer: GGGTGTCAAAGAAGATTTTAAAG**,** reverse primer: TTCTATCTTGCAACATTATATTT

Statistical analysis - A two-tailed Chi squared test was performed to compare anti-ETX immunoreactivity in MS patients vs. controls and the prevalence of commensal Clostridium *perfringens* type A in MS patients vs. healthy controls.

DISCUSSION

While the above experiments do not prove a causal relationship between ETX and Multiple Sclerosis, they provide supporting evidence for the ETX/MS hypothesis. Moreover, we have gathered experimental evidence suggesting that our current method of culturing C. *perfringens* from stool samples can be improved.

Currently, we culture the samples at an increased temperature of 47C, rather than 37C, which has been shown to favor C. *perfringens* growth over competing organisms (Erickson and Deibel 1978). However, this temperature may not be optimal for all C. *perfringens* strains. We find that 47C actually inhibits the growth of our laboratory derived type B strain, but does not affect the growth of our type A strain (Figure 26).
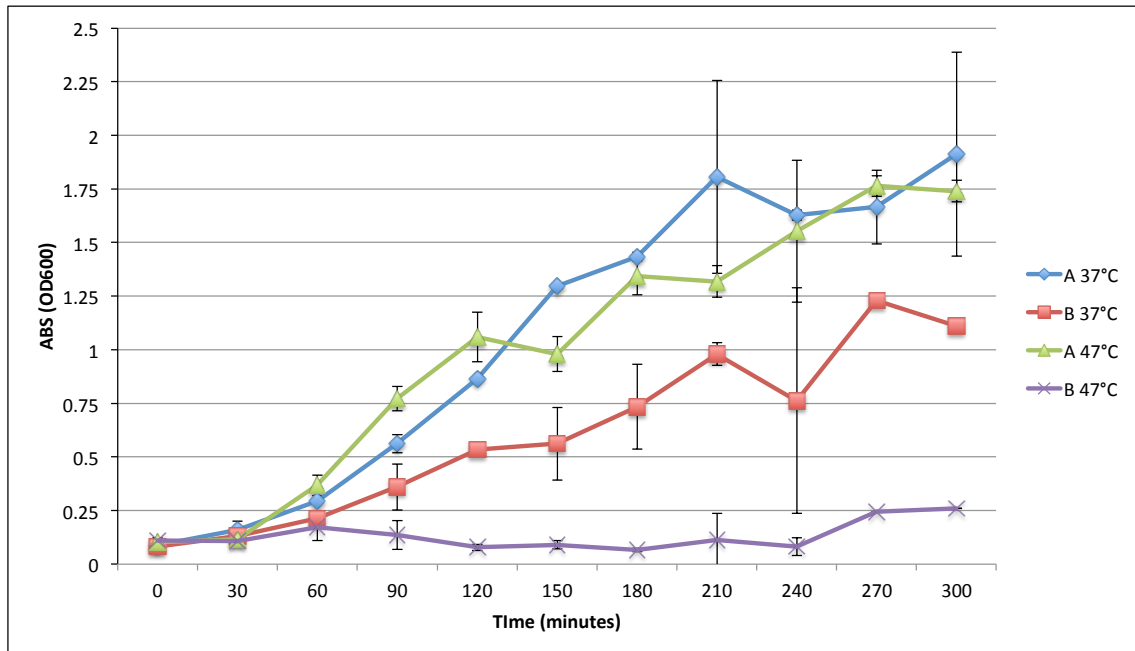
Figure 26. Growth Curves of C. *perfringens* types A and B. Overnight cultures of type A (ATCC 12915) and type B (ATCC 3626) were cultured at 37 degrees C in RPM. The following morning, starter cultures were diluted in RPM to an approximate OD600 of 0.1 and incubated at 37C or 47C.

Furthermore, our culture method seeks to expand C. *perfringens* that exist as both vegetative cells and dormant spores in the stool sample. However, selecting for bacterial spores before culture may increase our sensitivity, as vegetative cells from other species may outcompete C. *perfringens* and inhibit its growth. To test this idea, we re-suspended a patient's stool sample in rapid *perfringens* media and split the diluted sample into two vials of equal volume. One vial was heated at 80C for 15 minutes to kill the vegetative cells, such that only dormant spores would remain viable, while the other vial was kept at 25C. Both samples were then cultured overnight at 47C and we found that the patient was C. *perfringens* type A negative by our standard culture method, but C. *perfringens* type A positive when dormant spores we selected for by heat treatment (Figure 27).

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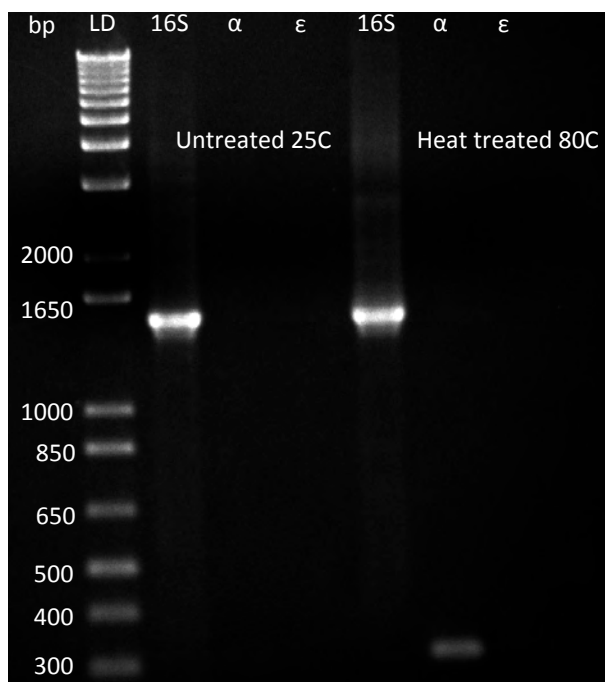
Figure 27. Spore Selection Improves Sensitivity. (Left) a stool sample from patient 167M that has been kept at 25C before culture. (Right) the same stool sample heated at 80C for 15 mins. to kill vegetative cells and select for dormant spores before culture. 16S rRNA PCR product (1600bps) is present in both as a positive control. Alpha toxin (300bps) is absent in the 25C sample, but present in the 80C sample. Epsilon toxin (650bps) is absent in both samples. Patient 167M is determined to be type A positive only after spore selection.

We also sought to experimentally determine if C. *perfringens* toxinotypes could inhibit each other's growth as proposed by McDonel and Itodo et al. based on soil studies showing C. *perfringens* type A dominance (McDonel 1980; Itodo, Adesiyun et al. 1986). The fact that healthy controls were twice as likely to carry C. *perfringens* type A than MS patients (Figure 23) led to the hypothesis that type A may also outcompete toxinogenic types B and D for crucial resources in the human gut. We found that soluble factors from C. *perfringens* conditioned media were able to inhibit the growth of a non-isogenic strain (Figure 28). However, when cultured together, our laboratory type B strain (ATCC 3626) outcompeted our laboratory type A strain (ATCC 12915) (Figures 29 and 30). Although seemingly contradictory to our hypothesis of type A dominance, it must be noted that this type A strain is pathogenic, and secretes enterotoxin (CPE) causing food

borne diarrhea in humans. Therefore, it is not a human commensal. Furthermore, this experiment has been conducted between just two of the many different strains of C. *perfringens* types A and B that exist in the environment. This particular result may be restricted to how these two unique strains interact with each other. The more important point to glean from this experiment is that C. *perfringens* secretes molecules that functionally inhibit growth of non-isogenic *perfringens* strains.
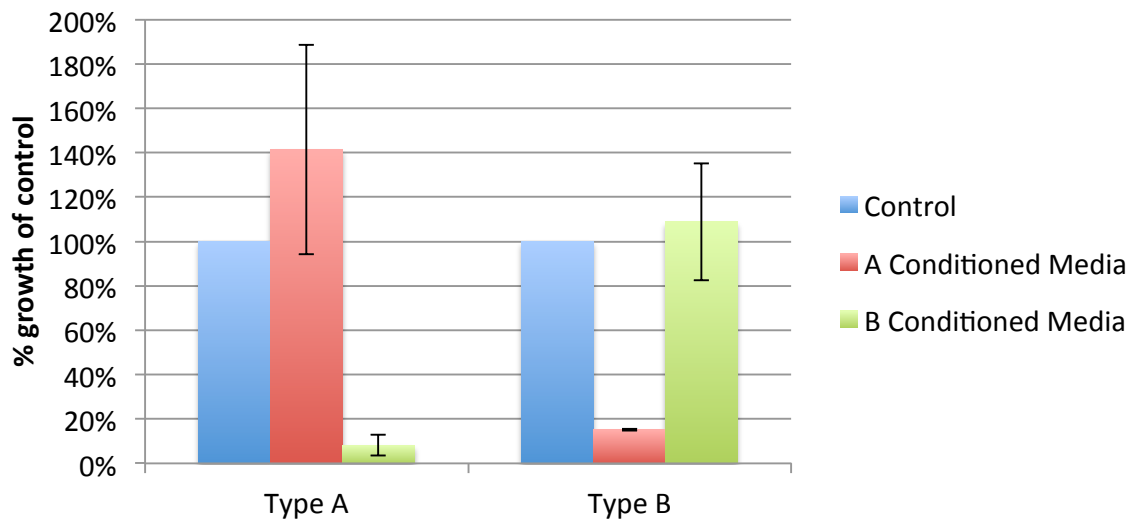
Figure 28. Non-Isogenic Supernatants Inhibit Growth. (Left) C. *perfringens* type A (ATCC 12915) cultured for 4 hrs in control media (blue), isogenic type A conditioned media (red) and non-isogenic type B media (green). (Right) C. *perfringens* type B (ATCC 3626) cultured for 4 hrs in control media (blue), non-isogenic type A conditioned media (red) and isogenic type B conditioned media (green).

Type A Control	1	1	1	0
A Conditioned Media	1.080364075	1.746341465	1.413932718	0.470917125
B Conditioned Media	0.091132539	0.114634146	0.108188343	0.046316631
Type B Control	1	1	1	0
A Conditioned Media	0.154901265	0.148288973	0.151595119	0.004675596
B Conditioned Media	1.275237775	0.901140684	1.08818923	0.26452659

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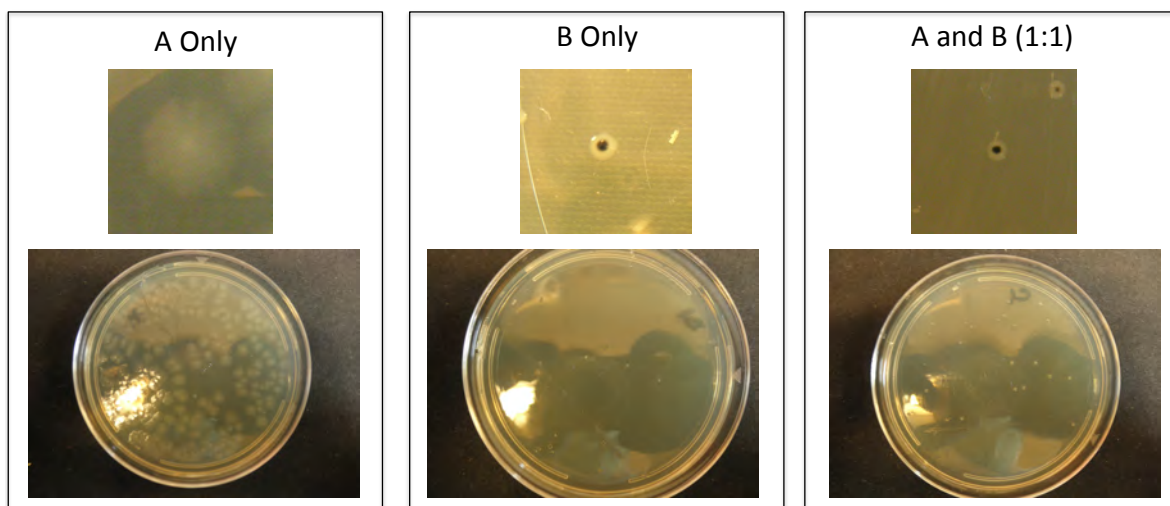
Figure 29. A Competitive Growth Assay Between Type A and Type B. (Left) type A colonies are large and flat and non-sulfur reducing (clear) on PAB agar. (Middle) type B colonies are small, raised and sulfur reducing (black) on PAB agar. (Right) when added in equal concentrations, type B colonies inhibit type A growth and are the dominant presence on the PAB plate.

% Control at 4 hrs
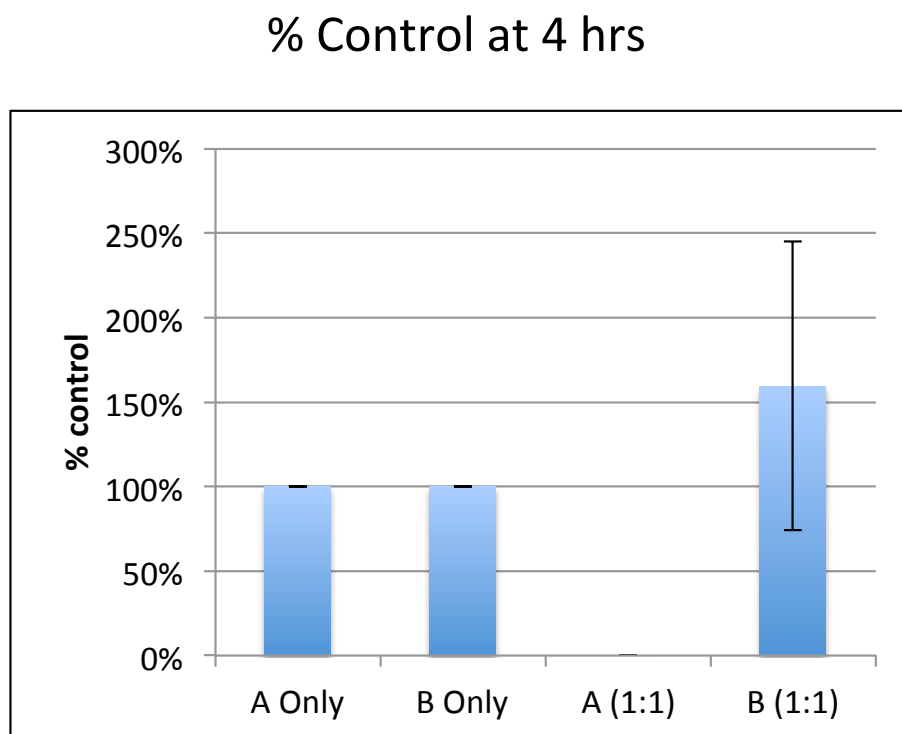
Figure 30. Quantification of the Competition Assay. Type A growth is completely abolished when mixed with type B. Type B growth is much less affected by the presence of type A in the culture media.

					Stdev
A Only	1.00	1.00	1.00	1.00	0.00
B Only	1.00	1.00	1.00	1.00	0.00
A (1:1)	0.00	0.00	0.00	0.00	0.00
B (1:1)	0.81	2.50	1.48	1.60	0.85

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Patient 73F, who was determined to be type B positive three months after her first clinical episode, became negative for all C. *perfringens* strains upon a second PCR stool analysis eight months later during her second visit to the MS clinic. Repeat MRI showed that she had developed new lesions since her first visit. While it is not possible to pin point when these new lesions may have occurred relative to her negative ETX PCR, the finding that she had converted from CIS to MS without stable detection of C. *perfringens* type B gives rise to many questions. 1) Could ETX be an initial trigger that catalyzes the MS disease process but is not required for propagation of the disease? This model has been termed, the "hit-and-run" hypothesis. If we suspect that ETX causes the BBB breakdown and non-immune mediated oligodendrocyte apoptosis found in each newly forming lesion by Barnett et al. and Adams et al. (Adams RD 1952; Barnett and Prineas 2004), then the ETX gene and toxin must be present in the MS patient at the time of relapse. Therefore, the hit-and-run model is not consistent with the ETX/MS hypothesis.

It is possible that our detection methods may not have been sensitive enough to detect type B later on in her disease course. Perhaps a larger/detectable bacterial load is necessary early on in MS to establish persistent carriage, which is below our detection limits. Alternatively, it is possible that type B/D carriage is, in fact, transient and we are often exposed to type B/D spores or vegetative cells, but only a small percentage of individuals possess a host environment that allows for transient colonization, toxin production and subsequent neurologic disease.

There is yet one other possibility, which may significantly add to the complexity of this potential host-pathogen interaction. Could it be possible that

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C. *perfringens* type B/D is necessary for the introduction the ETX gene to the human gut early in MS, and that mobilization of the ETX gene via horizontal gene transfer allows ETX to persist in the host after type B/D has been evacuated? Interspecies horizontal transfer of the ETX gene would be of enormous consequence to this study as our culture techniques are aimed at selectively culturing C. *perfringens*. Indeed, we could be reducing our ability to detect the ETX gene in MS patients by precluding the growth of other gut species. Although interspecies horizontal transfer of C. *perfringens* mobile elements is yet to be observed in a laboratory setting, many factors suggest that it is at least possible. Furthermore, there is evidence that C. *perfringens* and C. *difficile* have transferred genes in the past (Lyras, Storie et al. 1998).

The ETX gene is encoded on two distinct plasmids, a type B and type D plasmid (Havard, Hunter et al. 1992). These plasmids carry the tcp (transfer clostridial plasmid) genes, which encode the structural proteins required for conjugative pilus formation. Not only is ETX on this replicative mobile element, but it is also flanked by Insertion Sequences (IS), simple transposons, at both the 5′ and 3′ ends. These flanking mobile elements make this entire unit of DNA (**IS**1151-ETX-**IS**406) a composite transposon that is capable of excision and transposition into a new locus (Figure 31A). Furthermore, it has been shown that the ETX gene can exist as a conformational intermediate of the transposition process suggesting that transposition indeed occurs (Sayeed, Li et al. 2007) (Figure 31B).
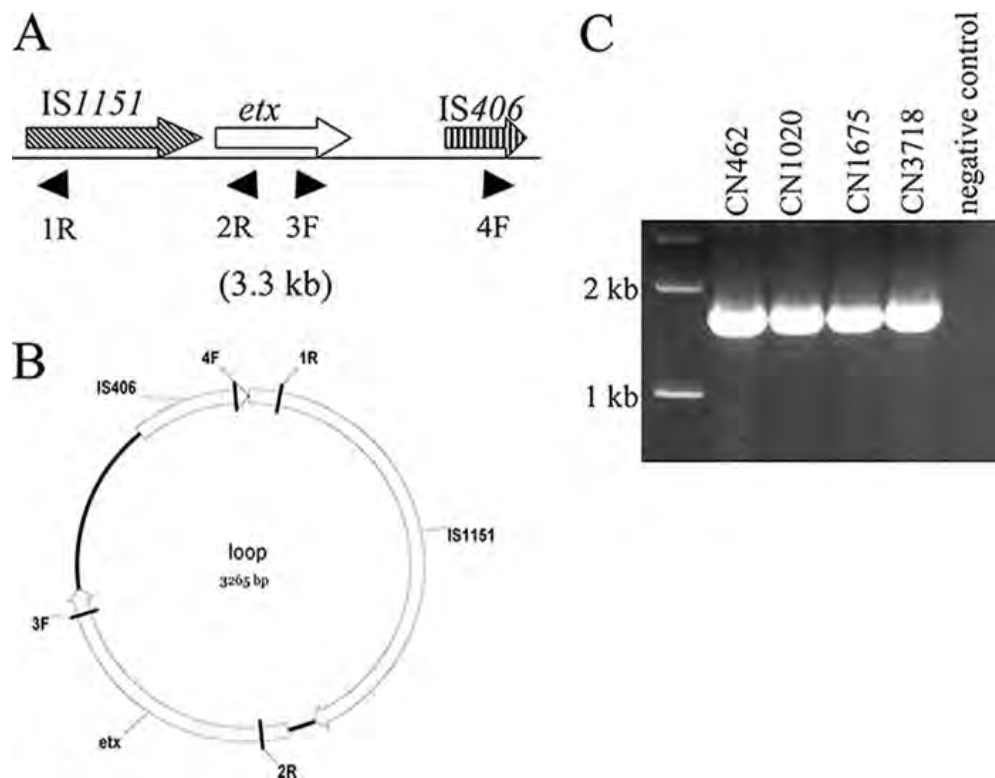
Figure 31. ETX Forms Circular Transposition Intermediates. (A) Arrangement of the etx locus in type D isolates (CN462, CN1020, CN1675 and CN3718). The designations below the line indicate the primers used to evaluate circular intermediate formation. (B) Map of the circular intermediate based on PCR and sequencing. (C) PCR amplification of the circular intermediate. The positions of molecular size markers are indicated on the left, and isolate designations are indicated above the lanes. (Sayeed, Li et al. 2007)

Composite transposons are well studied in the spread of antibiotic resistance. These mobile elements excise structural genes, such as toxins and antibiotic resistance genes, from donor DNA (chromosomal or plasmid) and incorporate them into recipient DNA (chromosomal or plasmid). Transposition can occur within the same cell or it can occur in a different cell that has acquired

the mobile element on a transferred plasmid or circular transposition intermediate (Puopolo, Klinzing et al. 2007). ETX's ability to be transposed is incredibly significant because transfer of an ETX plasmid alone would not be sufficient for a successful horizontal transfer. Foreign plasmids are often unable to replicate and are unstable in bacteria of different species, and this is true for C. *perfringens* plasmids, as they require the presence of the C. *perfringens* specific *Rep* protein for stability (Bannam, Teng et al. 2006). Therefore, ETX would have to be excised and transposed into the recipient's endogenous DNA to replicate and function. The fact that ETX is part of a composite transposon makes this possible.

There is another significant hurdle to interspecies horizontal transfer of ETX that must be addressed. Although the ETX plasmid is conjugative between C. *perfringens* strains, there is no evidence that C. *perfringens* can conjugate with another species by the normal plasmid mediated conjugative mechanisms. However, there is evidence that C. *perfringens* may be capable of horizontal transfer with C. *difficile* via an alternate and less well understood conjugative process. Investigators suspect that horizontal gene transfer has actually occurred between C. *perfringens* and C. *difficile* in the past, most likely via conjugative transposons. Rood et al. compared chloramphenicol resistance genes and other mobile elements in C. *perfringens* and C. *difficile.* They concluded that these genes were so similar that horizontal transfer by a conjugative transposon was the most likely mechanism (Lyras, Storie et al. 1998).

Conjugative transposons differ from regular transposons in that, in addition to being transposable mobile elements, they also encode their own conjugation proteins. Importantly, the conjugative pili formed by conjugative transposons

are promiscuous in their recipient targeting and will frequently conjugate with bacteria of different species. Additionally, while plasmid conjugative pili are exclusive for the transfer of the encoding plasmid, transposon conjugative pili allow the transfer of mobile elements in a non-specific fashion. Moreover, conjugative transposons have actually been shown to actively mobilize neighboring mobile elements for interspecies transfer (Salyers, Shoemaker et al. 1995).

Tn916 is a prototypic conjugative transposon that originates from Enterococcus *faecalis* and is thought to play a major role in the interspecies spread of antibiotic resistance genes. Once transposed into another bacterial species, Tn916 confers the ability to perform promiscuous interspecies conjugations to the recipient species (Roberts and Mullany 2011). Once the mating pore is formed, other mobile elements, such as plasmids, are mobilized and transferred. Haack et al. showed that a Tn916 carrying Bacillus *subtilis* transferred its pC194 plasmid to Bacillus thuringiensis via the Tn916 mating pore (Haack and Andrews 2000). Importantly, Tn916 easily transposes C. *perfringens* in a laboratory setting (Awad and Rood 1997), however co-transfer of C. *perfringens* plasmids/circular transposition intermediates via the Tn916 mating pore has not been investigated.

The fact that C. *perfringens* and E. *faecalis* share the same habitats (soil and animal GI tracts) and the ease with which Tn916 transposes C. *perfringens* support the idea that such a transposition event may occur in the natural environment. Although no functional conjugative transposons of C. *perfringens* origin have been identified, it is of interest that a defective Tn916-like transposon has been identified in C. *perfringens* (strain CW459). Although classified a Tn916-

like transposon, the CW459tet(M) element has lost the xis gene that is necessary for self-excision from its chromosomal locus. However, it encodes other proteins present in the functional Tn916 transposon, including the conjugation pilus (Roberts, Johanesen et al. 2001) (Figure 32). It is unknown if excision is necessary for the expression of mating pore proteins and subsequent interspecies conjugation. We must consider the context of this finding as the "defective" CW459tet(M) element was identified in one C. *perfringens* isolate. It is possible that other conjugative transposons exist in other C. *perfringens* strains, some of which may be fully functional. This may be a minor point however, as a neighboring E. *faecalis* could donate the prototypic Tn916 conjugative transposon to C. *perfringens*, thus conferring the ability for interspecies conjugation and horizontal gene transfer.
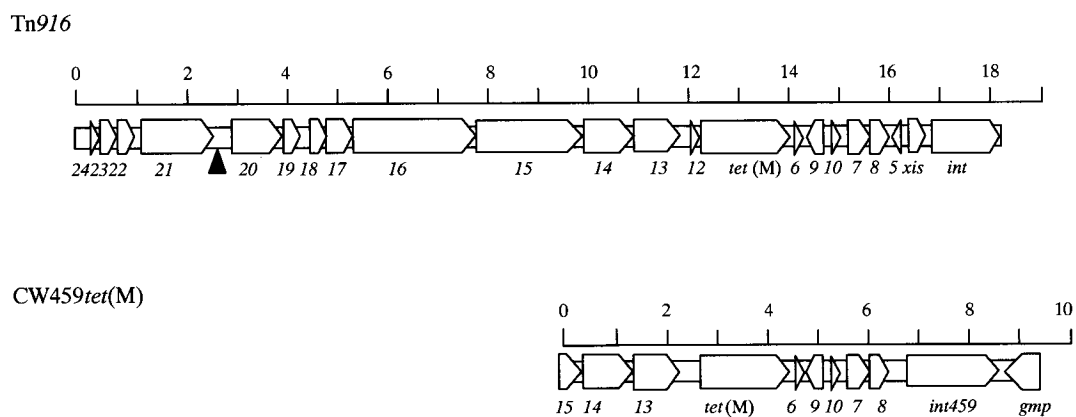
Tn*916*

CW459*tet*(M)

Figure 32. A Modular Comparison of Transposons: the prototypic conjugative transposon, Tn916, from Enterococcus *faecalis* (above) and the defective CW459tet(M) element from C. *perfringens* str. CW459 (below) (Roberts, Johanesen et al. 2001). The black arrow indicates the origin of transfer (oriT). Please note that CW459tet(M) may extend past module 15, as this is the point at which the sequencing probe ended.

Although no functional conjugative transposons have been identified in C. *perfringens,* from probing the whole genome sequence of C. *perfringens* type B ATCC 3626, we have discovered a previously unidentified conjugative transposon. This novel C. *perfringens* conjugative transposon shares an almost identical modular identity to the prototypic Tn5397 transposon found in C. *difficile* str. 630 (Figure 33). However, most of the modular proteins are quite divergent in their amino acid identity when compared to Tn5397. Surprisingly, most of these proteins share a high degree of amino acid identity (> 90 identity for 8/13 proteins) with another unidentified conjugative transposon that is also found in C. *difficile (*ATCC 43255 strain) (Table 5). The fact that the C. *difficile* proteins are of equal or greater identity than homologues from C. *perfringens* type D strain JGS1721, suggests that these homologues are the result of a conjugative transposition between C. *perfringens* and C. *difficile,* rather than divergent evolution from a common Clostridial ancestor. Perhaps the most important feature of this novel C. *perfringens* conjugative transposon is that it encodes a putative resolvase protein that is homologous to the TndX resolvase encoded by Tn5397. Resolvase is necessary and sufficient for both DNA excision and integration. Therefore this novel conjugative transposon may be functional.

A.

Conjugative Transp.	**typeB 3626**	**Tn5397**
GenBank Accession #	ABDV01000002.1	AF333235.1
	Resolvase	TndX
	Sigma 70 region 4	ORF7
	Macrolide Efflux Transp.	tet(M)
	TcpC	ORF13
	TraC F type	ORF16
	TcpE	ORF17
	ArdA antirestriction	ORF18
	Cro/CI DNA rep. initiation	ORF20
	ftsk/SpoIIIE	ORF21
	DUF916 famiily	ORF22
	DUF916 family	ORF23

B.
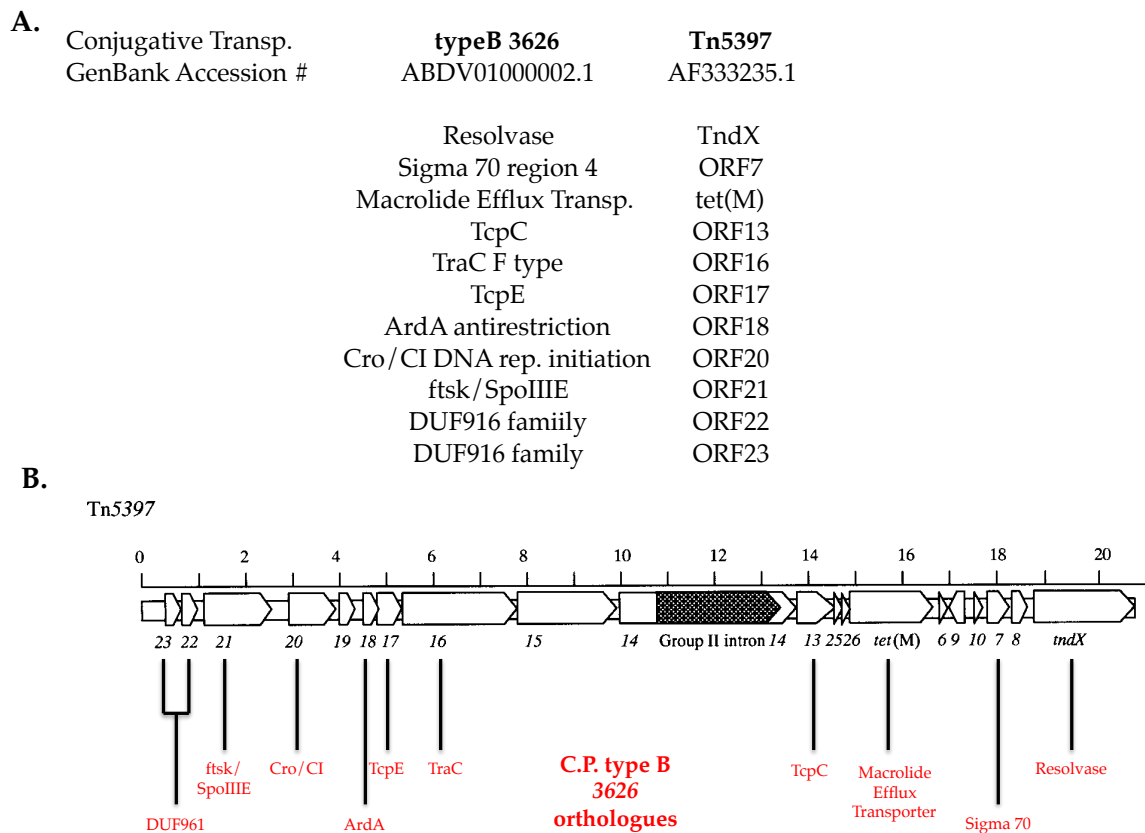
Figure 33. Identification of a Novel C. *perfringens* Conjugative Transposon.
A) Novel conjugative transposon proteins from C. *perfringens* type B ATCC 3262 and the corresponding ORFs in Tn5397 (C. *difficile* str. 630) in a tabular format. B) A schematic of prototypic Tn5397 and the corresponding C. *perfringens* type B ATCC 3626 proteins listed below. Adapted from (Roberts, Johanesen et al. 2001).

Table 5. A Comparison of Amino Acid Identities Between Conjugative Transposon Genes in C. *perfringens* **Type B, Type D and C.** *difficile.*

				%Ident.	
Gene	protein ID number	Function	type B	C. *diff*	type D
sigma-70, region 4 family	EDT25038.1	RNA polymerase subunit	100	81	N/A
TcpC type IV secretion protein	EDT24937.1	conjugative transfer	100	90	N/A
NLP/P60 family protein	EDT25012.1	cell wall hydrolysis	100	94	N/A
conjugative transposon protein	EDT25143.1	unknown	100	84	N/A
type-IV secretion system protein TraC	EDT24978.1	conjugative transfer	100	97	N/A
TcpE type IV secretion protein	EDT25106.1	conjugative transfer	100	80	N/A
antirestriction protein (ArdA)	EDT25111.1	bacterial conjugation	100	96	N/A
conserved domain protein	EDT25139.1	unknown	100	93	N/A
transcriptional regulator, Cro/CI family	EDT25017.1	DNA replication initiation	100	84	N/A
ftsk/SpoIIIE/ EssC type VII secretion protein	EDT24948.1	DNA segregation	100	89	78
conjugative transposon protein	EDT25129.1	unknown	100	91	91
conjugative transposon protein	EDT25010.1	unknown	100	94	88
cell wall-associated surface protein	EDT25119.1	cell envelope biogenesis	100	91	N/A

Future Directions

Identify the ETX pore in MS brain tissue: While suggestive, this preliminary study fails to prove that exposure to Clostridium *perfringens* epsilon toxin triggers the initial demyelinating event in MS. We propose that a definitive experiment will involve identifying the toxin, in situ, in the MS brain. In pursuit of this experiment we have generated affinity purified polyclonal antibodies against ETX, but have found that these antibodies are unable to bind to ETX once it has made contact with the cell membrane. ETX is pore-forming protein that attaches to susceptible cells, changes conformation as it inserts into the plasma membrane, and kills cells by osmotic lysis. Because a significant portion of the ETX molecule inserts into the membrane, antibody accessibility is likely to be greatly reduced when ETX is cell bound. Additionally, the conformational change may also reduce antibody binding by altering the structure of immunoreactive epitopes.

Finally, ETX inserts into lipid raft structures, which are densely decorated with carbohydrates and GPI linked proteins that may mask the ETX pore from antibody recognition. We will address these experimental hurdles in two ways:

1) Try different methods of antigen retrieval e.g. proteinase K, urea and citrate treatment of brain sections from ETX treated rodents to determine if these any of these treatments will unmask immunoreactive epitopes.

2) Design a DNA aptamer that will be small enough to access and specifically bind to the membrane spanning ETX pore.

A brief review of DNA aptamers and aptamer development: Aptamers are small oligonucleotides that specifically bind to a wide range of target molecules, such as drugs, proteins, or other inorganic or organic molecules, with high affinity and specificity. The concept of aptamers is based on the ability of small oligonucleotides (typically 80-100mers) to fold into unique three-dimensional structures that can interact with a specific target with high specificity and affinity (Mallikaratchy, Liu et al. 2009). As aptamer development is not our expertise, we will be collaborating with Dr. Probodhika Mallikaratchy of Lehman College. Dr. Mallikaratchy is an accomplished scientist in the aptamer development field. She has successfully developed aptamers that recognize protein kinase C-delta5, IgM heavy chain in Burkitt's lymphoma cells (Mallikaratchy, Tang et al. 2007), and the B cell receptor on human leukemia and lymphoma cells (Mallikaratchy, Ruggiero et al. 2011).

An ETX pore specific aptamer, will allow us to design and execute experiments that will definitively address whether ETX is indeed a trigger for nascent MS lesions. In addition to routine histology, aptamers can also be used in a clinical setting for both research and therapeutic purposes. For example, during an MS exacerbation, a radiolabeled aptamer could identify ETX in living patients via PET imaging. Furthermore, aptamers could be coupled to molecules capable of capping the ETX pore, thus preventing cell lysis and ameliorating MS symptoms.

Cell based aptamer selection: We will use MDCK cells, which are known to be susceptible to ETX, to develop a Flow Cytometry based assay for aptamer selection. Aptamers that bind to the ETX pore will be subjected to multiple rounds of enrichment and PCR amplification until highly pore-specific sequences are identified. Briefly, We will perform a titration of MDCK cells (500,000 cells/ml) with increasing concentrations of ETX and measure propidium iodide uptake after 1hr of incubation. We will select a concentration that corresponds to the beginning of the PI uptake plateau. This early plateau concentration will be used for the remainder of the study to ensure that the entire quantity of toxin present exists in the heptameric pore conformation. It will be important to avoid saturating the cells with toxin such that some of the bound ETX remains monomeric, as we only wish to select aptamers that recognize the ETX pore. Toxin treated MDCK cells will be incubated with a commercially available DNA library. Each unique DNA sequence will be flanked by conserved sequences at both the 5' and 3' ends. These conserved sequences will be used as recognition sites for PCR primers and subsequent PCR amplification. The DNA oligomers

will also be fluorescently labeled so that we may visualize aptamer binding. After the first round of incubation and washing of the cells, heating the sample will elute the bound DNA sequences. We will cool the eluted DNA, thus allowing it to regain its original structure, and incubate with non-treated MDCK cells as a negative selection measure. The unbound sequences (the sequences of interest) will be isolated and PCR amplified. We will repeat these selection and amplification steps multiple times until we have generated a pool of oligomers that probe ETX treated cells exclusively. Finally, we will sequence the evolved oligomers to determine their primary structures. Each of the above steps is summarized below in Figures 34 and 35.
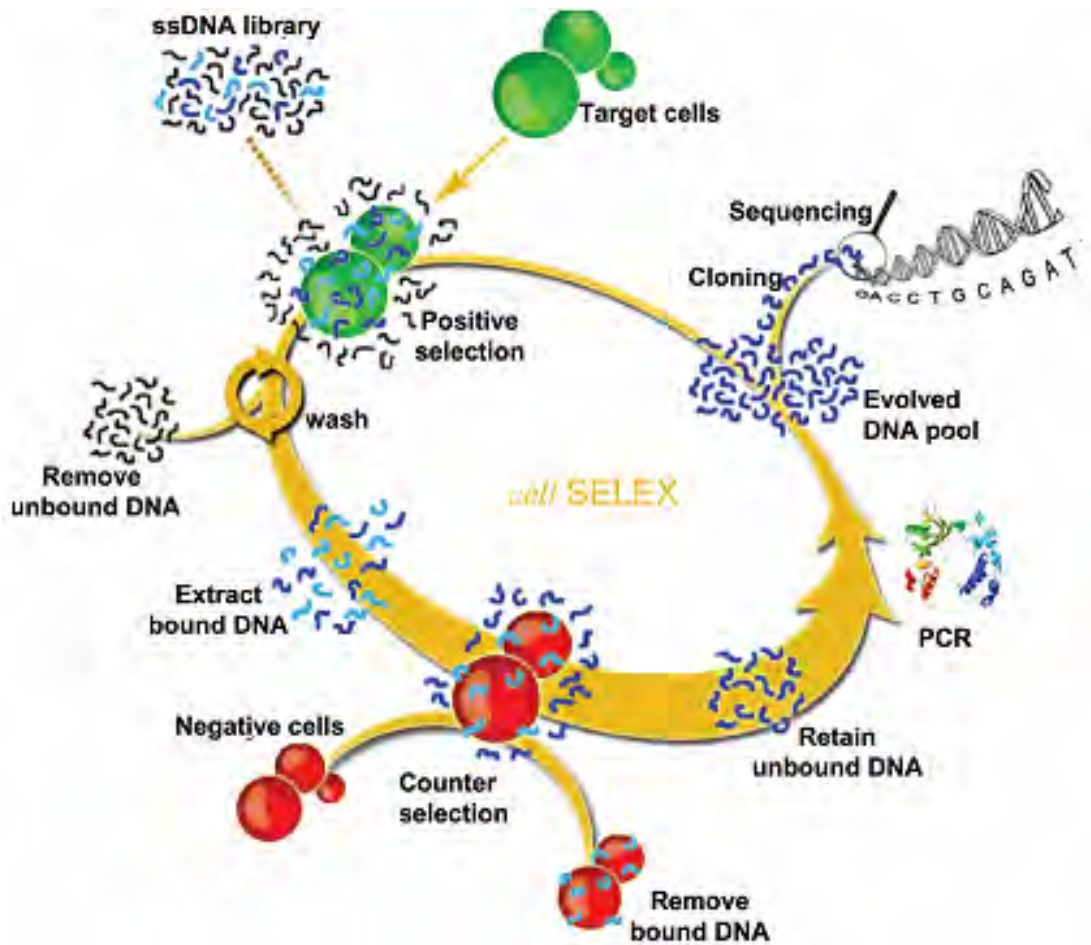
Figure 34. A Schematic of Cell Based Aptamer Selection. The cell-SELEX aptamer selection process developed by the Tan group. A ssDNA pool is incubated with target cells. After washing, the bound DNAs are eluted by heating to 95°C. The eluted DNAs are then incubated with negative cells for counter-selection. After centrifugation, the supernatant is collected and the selected DNA is amplified by PCR. The PCR products are separated into ssDNA for the next round of selection. Finally the enriched library with aptamer sequences is cloned and sequenced to obtain aptamer candidates. (Shangguan, Li et al. 2006)
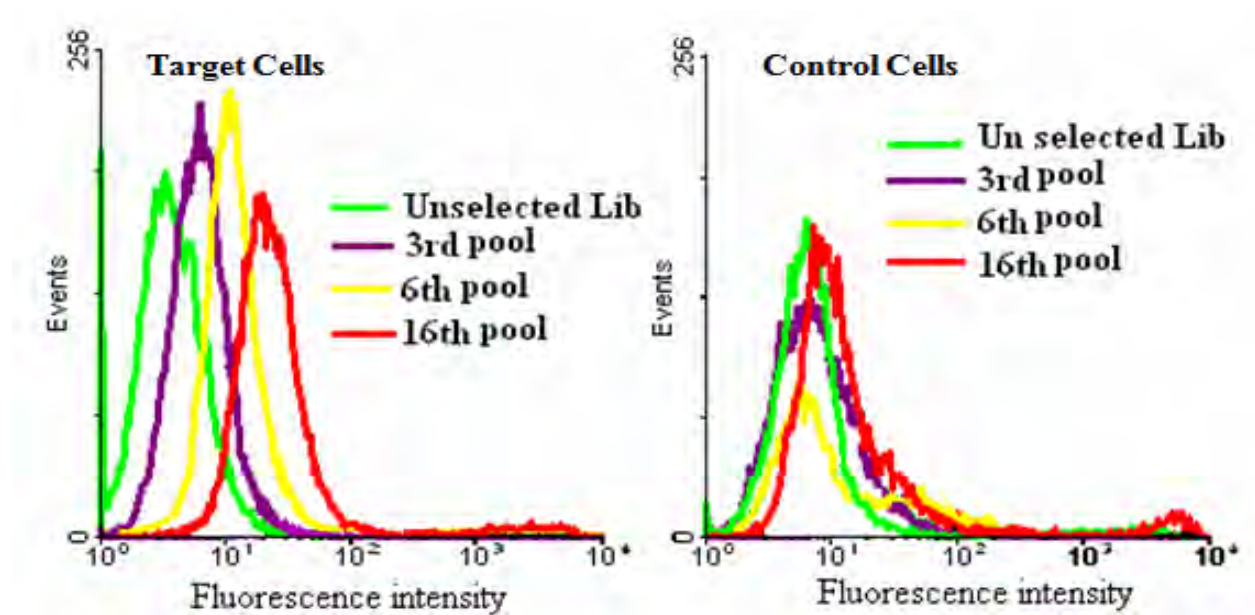
Figure 35. FACS Analysis of Aptamer Enrichment. (a) The green curve represents the background binding of the starting DNA library. An increase in fluorescence intensity with the number of cycles is indicative of enrichment of the affinity towards the target cells. (b) Little change is observed for the control cells. (Shangguan, Li et al. 2006)

A detailed microbiome assessment: We wish to 1) validate our preliminary observation with a larger cohort by expanding the numbers of patients and healthy controls analyzed for C. *perfringens* toxinotypes. 2) specifically interrogate distinct body habitats for C. *perfringens* toxinotypes, which have not previously been performed in MS or healthy controls using optimal culture techniques. 3) study a cohort of patients and controls longitudinally over a two-year period, and thus define variation in C. *perfringens* toxinotype abundance over time in individual patients. 4) compare the sensitivities of C. *perfringens*

specific culture methods to that of high-throughput technologies for identification of C. *perfringens.* 5) perform high throughput sequencing of 16S rRNA genes and characterize/compare microbiomes to identify the microbial communities that may be specific for MS patients and determine environmental pressures that may shape susceptibility to C. *perfringens* types B and D colonization.

Create a new animal model for MS: As previously mentioned, no animal model currently exists that accurately recapitulates the tissue damage and symptomatology of MS. ETX provides the unique opportunity to induce spastic paralysis, Dawson's fingers-like periventricular lesions, breakdown of the blood-retinal barrier in addition to the blood-brain barrier, and perhaps most importantly, cause damage to oligodendrocytes in the absence of an immune infiltrate; similar to what has been observed in newly forming MS lesions. We wish to model each of these characteristics in vivo, ex vivo and in vitro. Furthermore, we wish to supplement our in vivo histological analyses with small rodent MRI imaging. This will be particularly useful in assessing ETX's ability to recapitulate Dawson's fingers.

Address whether interspecies ETX gene can transfer occur: Interspecies horizontal gene transfer is frequently observed and has been well studied for antibiotic resistance genes. However, less attention has been paid to bacterial toxins despite the fact that toxins are often carried on the same types of mobile genetic elements. Evidence for interspecies toxin transfer would have an enormous impact on how we proceed with this study. It would change our

experimental design and may open new avenues for future research. With this in mind, we wish to determine whether the introduction of a conjugative plasmid such as Tn916, derived from Enterococcus *faecalis,* can facilitate interspecies mating and transposition of the ETX gene from C. *perfringens* to a distantly related organism. It may also be fruitful to isolate commensal C. *perfringens* type A strains from MS patients and controls, and determine their relative abilities to acquire the ETX gene through conjugation with a type B or D toxinotype. Perhaps commensal type A strains from MS patients more readily acquire the ETX gene, thus obviating the need for true colonization by a toxinogenic type B or D strain.

CHAPTER 3

A Review of Protein Toxins, ETX and The Elusive ETX Receptor

Toxins are any poisonous substance produced by a living organism that is capable of causing disease or death in other organisms (Elisabete Valério 2010). These molecules come in many different flavors; proteins, small molecules, alkaloids etc. and may disrupt cellular function in a myriad of ways. Indeed, the most toxic molecules known to man are bacterial toxins derived from the Clostridium genus; Clostridium *botulinum*, *tentanus* and *perfringens* epsilon toxins being the three most toxic molecules in descending order. It is likely that these Clostridial toxins display such devastating toxicities because they each affect the central nervous system (CNS), which is critically important for sustaining life. Thus, it is important to note that a toxin's potency is not only dependent on its mechanism of action, but also its target tissue, and the importance of that target tissue to the organism's viability.

Although evolved to harm other organisms and to confer an evolutionary advantage over competitors, studying the mechanisms by which toxins work has already yielded therapeutic dividends; perhaps the best example being the use of botox to treat ailments of muscle spasticity (Brin 2009). Further studies are currently underway, exploring the potential use of bacterial toxins to selectively kill cells (Walther, Petkov et al. 2011). The success of these emerging technologies rests not on how the toxin affects the target cell, but more so on how

the toxin initiates contact with its target. Indeed, the specificity of bacterial toxins may prove to be their greatest benefit to medicine.

After binding to the target cell receptor, toxins usually derail cellular function in one of six ways (Elisabete Valério 2010):

Membrane permeabilization: These toxins start binding to the membrane in their monomeric form. Afterwards, self-oligomerization occurs resulting in the formation of pores that are permeable to ions and small metabolites. Ultimately, this leads to membrane disruption, membrane permeability and osmotic lysis of the cell (Zitzer 1997).

Toxins affecting membrane trafficking: Some toxins can interfere with several components of vesicle-associated membrane protein system, altering the trafficking of molecules like neurotransmitters across the membrane e.g., botulinum toxin. (Martinez-Arca 2000; Chen 2009).

Toxins affecting signal transduction: There are toxins that target the intestinal epithelial cells where, after a complex series of events, they activate adenylate cyclase, interfering with signal transduction e.g., cholera toxin (Lencer 1995). Other natural toxins act by modifying key functions of the phosphorylation-based signaling machinery, thus affecting the signal transduction pathways e.g., microcystins (Sivonen 1999).

Toxins affecting protein synthesis: This group of toxins can present more than one mechanism to inhibit protein synthesis. Two examples are the cleavage of several

nucleobases from the 28S rRNA e.g., Shiga toxins (Cherla 2006) or the inactivation of elongation factor 2 (eEF-2) by transferring the adenosine diphosphate ribose moiety (ADP-ribose) of NAD to eEF-2 e.g., *Pseudomonas exotoxin* A (Menestrina 1991).

Cytoskeleton-affecting toxins: These toxins can induce structural changes in the cytoskeleton and consequently inhibit its functions. Cytoskeleton modifications include the disaggregation of actin microfilaments e.g., Toxin B from *Clostridium difficile* (Pothoulakis 1991) or the induction of the formation of giant multinucleated cells, leading to changes in actin and tubulin organization e.g., cytotoxic necrotizing factor of *Escherichia coli* (Fiorentini 1988).

Voltage-gated ions channels blockers: These toxins have the ability to interact with the specific receptors associated with neurotransmitter receptors, or with voltage-sensitive ion channels, therefore inhibiting the nervous signaling e.g., saxitoxin, kalkitoxin and jamaicamides (Wang 2008; Aráoz 2010).

Despite the great diversity of toxic mechanism, at least a third of the more than 300 protein toxins characterized to date damage cells by pore formation and membrane permeabilization (Alouf 2001). These pore forming proteins often share similarities in their membrane spanning domains and mechanisms of oligomerization. However, they are able to target individually specific tissues due to unique receptor binding domains.

Pore Forming Toxins

The pore-forming toxin has the unique ability to exist as either a hydrophilic solute or a hydrophobic, membrane-spanning pore depending on its surrounding environment. Once released into the host, the toxin must circulate in solution to find its target cell/tissue; this requires a soluble existence. However, once bound to its cellular receptor, the toxin must develop the ability to wedge itself into the lipid bilayer to form a channel; this requires a conformational change that either exposes buried hydrophobic surfaces or that oligomerizes the molecule such that a hydrophobic surface is formed cooperatively. These two distinct mechanisms of toxic pore formation have arisen in many different organisms, prokaryote and eukaryote alike, by convergent evolution. Below we will explore how each of these strategies work on a mechanistic level:

α Pore Formers: α-Pore Forming Toxins (α-PTFs) contain stretches of hydrophobicity that are predicted to be α helical (Figure 36) that can span the lipid bilayer resulting in a pore (Iacovache, van der Goot et al. 2008). These long hydrophobic stretches must be protected such that the molecule does not aggregate and precipitate before it reaches its cellular target. To avoid such an occurrence, some α-PTF require a change in the microenvironment, such as pH, which can destabilize the molecule and expose its hydrophobic regions e.g. colicin from E. *coli*. Diphtheria toxin, Cry δ-endotoxins produced from Bacillus *thuringiensis* and Pseudomonas *aeruginosa* exotoxin A are some other exotoxins that form pores that employ hydrophobic α helices (Parker 2010).
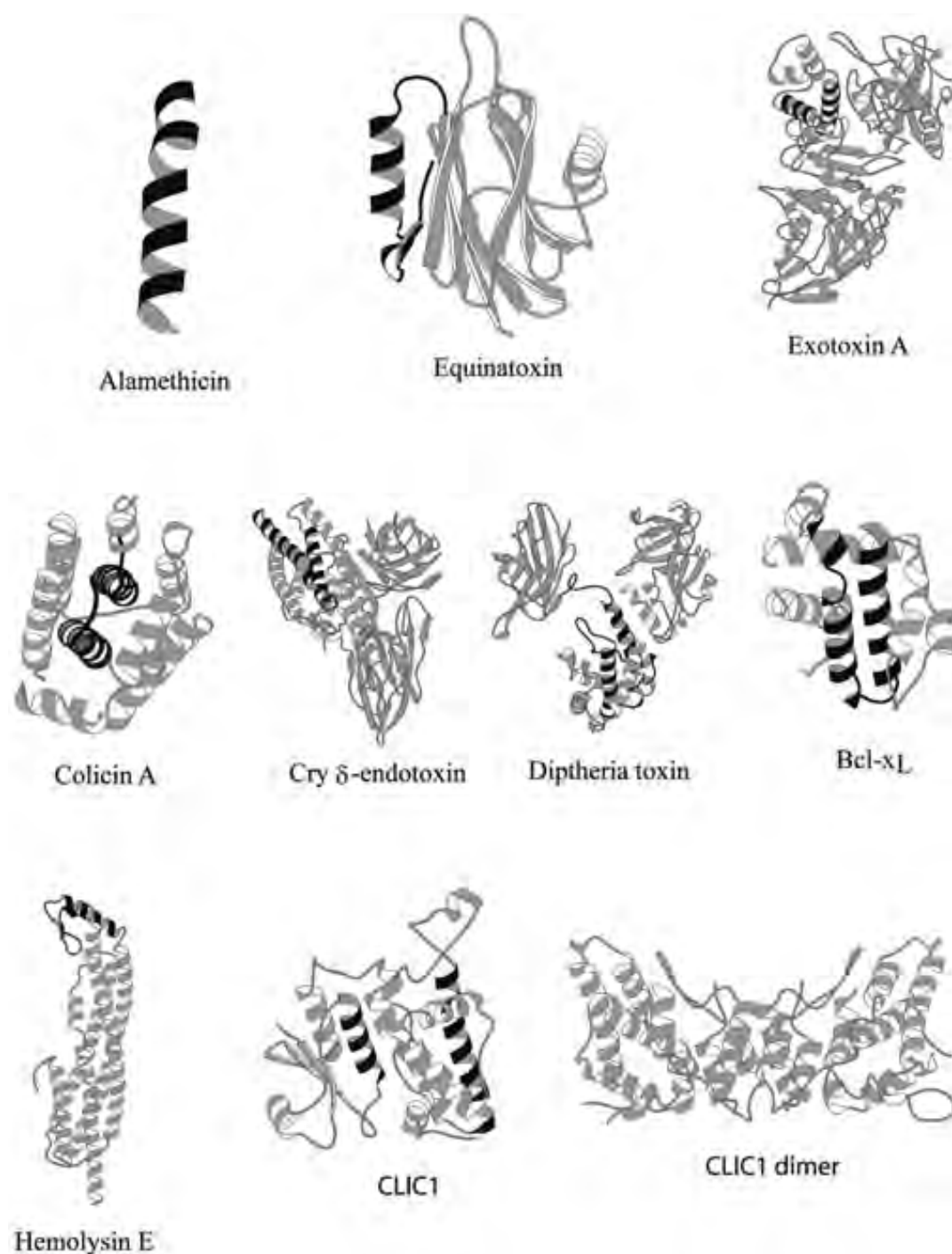
Figure 36. Ribbon Images of α-PFTs. The regions thought to form the transmembrane stretches that take part in the initial step in membrane insertion are highlighted in dark shade. The PFTs with colicin-like folds are shown in the second row. For some of the PFTs shown (equinatoxin, exotoxin, hemolysin E, CLIC1) their categorization as α-PTFs is only tentative. (Parker 2010)

β Pore Formers: The majority of bacterial PFTs known to date are β-PTFs. (Iacovache, van der Goot et al. 2008). β-PTFs are predicted to insert into membranes to form β-barrels. β-PTFs are released as soluble monomers or dimers that oligomerize before forming active pores. The final insertion step often requires significant protein refolding, so that subunits can donate β-strands to form a transmembrane β-barrel (Tilley and Saibil 2006). Unlike α-PTFs, there are no long stretches of hydrophobicity. Instead, β-PTF monomers each contain a β hairpin consisting of alternating hydrophobic and hydrophilic residues so that when assembled in the membrane, the hydrophilic residues form the lumen of the pore whereas the hydrophobic residues interact with the membrane core (Parker 2010). The lack of long hydrophobic regions helps avoid precipitation but as an additional regulatory step, β-PTFs are secreted as protoxins, e.g. ETX prototoxin, and must be enzymatically activated for oligomerization to occur (Parker and Feil 2005). Figure 37 illustrates the ribbon of β-PTFs.

Aerolysin

HlgB

α-Hemolysin

Defensin HNP-3

Anthrax Protective Antigen

CytB
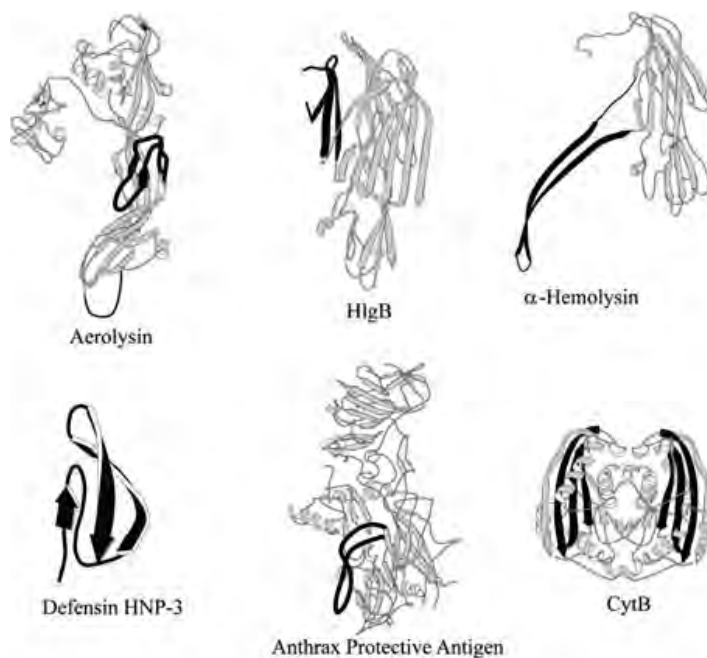
Figure 37. Ribbon Images of β-PFTs. The regions thought to form the transmembrane stretches are highlighted in dark shade. The archetypical toxins of the family are shown in the first row. (Parker 2010)

The Aerolysin Family of β Pore Formers

C. *perfringens* ETX is a member of the aerolysin family of β-PTFs, which also consists of aerolysin (Aeromonas *hydrophila*), α-toxin (Clostridium *septicum*), Enterolobin (Enterolobium *contortisiliquum,* a Brazilian tree), LSL (Laetiporus *sulphureus*, a mushroom), Mtx2/Mtx 3 (Bacillus *sphaericus*), Hydralysins (Chlorohydra *viridis*, an aquatic Cnidarian) and parasporin-2 (Bacillus *thuringiensis).* Despite the fact that these PFT producers originate from diverse kingdoms represented by plants, fungi and bacteria, most of the PFTs share remarkable sequence identity with aerolysin. Contrastingly, ETX and parasporin-2 share very little sequence identity with aerolysin, 13.9% and 25.7% respectively, but are still classified in the aerolysin family because of structural similarities within their domain structure (Knapp 2010). As the prototypic member, aerolysin has been the best characterized of these toxins.

Members of the aerolysin-like toxin family use amphipathic β-hairpins for membrane insertion and are therefore rich in β-sheets. Although β-PFTs share a common mode of pore formation, several types can be distinguished according to their individual receptors, number of monomers forming oligomers, pore size, and functionality. Like other β-PFTs, aerolysin-like toxins bind to a cell-surface receptor, oligomerize, and generate β-hairpins from 6 to 7 individual monomers to form the pore (Table 6) (Knapp 2010).

Aerolysin-like toxins are secreted as protoxins, requiring enzymatic activation. They bind to high affinity receptors located in lipid rafts, e.g. GPI-linked proteins for aerolysin. The spatial concentration of these receptors within

the lipid raft is thought to aid in concentrating the toxin such that it can oligomerize and cooperatively form a hydrophobic surface that can insert into the membrane. Activated monomers assemble into heptamers (Figure 38) as well as form channels through insertion into lipid bilayers, as observed indirectly by electron microscopy since only the mushroom cap and not the transmembrane region is visible (Wilmsen, Leonard et al. 1992). Heptamers inserted into the membrane form voltage-dependent, anion selective channels with a single channel conductance of about 70 pS in 0.1 M KCl which corresponds to a pore diameter of at least 7 Angstroms (Knapp 2010).

As a consequence of anion-selective transmembrane channels being formed, a cell can readily lose ions, water, and small molecules (i.e. sugars, amino acids, nucleic acids) that do not include proteins (Chakraborty, Schmid et al. 1990; Abrami, Fivaz et al. 1998). Besides breakdown of the Na^+/K^+ gradient, an influx of Ca^{+2} can be observed in cells, which seemingly mediates cell signaling (Krause, Fivaz et al. 1998). Loss of K^+ facilitates formation and activation of the inflammasome, cytoplasmic multiprotein complexes harboring Nod-like receptors such as IPAF and NALP3, as well as caspase-1 (Abrami, Fivaz et al. 1998). Finally, via caspase-1, the K^+ efflux mobilizes Sterol Regulatory Element Binding Protein (SREBP)-1 and -2. Both proteins are involved in the biogenesis of membranes and their activation enhances cell survival (Gurcel, Abrami et al. 2006). Caspase-1 is also responsible for the processing and activation of cytokines IL-1β and IL-18 (Krause, Fivaz et al. 1998), which are also observed during aerolysin intoxication.

Table 6. The Main Characteristics of Aerolysin-Like Toxin Family Members. (Knapp 2010)

	Aerolysin (*Aeromonas species*)	Septicum-α-Toxin (*Clostridium Septicum*)	Enterolobin (*Enterolobium Contortisiliquum*)	Epsilon-Toxin Type B and D (*Clostridium Perfringens*)	LSL (*Laetiporus Sulphureus*)	MTXs (Mtx2/Mtx 3) *Bacillus Sphaericus*	Hydralysins (*Chlorohydra Viridis*)	Parasporin-2 Bacillus Thuringiensis
Size in kDA: (monomer unprocessed)	52	47.5	55	32.5	35	31.8/35.8	27	37
Propeptide:	yes, N-terminal	yes, C-terminal	unknown	yes, N- and C-terminal	unknown	possibly	unknown	Yes, N- and C-terminal
Pore formation:	yes	yes	possibly	yes	possibly	possibly	yes	possibly
Oligomerization (number of monomers):	7	6,7	unknown	7	4,6	unknown	possibly	unknown
Channel conductance G [pS]:								
0.1 M KCl	72	175	n.m	60	n.m	n.m	n.m	n.m
0.3 M "	220	450	n.m	160	n.m	n.m	n.m	n.m
1 M "	650	1250	n.m	550	n.m	n.m	n.m	n.m
3 M "	1700	3750	n.m	1700	n.m	n.m	n.m	n.m
P_{cation}/P_{anion}:	0.21 (-24 mV) anion selective	0.38 (-14 mV) anion selective	n.m	0.3 (-19 mV) anion selective	n.m	n.m	n.m	n.m
Pore size:	0.7 nm	1.3-1.6 nm	unknown	1 nm	>3.8 nm	unknown	1.2 nm	unknown
Receptor	GPI-anchored proteins	GPI-anchored proteins, including human folate receptor (hFR), Thy-1 (CD90), SAG family	unknown	membrane protein, not GPI-anchored	unknown, contains N-acetyllactosamine glycoconjugates	unknown	unknown	unknown
Target cells	GPI-anchored protein expressing cells	GPI-anchored protein expressing cells	arthropod and mammalian cells	only a few cell lines including kidney cells (MDCK, mpkCCD$_{C14}$, human renal leiomyoblastoma, and G-402 cells) and glutamatergic neurons	unknown	mainly insect cells, larvae of the genus *Culex*, *Aedes*, and *Anopheles*	arthropod and eukaryotic cells	Liver and colon cancer cells and cell lines (HepG2, CACO-2) also human leukemia T-cell lines (Molt-4, Jurkat, Hl-60)

n. m. Means not Measured.
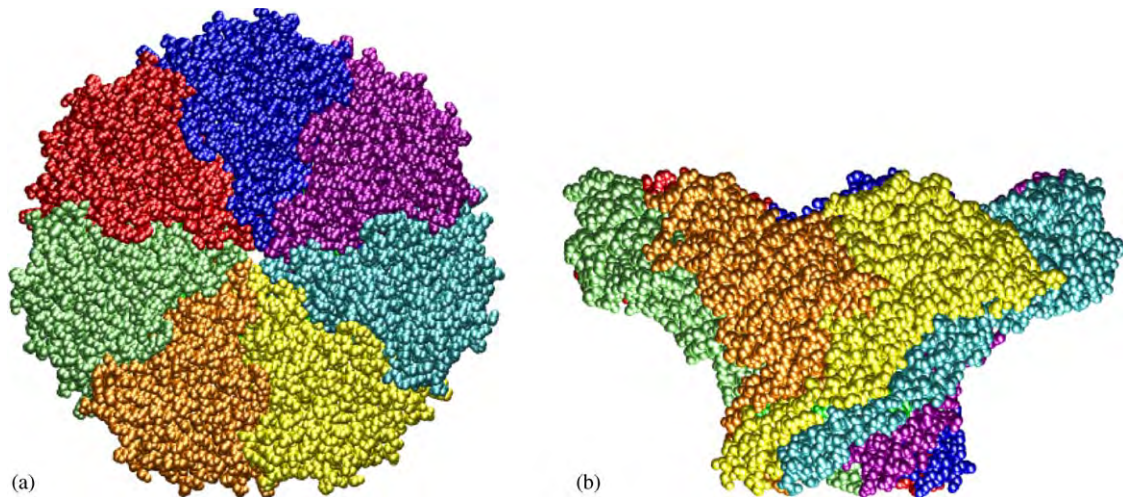
Figure 38. A Model of the Aerolysin Pore. The aerolysin heptamer modeled from electron microscopy images and the crystal structure of the water-soluble dimer form (Tsitrin, Morton et al. 2002). Each monomer is shown in a different color. (a) View down heptameric pore towards membrane plane. (b) Side view of heptamer. (Figures courtesy of Dr Craig Morton). (Parker and Feil 2005)

The Potent Epsilon Toxin: structure and function

As previously mentioned, ETX is part of the aerolysin family of β-PTFs and is the third most toxic molecule known to man, following only Clostridium *botulinum* and *tetanus* toxins. ETX has a minimum recorded LD_{50} of 50-65 ng/kg in mice (Bokori-Brown, Savva et al. 2011), but is commonly listed as has having an LD_{50} of 100ng/kg . While sharing many similarities with aerolysin, there are several important differences worth mention.

Similarities to aerolysin: Although not sharing a high degree sequence identity (13.9%) with aerolysin, the two molecules are organized in much the same way structurally (Figure 39). The majority of these two molecules is comprised of beta sheets, with an N terminal domain that is alpha helical. This helical domain, domain I in ETX and domain II in aerolysin, is the putative binding domain as it is rich in aromatic residues, which have been shown to be important for receptor binding in both molecules. Domain II of aerolysin is thought to be important for interacting with GPI anchors (MacKenzie, Hirama et al. 1999). There is a cluster of aromatic residues in this region, which is observed for other sugar-binding proteins. A similar region with a cluster of aromatic residues (Tyr49, Tyr43, Tyr42, Tyr209 and Phe212) is present in domain I of ETX, along with the only tryptophan, which has been previously implicated in receptor binding (Nagahama and Sakurai 1992). Interestingly, mutation of each of the above ETX tyrosine residues (Y to E) significantly impairs receptor binding (Ivie and McClain 2012). Both molecules form a heptameric, anion selective pore, oligomerization seemingly inhibited by the C terminus of the precursor molecule. It is likely that the C terminus of each protein sterically hinders oligomerization of both toxins and their removal likely reveals residues of importance for oligomerization (Cole, Gibert et al. 2004).
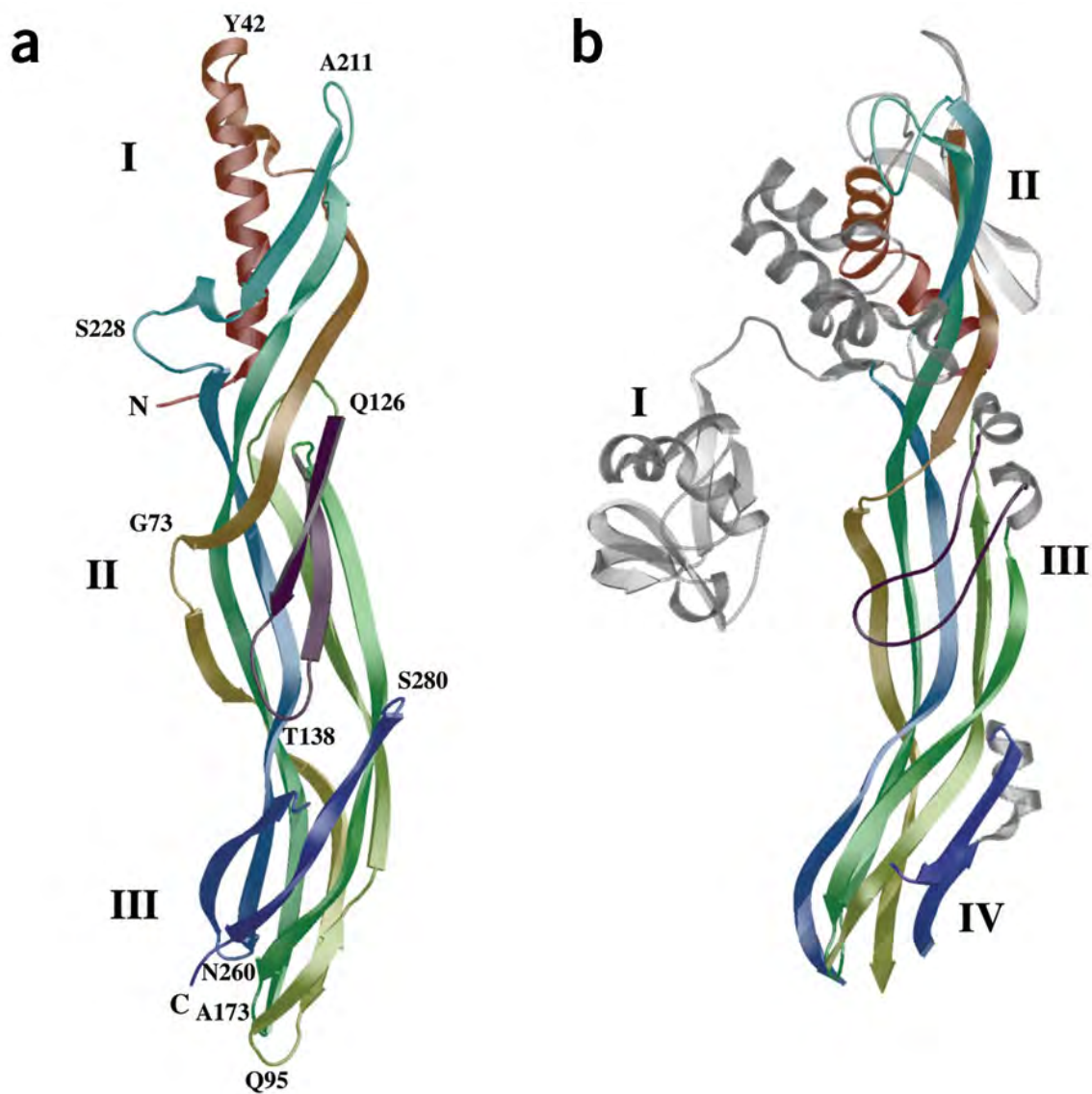
Figure 39. Structures of ETX and Aerolysin with Domains Labeled. (a,b)
Ribbon diagrams of ETX (a) and aerolysin (b) colored from red at the N terminus
to blue at the C terminus. Extra helices and loops in aerolysin are gray and the
region possibly inserted into the membrane is highlighted in purple. The
coordinates of ETX have been deposited in the Protein Data Bank (accession code
1UYJ). (Cole, Gibert et al. 2004)

Differences with aerolysin: While aerolysin circulates as a dimer in solution, ETX exists as monomer. Furthermore, furin activation of aerolysin occurs after the molecule has made contact with the cell membrane. In contrast, ETX is activated in the lumen of the gut by trypsin, chymotrypsin or Clostridium lambda toxin before reaching target cells such as brain endothelium. Aerolysin causes apoptosis as does ETX, however, ETX can also induce necrosis. Most importantly, unlike aerolysin, ETX fails to bind to GPI-liked proteins in lipid rafts and is thus non-hemolytic. Although the putative ETX receptor has been shown to localize to lipid rafts, its identity remains unknown.

Another important difference with aerolysin is ETX's ability to form a prepore intermediate before membrane insertion. In this way, ETX is the rule while aerolysin is the exception, as most β-PTFs form prepores (Parker 2010). The prepore comprises a ring of subunits on the surface of the membrane that contains largely unperturbed monomers. By contrast, the subunits in the pore have undergone a striking reorganization, resulting in a structure with several unusual features, including a hollow rim, vertical β-strands and a locally distorted lipid bilayer (Bayley, Jayasinghe et al. 2005). Robertson et al. found that at 4 degrees C, treating MDCK cells with ETX led to the formation of a non-toxic heptameric prepore. If the treated cells were then moved to 37C, the prepore was able to insert into the membrane and lyse the cells (Figure 40). To evince membrane insertion, Robertson treated both the 4C and 37C preparations with pronase, and only observed pronase degradation of the ETX pore in the 4C circumstance. The investigators concluded that at 4C, pronase can access the

ETX prepore, however, pronase cannot access the pore once it has inserted into the membrane at 37C (Robertson, Li et al. 2011). Although low temperatures can inhibit membrane insertion of the ETX prepore, it is interesting that the toxin-receptor interaction is unaffected.
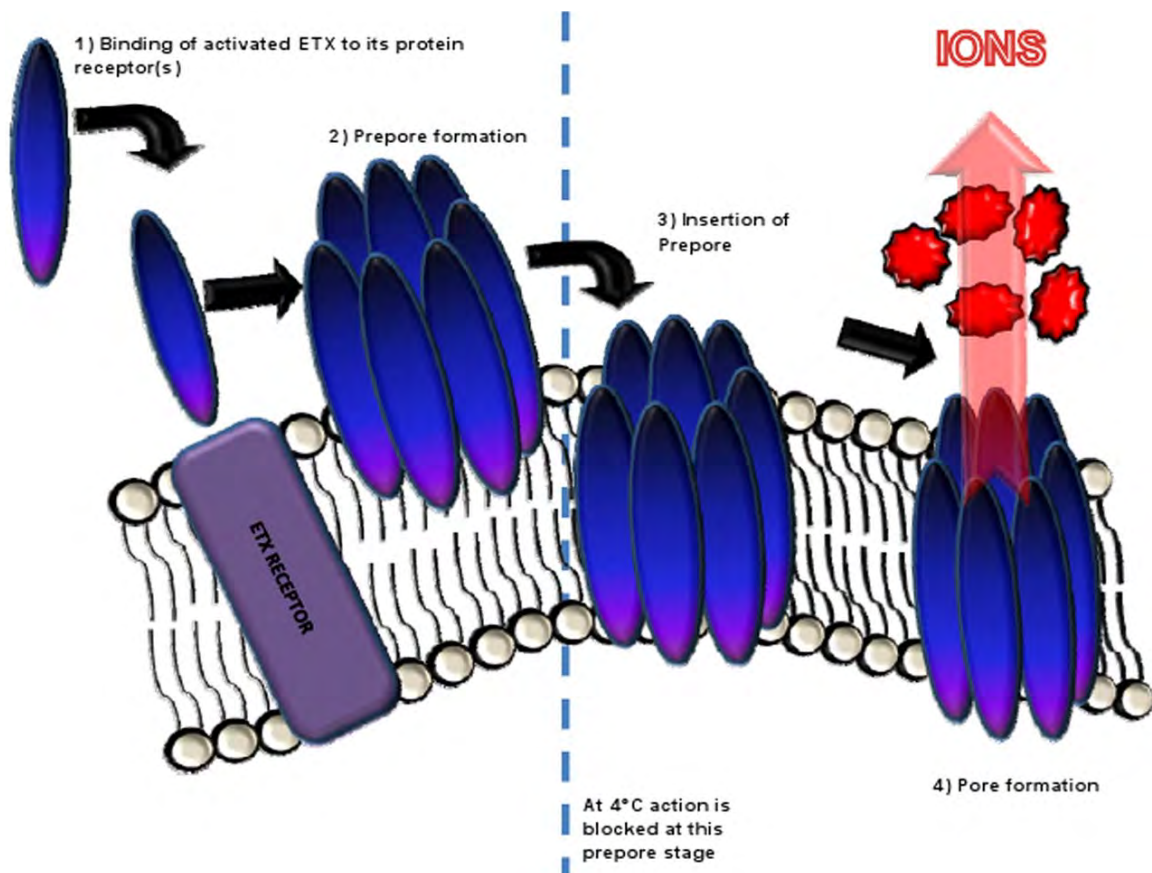
Figure 40. A Schematic of ETX Pore Formation. Mode of action begins with activated ETX binding to proteinacious membrane receptor. Bound ETX then oligomerizes to form a heptameric prepore on the membrane surface. At 4C, the process stops here, but at 37C the prepore intermediate inserts into the membrane to complete pore formation. (Robertson, Li et al. 2011)

Knapp et al. have demonstrated that an amino acid stretch in domain II of ETX, position 151-180, is critical for channel formation. Mutations along this region can impair pore formation and even change pore characteristics from anion selectivity to cation selectivity (Knapp, Maier et al. 2009). Surprisingly, this change in ion selectivity has minimal impact on ATP depletion and ETX toxicity, suggesting that the influx of Na^+ and Ca^{2+} are not significantly involved in ETX mediated cell death.

It seems that ETX toxicity may not be entirely due to pore formation and reduction in membrane potential. Chassin et al. have demonstrated that disruption of lipid rafts with methyl-β-cyclodextrin inhibits ETX heptamerization and pore formation, but cellular ATP depletion still occurs. There is an apopotosis-like signaling cascade that involves stimulation of the AMP-activated protein kinase, induction of mitochondrial membrane permeabilization and mitochondrial nuclear translocation of apoptosis-inducing factor (AIF), which is a potent caspase-independent cell death effector. ETX can also lead to cellular necrosis evidenced by nuclear shrinkage without DNA fragmentation (Chassin, Bens et al. 2007).

Contrastingly, Fennessey et al. present a model of ATP depletion that argues for the importance of pore formation. By their model, ETX pores allow the escape of essential coenzymes that are essential for ATP production (CoA, and its precursor, pantothenate, NAD+ and NADH, and carnitine). This leads to a dissipation of mitochondrial membrane potential and release of mitochondrial apoptosis mediators such as AIF. The same mechanism seems to be true for aerolysin mediated apoptosis (Fennessey, Ivie et al. 2012).

Efforts have been made to generate non-toxic ETX mutants, which can serve as antigens for vaccination or dominant negatives. Mutations of key amino acids have been identified. Oyston et al. showed that mutating histidine 106 to proline, complete abolishes ETX toxicity without destabilizing the molecule (Oyston, Payne et al. 1998). The exact mechanism by which this mutation renders ETX non-toxic remains unknown. These investigators hope to develop the H106P mutant as a cost effective recombinant vaccine.

Dominant negatives have also been explored. ETX-I51C/A114C and ETX-V56C/F118C have been identified as two mutants that lack observable cytotoxicity. The ability to reconstitute the cytotoxic activity of the ETX-I51C/A114C and ETX-V56C/F118C mutant proteins by treatment with DTT suggests that the cysteine substitutions per se are not responsible for the lack of cytotoxicity. Rather, it is likely that disulfide bonds formed between the introduced cysteines are responsible for the lack of cytotoxicity. These results are consistent with the hypothesis that the membrane insertion domain of the ETX undergoes a structural transition in the process of intoxicating host cells. Furthermore, insertion of a disulfide bond between an amino acid within the membrane insertion domain and an amino acid in the protein backbone blocks the structural transition required for ETX mediated cytotoxicity (Pelish and McClain 2009).

Finally, neutralizing antibodies have also been developed, identifying a region of ETX that seems to be critical for membrane insertion. Both 4D7 and 5B7 monoclonals target the amino acid stretch 134 to 145 (SFANTNTNTNSK) (McClain and Cover 2007). These amino acids map to the β hairpin, amphipathic

loop of ETX (Figure 41) that has been proposed to oligomerize and insert into the cell membrane.
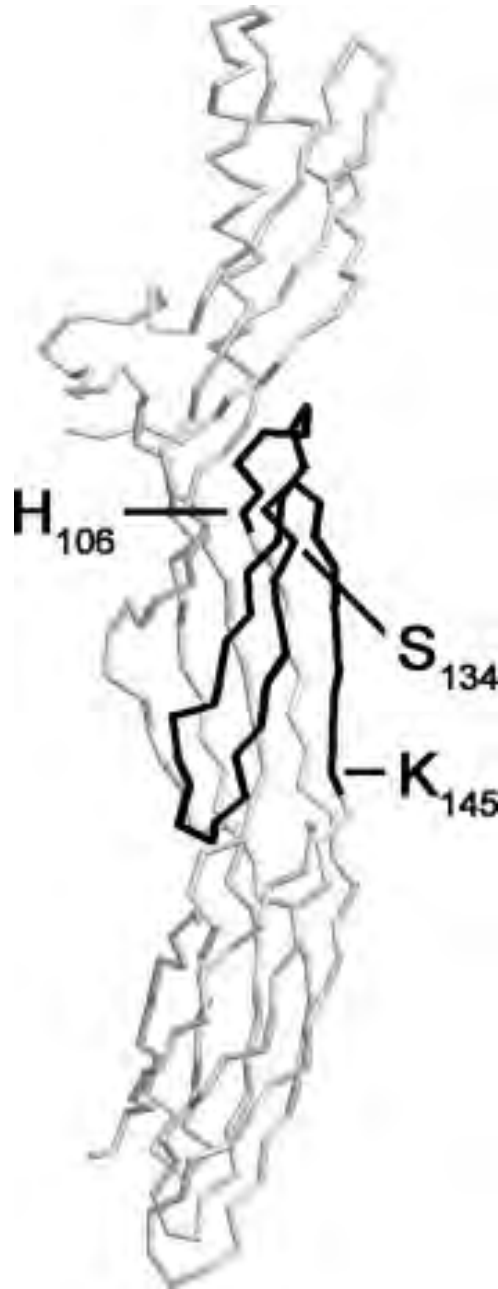
Figure 41. Mapping of the Neutralizing Epitope. The structure of the epsilon-toxin is shown. The region from the histidine at amino acid position 106 through the predicted membrane-inserting loop (amino acids 111 to 139) to the epitope recognized by neutralizing monoclonal antibodies 4D7 and 5B7 (amino acids 134 to 145) is highlighted. (McClain and Cover 2007)

The Elusive ETX Receptor

Despite its importance, the mammalian receptor for ETX remains unknown. However, it has been shown that ETX targets a remarkably small number of tissues and cell types; namely renal cell lines (canine MDCK, human ACHN, human G-402 and murine mpkCCDc14 cells), brain endothelial cells, the distal tubule of the kidney/renal collecting ducts, granule cells of the cerebellum, CNS myelin and PNS myelin (Popoff 2011) and peritoneal macrophages harvested from guinea pigs and rabbits (Buxton 1978). In addition to being expressed by an exclusive list of cell types, the ETX receptor has also been shown to localize to Detergent Resistant Membranes or lipid rafts (Miyata 2002).

Although causing cell death in the majority of cell types studied, in some instances, there seems to be greater nuance to the target cell-ETX interaction. In cerebellar granule cells, ETX exposure does not lead to cell death, but rather results in glutamate release. Using both primary cell culture and cerebellar slice culture methods, Lonchamp et al. found that ETX bound to the soma of the cerebellar granule cells and depolarized these neurons. The drop in membrane potential, presumably by pore formation, triggered Ca^{2+} influx and glutamatergic synaptic release (Lonchamp, Dupont et al. 2010). Interestingly, the authors failed to observe cytotoxicity in the cerebellar slices, but observed late/delayed cytotoxicity in granule cell primary cultures.

 Other nuanced ETX interactions become clear when studying its effects on macrophages and lymphocytes from different species. Buxton harvested immune cells from mice, rabbits, guinea pigs and sheep and observed heterogeneous ETX cytotoxicity (Table 7).

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Table 7. The In Vitro Effects of ETX on Guinea Pig, Mouse, Rabbit and Sheep Cells. (Buxton 1978)

Animal species	Type of cell	Number of animals	Incubation time (min.)	Toxin (μg per ml)	Percentage of cells killed
Guinea pig	Peritoneal macrophages	5	30	0·001	3
	Peritoneal macrophages	5	30	0·01	4
	Peritoneal macrophages	5	30	0·1	5
	Peritoneal macrophages	5	30	1·0	33
	Peritoneal macrophages	5	30	10·0	79·5
	Pulmonary alveolar macrophages	2	180	20	0
	Lymphocytes	1	30	20	0
	Lymphocytes	1	60	20	0
Mouse	Peritoneal macrophages	2	30	20	0
	Peritoneal macrophages	3	165	20	0
	Lan Schultz ascites-tumour cells	1	150	20	0
	Lan Schultz ascites-tumour cells	1	180	20	0
Rabbit	Peritoneal macrophages	2	30	20	11·2
	Peritoneal macrophages	2	60	20	26·8
	Peritoneal macrophages	3	90	20	40·3
	Peritoneal macrophages	1	150	20	45·5
	Pulmonary alveolar macrophages	2	150	20	0
Sheep	Lymphocytes	1	30	20	0

There is a surprising intraspecies heterogeneity in ETX toxicity as peritoneal macrophages from different individuals of the same species show different degrees of susceptibility. Furthermore, only rabbit and guinea pig peritoneal macrophages are affected, pointing to interspecies differences. Finally, the effect seems to be restricted to peritoneal macrophages as pulmonary macrophages and lymphocytes seem to remain unaffected.

Once ETX encounters a susceptible cell type, it has been reported to localize to detergent resistant membranes or lipid rafts. Miyata et al. showed that radiolabeled ETX associated with lipid rafts that were isolated from MDCK

membranes using cold Triton 100X extraction and sucrose gradient separation (Miyata 2002).

From enzymatic digestion studies of ETX susceptible membranes, investigators have found that ETX binding is sensitive to pronase, sialidase and is completely abolished by lipase (Dorca-Arévalo, Soler-Jover et al. 2008), suggesting that the ETX receptor maybe a glycoprotein residing in a lipid environment that facilitates binding. An alternate and unexplored hypothesis is that the receptor may not be a glycoprotein, but rather an unmodified protein that simply resides in a carbohydrate and lipid rich environment. The lipid raft environment contains a high density of glycosphingolipids and fits this description.

Dorca-Areᶥvalo et al. demonstrate the importance of carbohydrate residues in ETX binding by showing that beta-elimination of O-liked carbohydrate groups abolishes ETX binding while removal of N-linked sugars with N-glycosidase F has no effect. They also show that pronase and detergent treatments also mitigate binding, leading to the conclusion that the receptor may be an O-linked glycoprotein residing in a lipid environment that is conducive to ETX binding (Dorca-Arevalo, Martin-Satue et al. 2012). One oversight in this hypothesis is that the carbohydrate moieties of lipid raft glycosphingolipids are also O-linked. Therefore beta-elimination may not be acting on a glycoprotein, but may instead target glycosphingolipids that are crucial for ETX to bind to its unmodified protein receptor that merely sits in the lipid raft.

Mutation of the putative ETX binding domain has revealed that aromatic amino acids are critical for binding. This has reinforced the idea that ETX, at

least in part, binds to sugar moieties because similar interactions have been observed for aerolysin and other sugar binding proteins (Cole, Gibert et al. 2004). Ivie et al. generated four ETX mutants, ETX-Y29E, ETX-Y30, ETX-Y36E and ETX-Y196E, all of which showed defects in ETX binding (Ivie and McClain 2012).

To date, only one molecule has been proposed to directly interact with ETX. Bruggemann et al. used gene-trap mutagenesis to identify mammalian proteins that modulate ETX toxicity. They identified, hepatitis A virus cellular receptor 1 (HAVCR1) and showed that ETX can bind to HAVCR1 by Western blot. However, when transfected into HEK293 cells, an ETX resistant cell line, HAVCR1 failed to induce ETX binding and/or confer ETX susceptibility (Bruggemann, Ivie et al. 2011). Although a provocative candidate receptor, this lack in vivo functionality suggests that a better candidate receptor may exist.

CHAPTER 4

Is Myelin and Lymphocyte Protein (MAL) a Functional ETX Receptor?

As a preliminary step toward identifying a candidate ETX receptor, we searched the literature for lipid raft associated proteins that are expressed by multiple ETX susceptible cell types and tissues e.g. oligodendrocytes and distal tubule of the kidney. Surprisingly, only one protein fit each of these specifications, Myelin and Lymphocyte protein (MAL). Because ETX targets the BBB and spares peripheral vasculature, we hypothesized that MAL may be enriched at the BBB. To test this idea we contacted Dr. Richard Daneman, a BBB investigator at UCSF. Dr. Daneman has performed a transcriptome comparison of BBB endothelial cells vs. lung and liver endothelial cells to determine BBB specific gene expression. Furthermore, upon our request, Dr. Daneman was able to select genes shared by BBB endothelium and myelin, as he was a member of Ben Barres' lab (Stanford University), which has performed similar transcriptome comparisons for glial cell (astrocyte and oligodendrocyte) specific gene expression. Below are the BBB specific genes that are also shared by the oligodendrocyte-myelin unit:

1. Mfsd2
2. Ddc
3. Prom1
4. **MAL**
5. Elovl7
6. Bche
7. Slc1a1
8. Dixdc1
9. Gstm7
10. Apod

11. Chn2
12. Mpzl1
13. Tbc1d14
14. Osbpl1a
15. Cobll1
16. RGC-32h
17. Acsl3
18. Cdc216
19. 5133401H06Rik

Indeed, MAL was on this short list of genes and was one of the few hits that trafficked to the membrane, which would be necessary to interact with ETX. Dr. Daneman also provided the ratios of BBB MAL expression to both lung and liver endothelial expression; 50x Brain/Lung and 7x Brain/Liver (personal communication).

A review of Myelin and Lymphocyte Protein (MAL)

MAL is a highly hydrophobic, 17kDa, tetraspan, membrane proteolipid that is expressed by myelin, MDCK cells, and the distal tubule of the kidney all of which have been shown to bind ETX. MAL is also found in human T lymphocytes, for which no association with ETX has ever been demonstrated, and a variety of epithelial cell types. This molecule contains two extracellular loops (ECLs), one of which has a high density of aromatic amino acid residues (Figure 42) much like the putative ETX binding domain. It is conceivable that an aromatic-aromatic pi stacking or T stacking interaction may facilitate a MAL-ETX binding event.
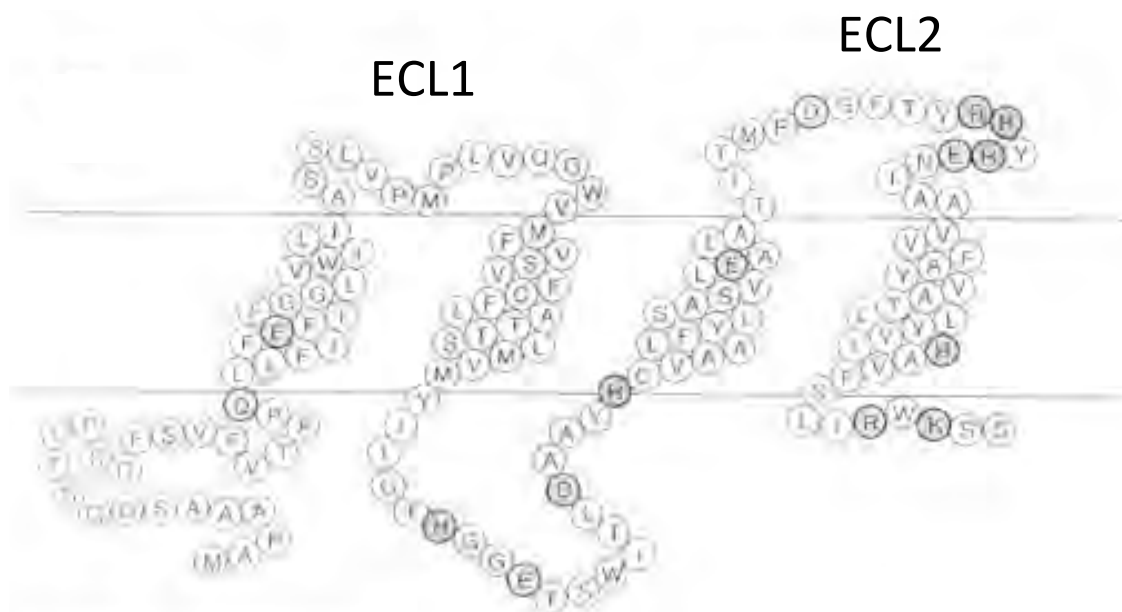
Figure 42. A Schematic of Full Length MAL. The presumed orientation of rat MAL within the plasma membrane. The primary structure is shown with charged amino acids colored in gray. Please note the high density of aromatic residues in ECL2, which may interact with the aromatic residues found in the ETX binding domain. (Schaeren-Wiemers, Valenzuela et al. 1995)

Dr. Miguel Alonso discovered MAL in human T cells in 1987 (Alonso and Weissman 1987). Initially thought to have channel like properties, further experiments showed that the MAL tetraspan was not a channel but associated with molecules critical to formation of the immunological synapse (IS) and subsequent T cell activation. Co-immunoprecipitation experiments showed that MAL associated with Lck, a Src family kinase, and GPI-linked CD59. It was later shown that MAL is necessary for the trafficking of Lck to the IS. (Millan and Alonso 1998; Anton, Batista et al. 2008). Alonso et al. showed that human

peripheral blood T cells, thymocytes and various T cell leukemia cell lines such as Jurkat, HPB-ALL and peer cell lines express MAL (Rancano, Rubio et al. 1994; Millan and Alonso 1998). He also showed that MAL could be expressed as four different isoforms, representing every combinatorial scenario for the presence and/or absence of ECLs 1 and 2 (Figure 43).
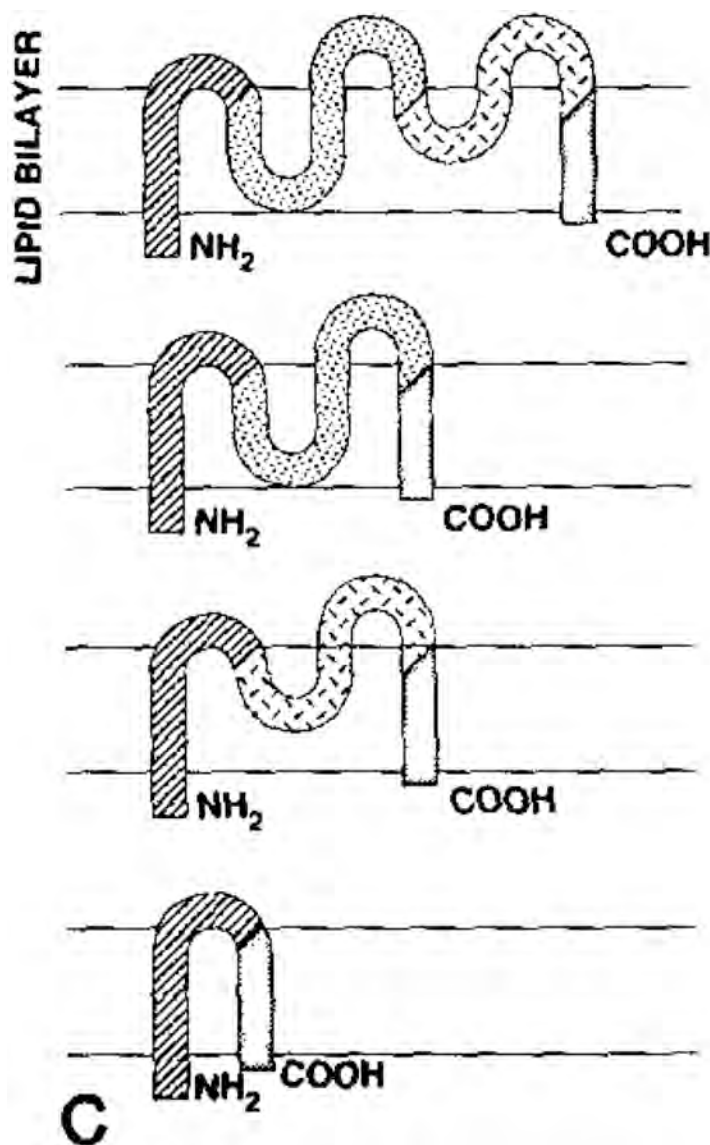
Figure 43. The Proposed Structure of the MAL Isoforms. Please note that the most N-terminal region (diagonal hash marks) is thought to reside completely within the plasma membrane and is not an ECL. (Rancano, Rubio et al. 1994)

Interestingly, mutation of the ECL2 results in MAL mislocalization within the IS, from the central IS to the peripheral IS. MAL mislocalization results in aberrant targeting of virtually all IS adapter proteins from the central IS to the peripheral IS (Figure 44). However, the T cell receptor (TCR) is still able to correctly traffic to the central IS (Anton, Andres-Delgado et al. 2011).
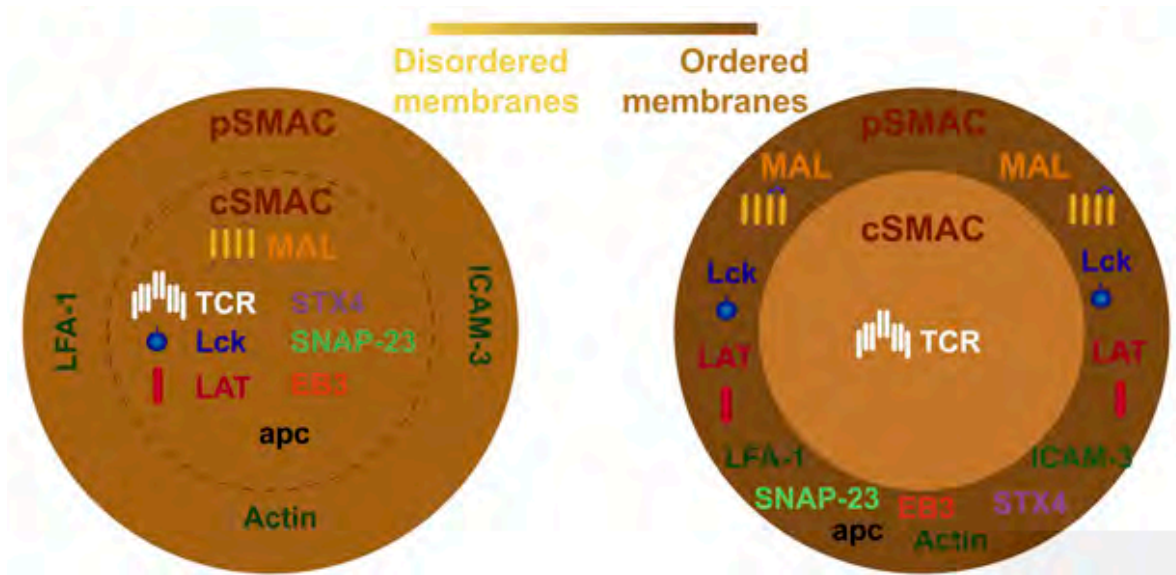
Figure 44. Schematics of MAL in the assembly of the IS (or Supramolecular Activation Cluster, SMAC). MAL is normally sorted to the central IS, where it colocalizes with highly ordered membranes. MAL mediates vesicular transport of Lck from pericentriolar endosomes directly to the central IS using microtubule tracks docked at the central IS. LAT, however, translocates to the central IS mainly from a plasma membrane pool. Mislocalization of MAL to the peripheral IS produces loss of highly ordered membranes from the central IS, increased membrane condensation at the peripheral IS, and missorting of Lck, LAT, and machinery for microtubule or vesicle transport docking to the peripheral IS. As a result of these changes, Lck is transported directly to the peripheral IS instead of to the central IS. (Anton, Andres-Delgado et al. 2011)

The authors conclude that this mistargeting phenotype at the IS may, in part, be due to MAL's ability to maintain highly ordered membranes, or lipid rafts (Anton, Andres-Delgado et al. 2011). Indeed, MAL has been shown to be an important player in the formation and maintenance of lipid rafts in T cells, oligodendrocytes and various epithelial cell types.

Robust T cell MAL expression may be limited to humans however, as no expression of MAL transcript was found in the rat thymus and only low levels were detected in the rat spleen. In line with these findings, Northern blots and in situ hybridization showed barely detectable MAL mRNA levels in the rat spleen, while protein was beyond detectable limits (Frank, van der Haar et al. 1998).

In the brain, MAL has been shown to be necessary for the maintenance, but not the initial formation of paranodal loops in CNS myelin. Dr. Nicole Schaeren-Wiemers developed and characterized the MAL KO mouse. Genetic ablation of MAL resulted in cytoplasmic inclusions within compact myelin, paranodal loops that were everted away from the axon, and disorganized transverse bands at the paranode-axon interface (Schaeren-Wiemers 2004). Schaeren Wiemers et al. hypothesized that this phenotype was the result of aberrant trafficking of oligodendrocyte membrane proteins such as neurofascin 155 and other paranodal proteins. Despite these myelin abnormalities, MAL KO mice show no signs of disability.

Nonetheless, most of the experimental MAL data have come from exploring MAL's role in epithelial cell function. A variety of tissues express MAL and expression is primarily of epithelial origin (Table 8). Absorptive cells (e.g. enterocytes), and many different types of specialized secretory cells, either organized in discrete clusters (e.g. endocrine cells in the pancreas), gathered

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together in an endocrine gland (e.g. thyroid), interspersed with other cells in glands (e.g. parietal cells), or dispersed singly among other cells (e.g. type 2 pneumocytes) are positive for MAL (Marazuela, Acevedo et al. 2003).

Table 8. A Summary of the Distribution of MAL in Different Human Tissues (Marazuela, Acevedo et al. 2003)

Tissue/organ	Strong expression	Weak or focal expression	No expression
Common structures	Axons		Endothelial cells
	Mast cells		Fibroblasts
			Muscle cells
Skin	Ductal eccrine cells		Keratinized epithelium
			Keratinocytes
			Hair follicles
			Melanocytes
Esophagus	Stratified squamous epithelium		Muscle cells
			Submucosa
Stomach	Parietal cells	Surface mucous cells	Muscle cells
	Chief cells		Submucosa
Small intestine	Crypt cells	Enterocytes with microvilli	Muscle cells
	Paneth cells		Submucosa
	Lymphocytes (Peyer's patches)		
Large intestine	Mucous cells		Muscle cells
			Submucosa
Liver	Centrilobular hepatocytes	Other hepatocytes	Kupffer cells
		Intrahepatic ductal epithelium	Endothelium
Pancreas	Acinar cells	Ductal cells	
		Endocrine cells	
Kidney	Distal convoluted tubules	Loop of Henle	Glomerulus
	Collecting tubules		Proximal convoluted tubules
			Bowman's capsule
			Juxtaglomerular apparatus
Ureter		Superficial cells from transitional epithelium	Other epithelial cells
Bladder			Muscle cells
Prostate	Ductal and acinar cells		
Lymph node	T-cells		B-cells
	HEV endothelium		Sinusoidal cells
			Dendritic cells
			Macrophages
			Other endothelia
Tonsil	T-cells		B-cells
	HEV endothelium		Sinusoidal cells
	Superficial and crypt epithelium		Macrophages
			Dendritic cells
Thymus	Cortical thymocytes	Medullary thymocytes	Epithelial cells
		Hassall's corpuscles	Dendritic cells
Bronchi and trachea	Goblet cells	Respiratory epithelium	
Lung	Type 2 pneumocytes	Mucous cells	Alveolar lining cells
Thyroid gland		Thymocytes	Parafollicular cells
Testis	Leydig cells	Sertoli cells	Germinal cells
Adrenal gland	Medullary cells	Zona glomerulosa	
	Zona reticularis	Zona fasciculata	

MAL localizes to the apical membrane, and co-purifies with detergent resistant lipid rafts of classical "barrier epithelia" e.g. kidney and stomach. In MDCK cells, MAL localizes to vesicular structures of the trans-Golgi network

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and in transport vesicles. Therefore, MAL is postulated to mainly subserve transport functions. Experimental findings support this suggestion convincingly: transfection of MDCK cells with antisense MAL cDNA strongly reduces the rate of apical transport in these cells. Both velocity and efficiency in the apical raft delivery are decreased and misrouting of apically sorted proteins to the basolateral compartment is observed. In addition, the targeting of other apically sorted proteins, e.g. caveolin, into lipid rafts is disturbed. Velocity and accuracy of the apical transport is also significantly increased in epithelial A498 cells that lack endogenous MAL after transfection with recombinant MAL. In MDCK cells, MAL overexpression leads to increased surface expression of apical proteins and to an apparent increase of the surface area of the apical membrane. These experiments suggest that the amount of MAL present is a rate-limiting component of apical transport in MDCK cells. In essence, the in vitro and in vivo observations show that MAL is an organizer and a stabilizer of glycosphingolipid enriched microdomains in epithelial cells, and it seems that MAL has an important function in sorting and apical transport (Frank 2000).

Finally, Magal et al. have proposed a mechanism for how MAL facilitates lipid raft formation and resultant protein trafficking to membrane microdomains on the apical surface (Figure 45). Site-directed mutagenesis and bimolecular fluorescence complementation analysis demonstrate that MAL forms oligomers via intramembrane protein-protein binding motifs. Furthermore, Magal et al. hypothesize that MAL's association with raft lipids is driven, at least in part, by positive hydrophobic mismatch between the lengths of the transmembrane helices of MAL and surrounding membrane lipids (Magal, Yaffe et al. 2009).

Figure 45. A Proposed Role for MAL in Lipid Raft Formation. Facilitation of protein clustering and domain formation by positive hydrophobic mismatch between MAL and membrane lipids. (a) Scheme demonstrating the possible consequence of positive hydrophobic mismatch between MAL transmembrane domains and the width of the hydrophobic core of the membrane bilayer. (b) Spontaneous MAL oligomerization (red bars) is facilitated during transport between thin-membrane organelles. In the apical membrane, where alleviated mismatch promotes monomer formation, MAL participates in determining membrane thickness. ARC, apical recycling compartment. (Magal, Yaffe et al. 2009)

a

Hydrophobic mismatching

Bending

Lateral segregation   Clustering

Domain formation

b

Membrane thickness anchoring

Apical

ARC

Basolateral

Mismatch

Membrane domain

Golgi

ER

MAL oligomers
Mismatched membrane
Matched membrane
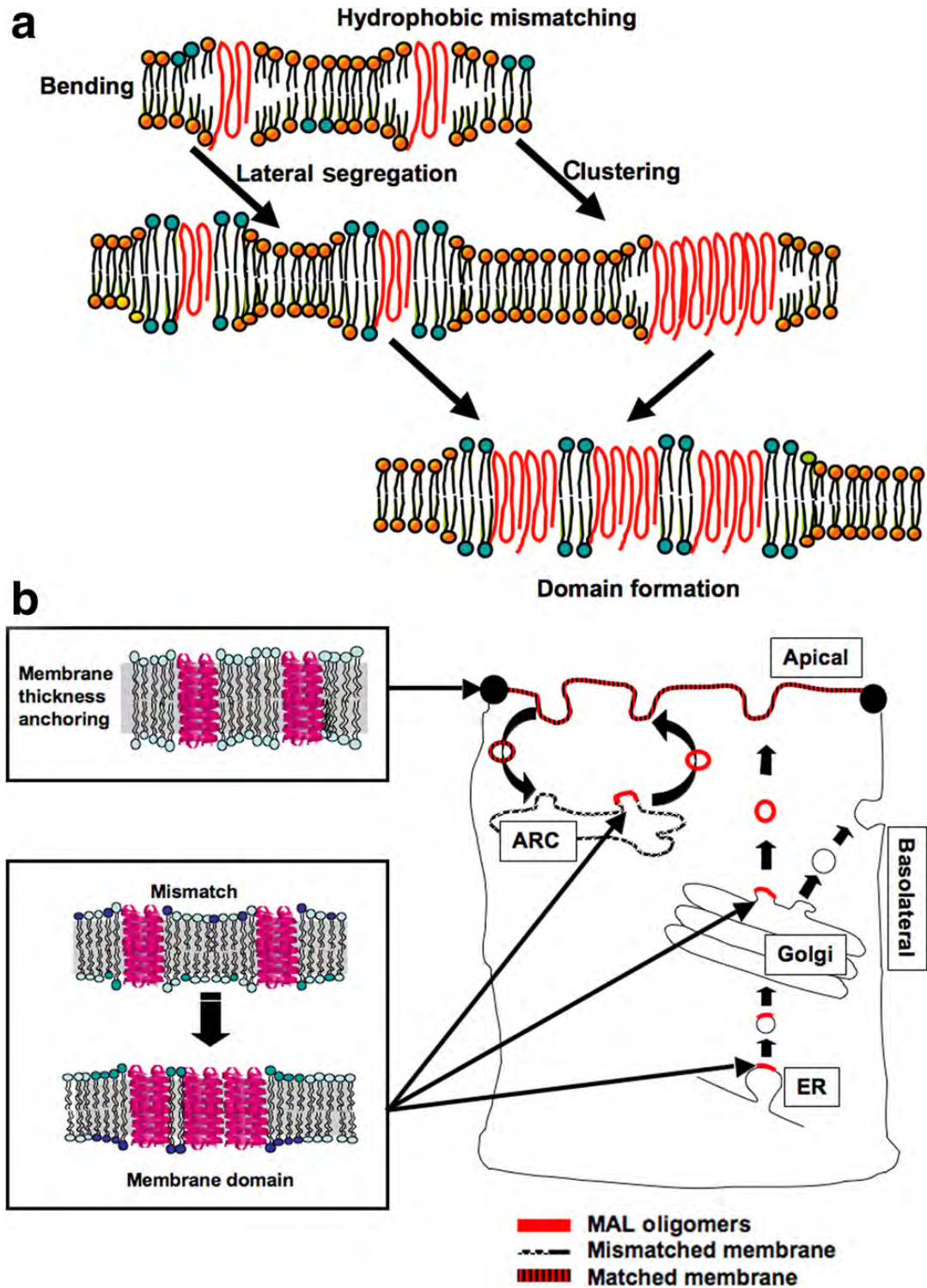
MAL's apical/lumenal distribution, ability to self oligomerize and high density of aromatic residues in its second extracellular loop make it an attractive candidate as an ETX receptor. As a barrier disruptor, ETX will likely target a molecule that is localized to the apical/lumenal surface. Furthermore, as ETX needs to heptamerize to form the pore complex, binding to a molecule such as MAL that naturally exists in an oligomeric state would easily concentrate the toxin and facilitate pore formation. Lastly, the aromatic residues of the ETX binding domain may be able to form pi or T stacking systems of electron delocalization with the aromatic residues in MAL's second extracellular loop.

MAL is a bone fide ETX receptor

How the potent neurotoxin, Clostridium *perfringens* epsilon toxin (ETX), targets mammalian cells remains unknown. We have identified the tetraspan proteolipid, myelin and lymphocyte protein (MAL), as an ETX receptor. Exogenous MAL expression renders ETX resistant CHO cells capable of ETX binding and confers ETX susceptibility. Insertion of FLAG sequences into either the first or second extracellular loops of MAL abolishes ETX mediated toxicity. ETX fails to stain tissues harvested from MAL knockout mice despite robustly staining tissues from wild type mice. Finally, MAL knockout mice exhibit resistance to ETX doses in excess of 1000 times our experimentally determined LD_{50}.

Expt 1. Is MAL sufficient to confer ETX binding and sensitivity?

RESULTS

ETX binds to and kills MAL expressing cells: We stably transfected ETX resistant Chinese Hamster Ovary (CHO) cells with a Green Fluorescent Protein rat MAL fusion protein construct (CHO-MAL), and found that these cells were able to bind Alexa 594 labeled ETX, while CHO cells transfected with Green Fluorescent Protein alone (CHO-GFP) remained unable to bind the toxin (Fig. 46A). This finding was further confirmed and quantified by assessing Alexa 647 labeled ETX binding to these two stable transfectants by Flow Cytometry. ETX showed robust binding to CHO-MAL cells, while failing to bind to CHO-GFP controls (Fig. 46B).

Figure 46. MAL Is Sufficient to Confer ETX Binding. A) EGFP-MAL transfected CHO cells (top) and GFP transfected CHO (bottom) stained with Hoechst and ETX-594. CHO-MAL cells stain positive for ETX-594, while CHO-GFP cells do not. Furthermore, ETX-594 colocalizes with the GFP signal in CHO-MAL cells. B) FACS analysis of ETX-647 binding shows that CHO-MAL cells become double positive for GFP and ETX-647 when incubated with toxin (top). However, CHO-GFP cells remain positive for GFP only, after incubation with ETX-647 (bottom).

A)

B)

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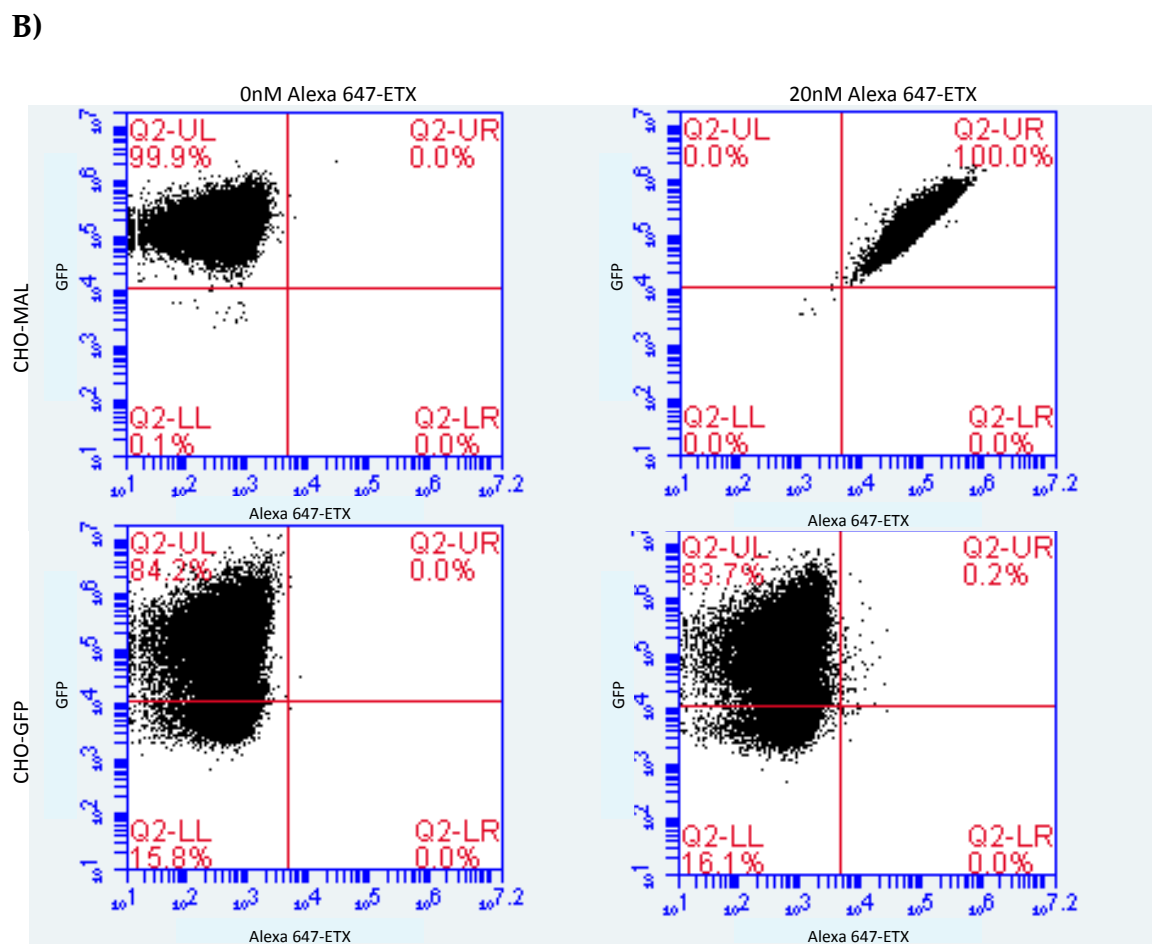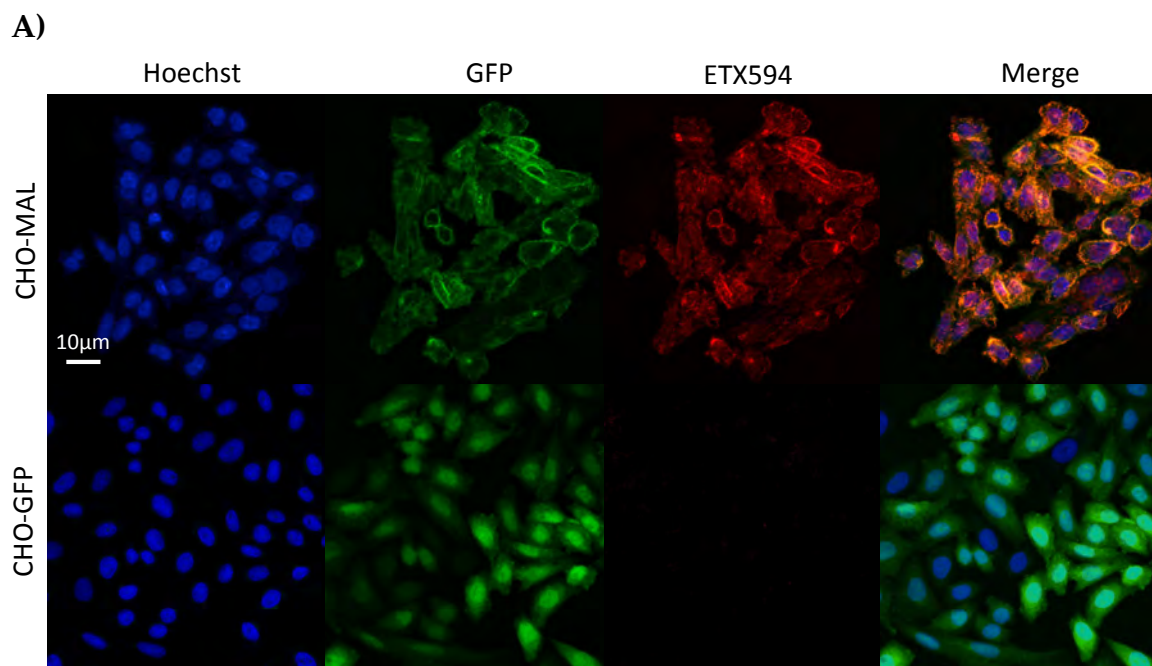
To determine if alterations to the primary structure of MAL would affect ETX binding, we transiently transfected CHO cells with different versions of the MAL tetraspan and incubated these cells with Alexa 647 labeled ETX. Flow Cytometry analysis showed that ETX binds to EGFP rat MAL and EGFP human MAL fusions, but fails to bind to EGFP human MAL with FLAG sequence insertions in either the first or second extracellular loops. Additionally, ETX fails to bind to the EGFP zebra fish MAL orthologue and to the GFP control (Fig. 47). It should be noted that while ETX bound both human and rat MAL, it displayed a ten fold increased affinity for rat MAL when compared to the human orthologue. Although HAVCR1 has been proposed as an ETX receptor, HEK293 cells, which are HAVCR1 deficient (Bruggemann, Ivie et al. 2011), are still able to bind ETX after rat and human MAL transfection despite lacking HAVCR1 (Fig. 48); thus HAVCR1 is not necessary for MAL mediated ETX binding.
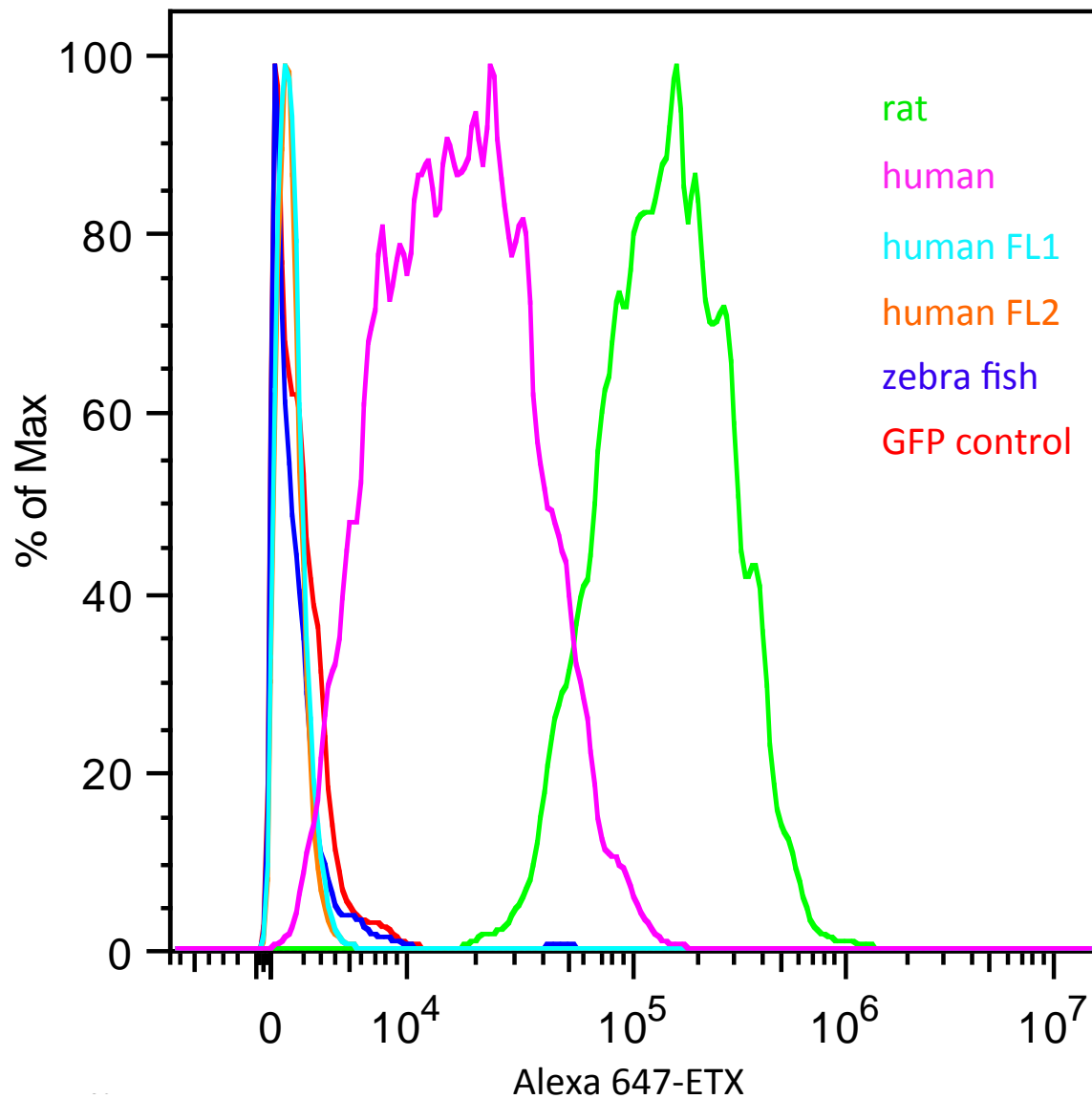
Figure 47. A FACS Histogram of ETX Binding to MAL. ETX-647 binds to rat MAL (green) and to human MAL (magenta). ETX-647 fails to bind to human MAL with a FLAG insertion in ECL1 (light blue), human MAL with a FLAG insertion in ECL2 (orange), zebra fish MAL (dark blue) and GFP alone (red).
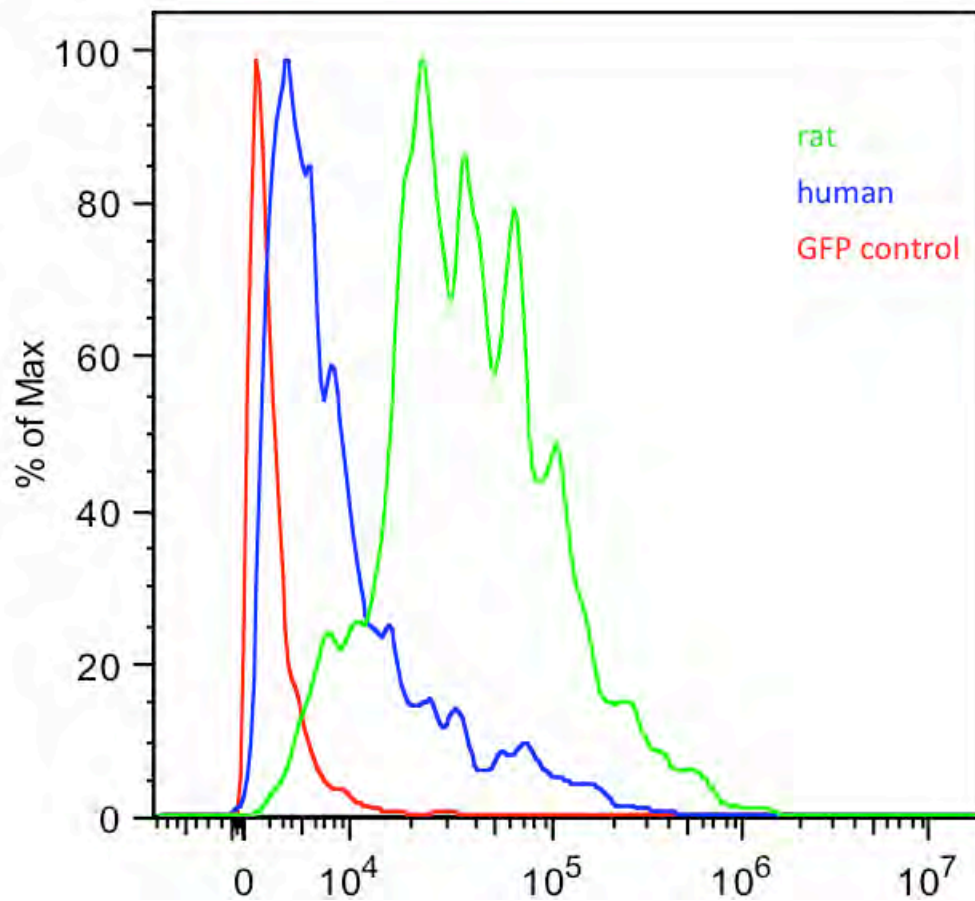
Figure 48. A FACS histogram of ETX binding to MAL transfected HAVCR1 Deficient HEK293 Cells. ETX-647 binds to both rat and human MAL transfected HAVCR1 deficient HEK 293 cells, but not to the GFP control.

ETX is a 31 kilodalton exotoxin that binds to target membranes, heptamerizes, generating a pore that allows the influx of ions and water, leading to osmotic cell death (Popoff 2011). To confirm the formation of a functional ETX pore, we assessed cell membrane permeability to propidium iodide (PI) by fluorescence microscopy. We treated CHO-MAL and CHO-GFP cells with ETX (500pM) and monitored PI uptake over a 24-hour period. By the 4-hour time point, we observed robust PI uptake in the CHO-MAL, but not the CHO-GFP controls (Fig. 49). After 24 hours, significant PI uptake remained undetectable in the CHO-GFP controls. We further determined that ETX kills MAL transfected cells in both a dose (Fig. 50A) and time dependent fashion (Fig. 50B) based on a presto-blue cell viability assay (Invitrogen), which assesses the ability of cells to reduce non-fluorescent resazurin to the fluorescent metabolite, resorufin.
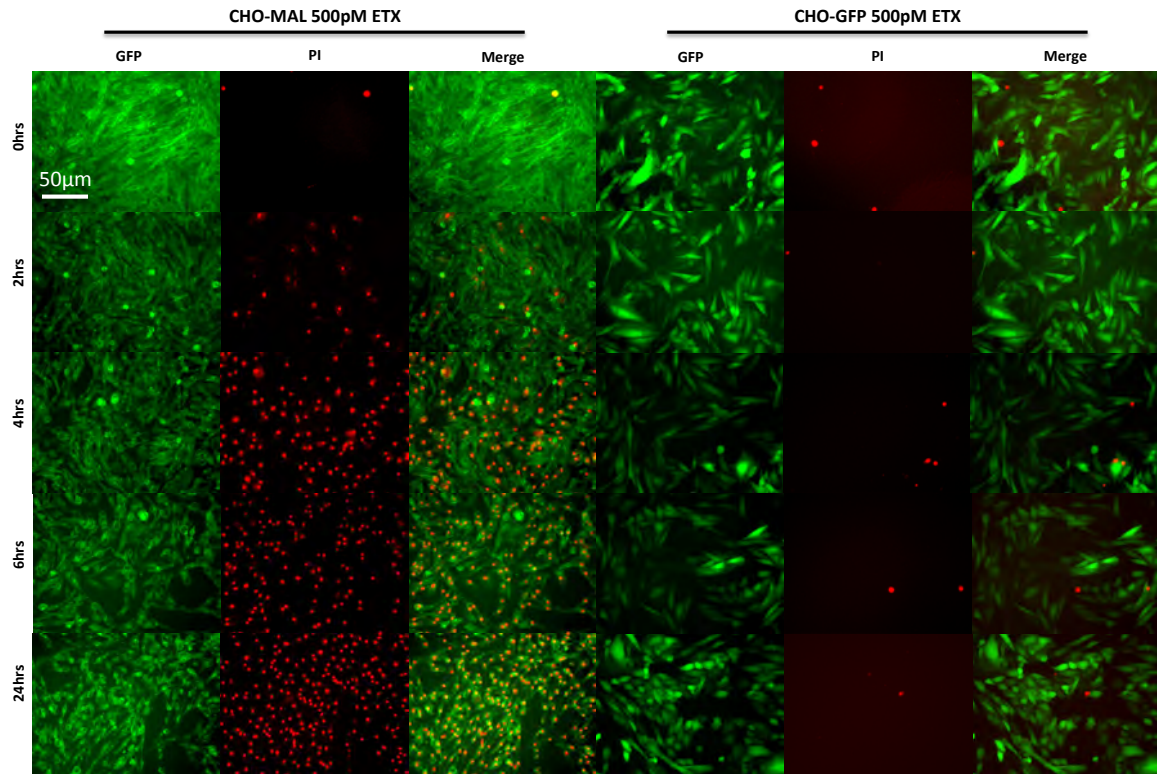
Figure 49. ETX Forms Pores in MAL Transfected Cells. Left) CHO-MAL cells become permeable to propidium iodide 2hrs after ETX treatment (500pm). Right) CHO-GFP cells remain resistant to ETX pore formation even after a 24-hour incubation with ETX.
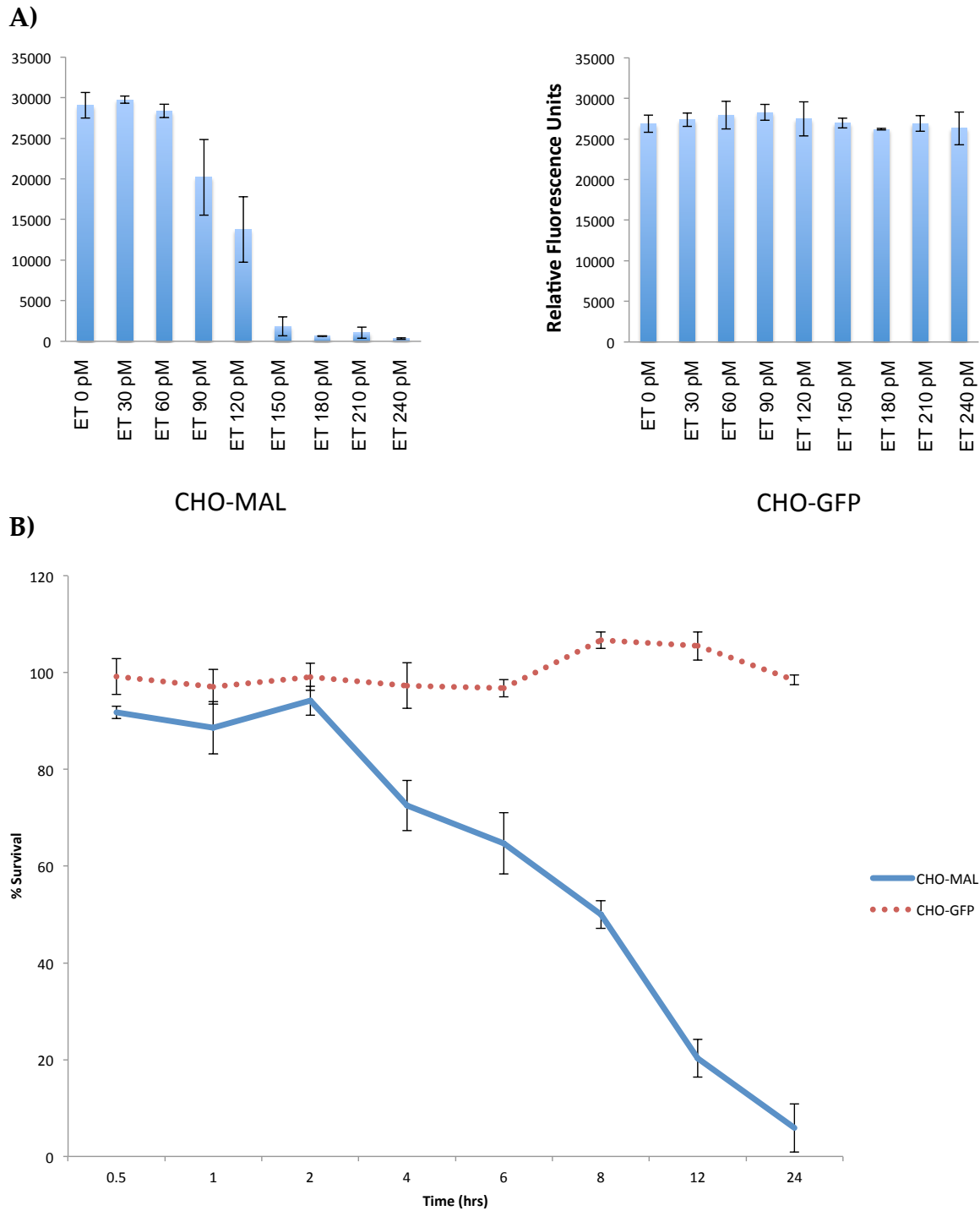
Figure 50. MAL Transfected Cells Are Susceptible to ETX. A) CHO-MAL exhibits ETX sensitivity in a dose dependent fashion (left), CHO-GFP fails to exhibit ETX sensitivity at any of the administered doses (right). B) ETX kills CHO-MAL cells in time dependent fashion, while CHO-GFP cells remain resistant at each time point.

Together, these data suggest that exogenous MAL expression is sufficient to confer ETX susceptibility. As additional confirmation that MAL is truly involved ETX toxicity, we tested the susceptibility of an endogenous MAL expressing cell line, which has no known ETX susceptibility; the human Jurkat T cell line (Anton, Andres-Delgado et al. 2011). Toxin treated cells lost their cell-cell adhesion/clustering characteristics and displayed marked swelling after treatment with ETX (Fig. 51), confirming their inherent ETX susceptibility.

Control 0nM ETX 8hrs 150nM ETX 8hrs
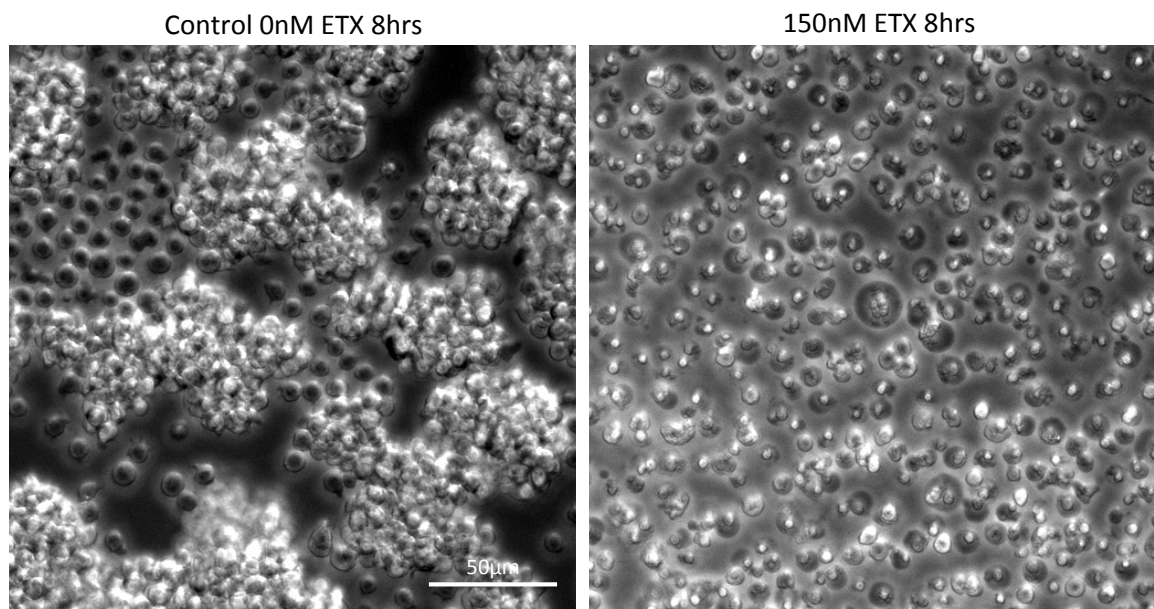
Figure 51. Jurkat T Cells Are Susceptible to ETX. Untreated Jurkat T cells display the typical clustered morphology (left). ETX treated Jurkat T cells dissociate for each other and exhibit marked swelling and osmolysis (right).

Expt 2. Is MAL necessary for ETX binding and sensitivity?

RESULTS

MAL ablation abolishes ETX binding and toxicity: Finally, we wished to determine if MAL is necessary for ETX binding and/or toxicity. We procured MAL knockout (KO) mice from Dr. Nicole Schaeren-Wiemers (University Hospital Basel, Switzerland) and harvested tissues known to express MAL, brain and kidney (Frank 2000). We also harvested eye tissue, as we postulated that the vessels comprising the blood-retinal barrier might also express MAL. MAL deficient mice show no overt physical or behavioral abnormalities, however, a defect in the maintenance of paranodal myelin loop structure has been observed upon examination of CNS myelin by electron microscopy.

In wild type (WT) mice, Alexa 594 labeled ETX stained CNS myelin (Fig. 52), retinal microvessels (Fig. 53), squamous epithelium of the cornea (Fig. 54) and renal tubules (Fig. 55). However, ETX failed to stain these same structures in the MAL KO mouse. Despite this lack of staining in KO mice, control markers for CNS white matter (PLP), retinal vessels and corneal epithelium (BSL1), and renal tubules (Alexa 488 labeled Shiga toxin 1 beta) showed no difference in staining for KO vs. WT mice. Of note, human T lymphocytes are known to express MAL and we have shown that the human Jurkat T cell line is ETX sensitive (Fig 51). However, we failed to observe ETX staining of splenic T lymphocytes harvested from wild type mice (data not shown).
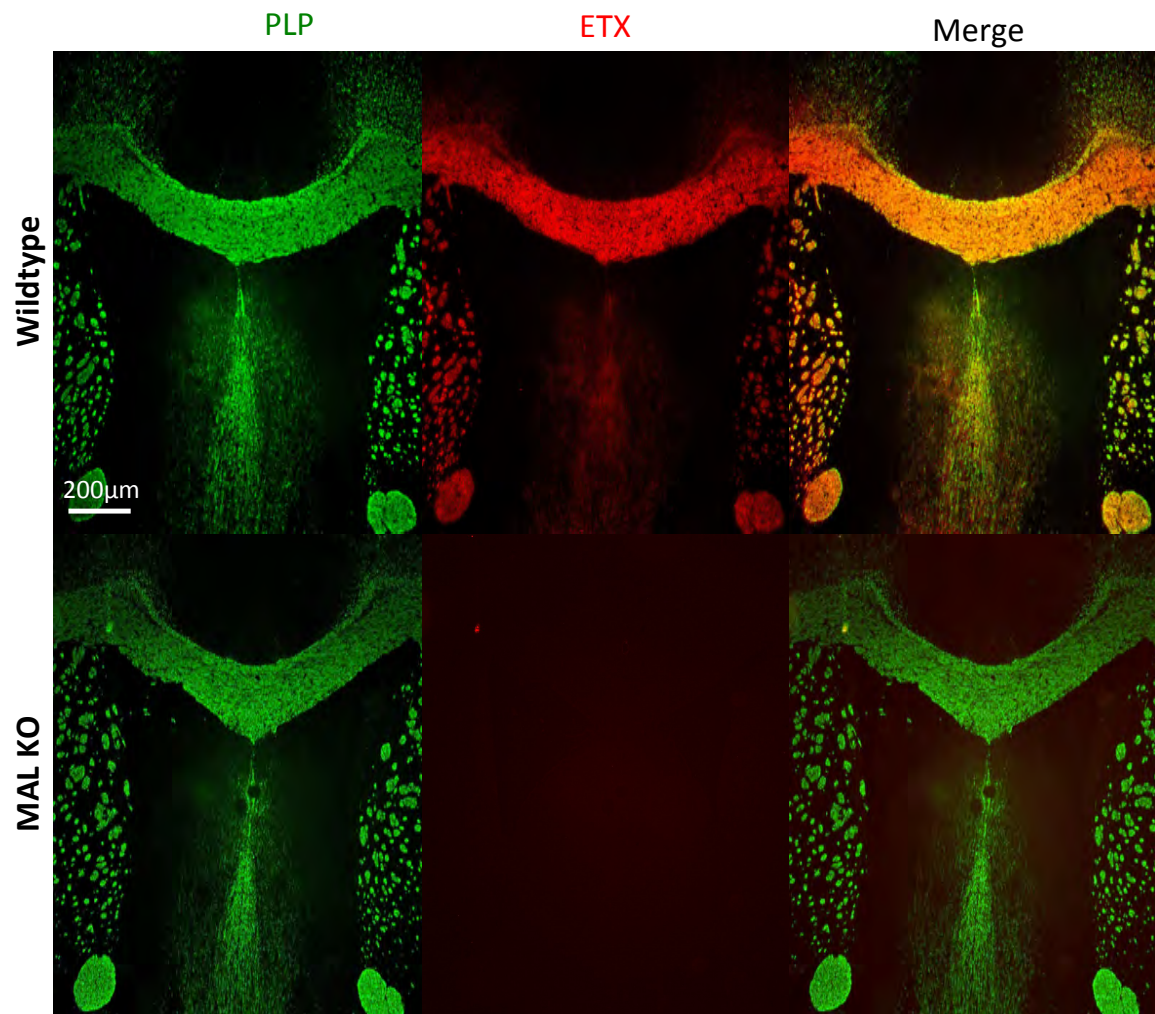
Figure 52. MAL Is Necessary for ETX to Bind to CNS White Matter. A brain section from a wild type mouse (top panel) stains for PLP (myelin marker) and ETX-594. The merged image shows almost compete overlap. A brain section from a MAL knockout mouse (bottom panel) stains for PLP, but not for ETX-594, thus there is no colocalization in the merged image.
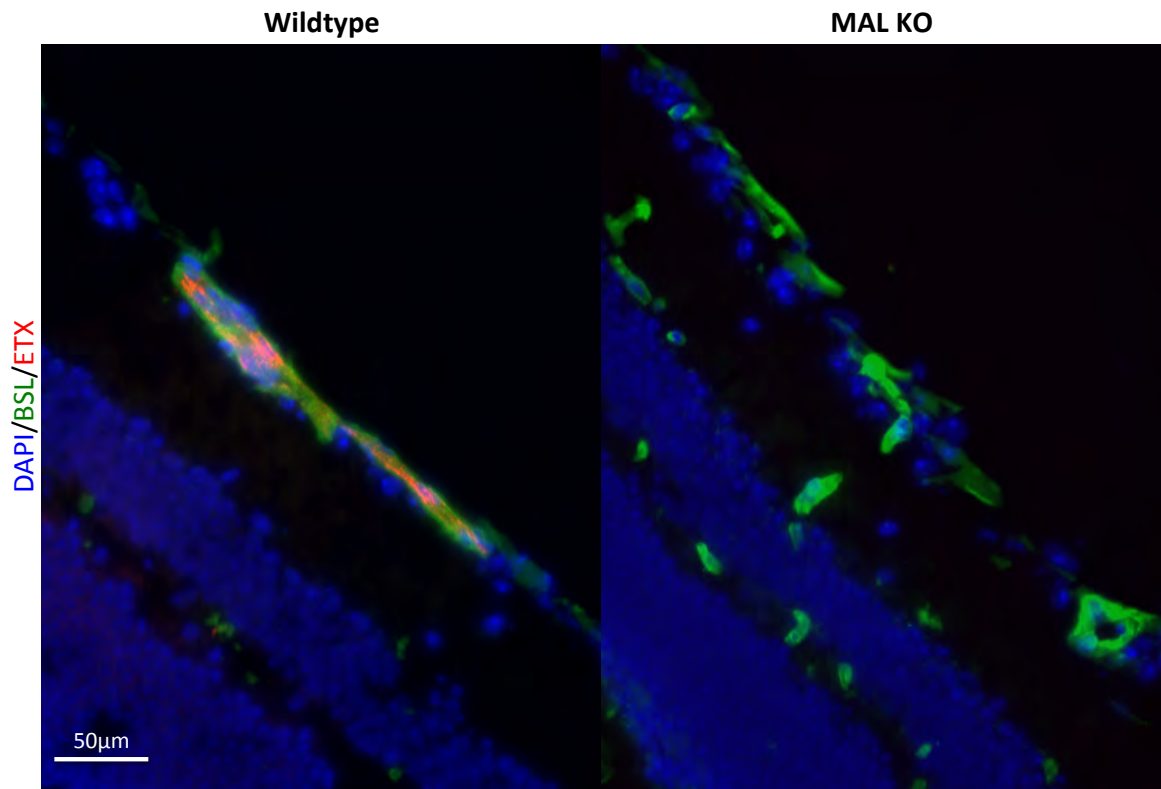
Figure 53. MAL Is Necessary for ETX to Bind to Retinal Vessels. (Left) retinal vessels from a wild type mouse stain positive for BSL (a pan vessel marker), ETX-594 and DAPI. (Right) retinal vessels from a MAL knockout mouse stains for BSL and DAPI, but fails to stain for ETX-594.
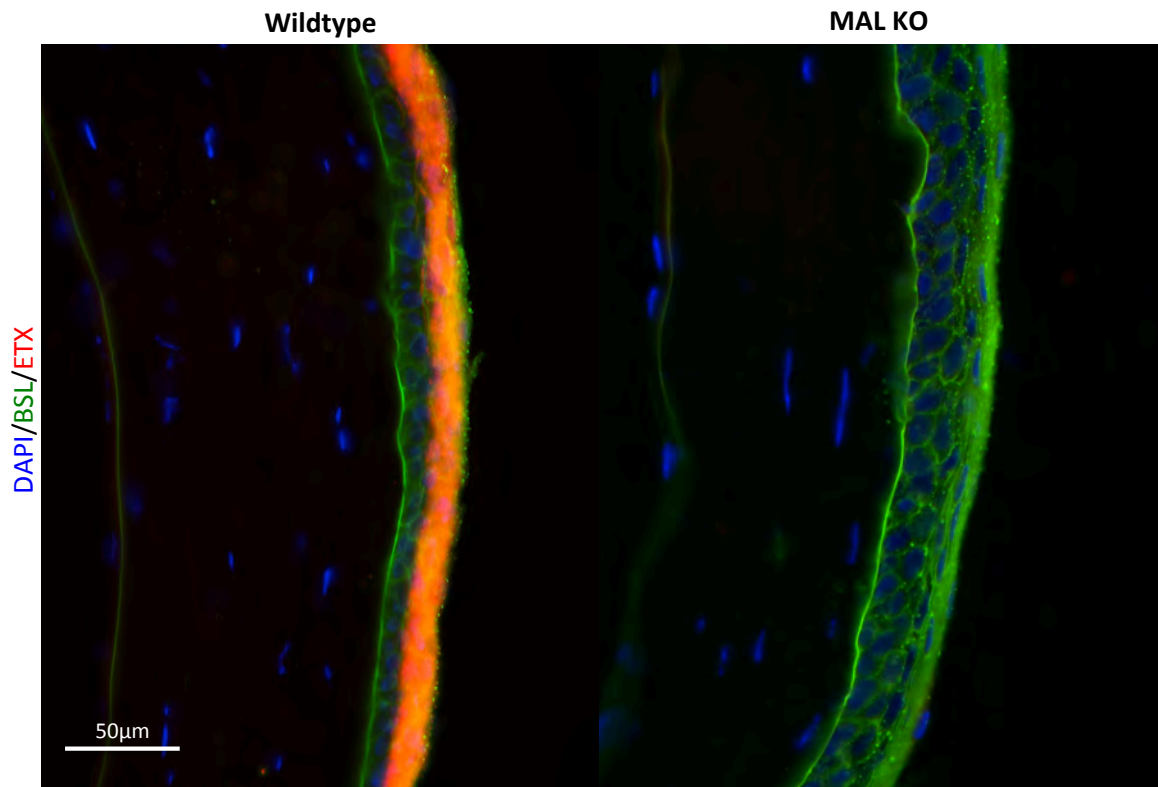
Figure 54. MAL Is Necessary for ETX to Bind to The Cornea. (Left) squamous epithelium of the cornea from a wild type mouse stains positive for BSL lectin, ETX-594 and DAPI. (Right) cornea from a MAL knockout mouse stains for BSL lectin and DAPI, but fails to stain for ETX-594.
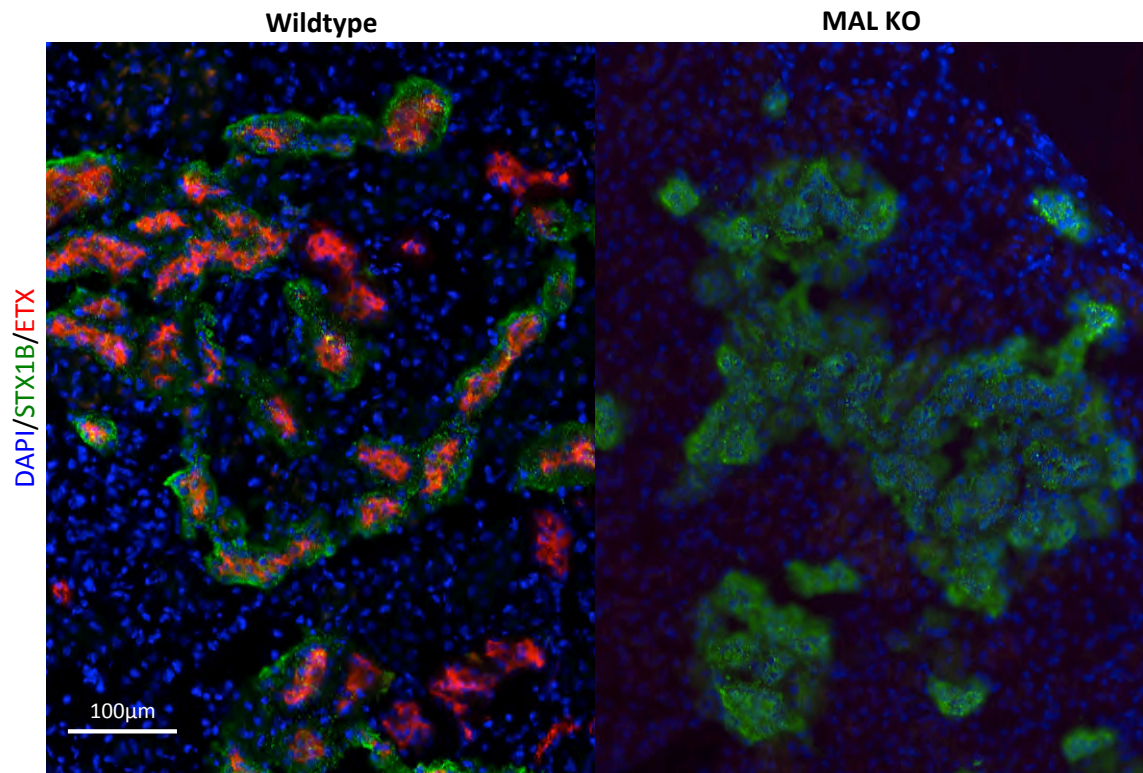
Figure 55. MAL Is Necessary for ETX to Bind to Renal Tubules. (Left) a kidney tissue section from a wild type mouse stains positive for shiga toxin-488 (green), ETX-594 (red) and DAPI (blue). (Right) a kidney tissue section from a MAL knockout mouse stains positive for shiga toxin-488 and DAPI, but is negative for ETX-594 binding.

The hallmark of ETX toxicity is cerebral edema as a result of blood-brain barrier breakdown (Bokori-Brown, Savva et al. 2011). However, we did not observe robust ETX staining in brain vasculature. This may be due to the fact that ETX has a predilection for white matter vessels (Finnie 1984; Finnie 1984) and it may be difficult to visualize vascular staining in white matter, as the vascular signal may be obscured by the surrounding myelin signal. The retina is a CNS tissue that is devoid of myelin, and we observed ETX staining of blood-retinal barrier vasculature, suggesting that these microvessels express MAL.

Because ETX failed to bind tissues harvested from MAL KO mice, we went on to compare ETX toxicity in MAL KO (n=5) versus WT (n=5) animals. We found that MAL KO mice were completely resistant to ETX (Figure 56). We also subjected MAL deficient mice to ETX doses that were in excess of 1000 times our experimentally determined LD_{50} and found that these animals remained unaffected even when injected with extremely high doses of toxin.

Figure 56. MAL Is Necessary for ETX Toxicity In Vivo. (Foreground), a wild type mouse injected with 50ng of ETX IP lays obtunded. (Background), a MAL KO mouse injected with 50ng of ETX IP remains fully alert and unaffected.

SUMMARY

In summary, we have shown that exogenous MAL expression confers ETX binding and susceptibility to an ETX resistant CHO cell line. We have identified the Jurkat T cell line, an endogenous MAL expresser, as a novel ETX sensitive cell line, further confirming that MAL is sufficient for ETX susceptibility. Furthermore, the inability of ETX to bind to tissues from MAL KO mice and the high degree of ETX resistance in KO animals demonstrate that MAL is also necessary for ETX toxicity. Finally, we have determined that both blood-retinal barrier and blood-brain barrier endothelial cells express MAL, a finding that supports the idea that MAL plays a role in barrier function.

MATERIALS AND METHODS

Generation of MAL constructs - pβ-actin-ECGFP-rat MAL construct was kindly provided by Nicole Schaeren-Wiemers of University Hospital Basel, Switzerland (Caduff, Sansano et al. 2001). Constructs for human MAL and human MAL with FLAG sequence insertion in the 2nd extracellular loop were gift of Miguel Alonso of Universidad Autonoma de Madrid, Spain. Human MAL and human MAL with the FLAG insertion in 2nd loop were subcloned and inserted into N-terminally GFP expressed pβ-actin-ECGFP vector. Human MAL with a FLAG insertion in the 1st extracellular loop was synthesized (RsrII/XbaI restriction sites included), and purchased from Genewiz, Inc. A plasmid encoding Zebra fish MAL was purchased from ATCC, subcloned and inserted into the pβ-actin-ECGFP vector. The GFP control vector for stable cell expression is pEGFP-C1 from Addgene. All recombinant plasmids were grown in One shot Top

10 Escherichia coli (Invitrogen). Sequence analysis and verification were performed (Macrogen USA). Plasmids were subsequently amplified and purified (Qiagen plasmid purification kit).

Cell Culture and Transfection - Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's Medium/Ham's F12 medium (gibco) with 10% heat-inactivated FBS, 50 units/ml penicillin and 50 µg/ml streptomycin (Invitrogen). CHO cells were transfected with the indicated expression plasmids using turbofect (Fermentas) according to the manufacturer's instructions. For stable cells, clumps of cells were collected after growing transfected cells in the growth medium with G418 400 µg per ml for 3 weeks. Cells were then analyzed under fluorescent microscope for enriched GFP expression. GFP enriched cells were FACS sorted by Becton-Dickinson Vantage cell sorter and clonally expanded.

Cell Viability Assays:

Presto Blue assay - PrestoBlue Reagent (molecular probes, invitrogen): CHO-EGFP cells and CHO-EGFP-MAL cells were grown together with ETX in 384 well plate for overnight. 10X PrestoBlue cell viability reagent, which is quickly reduced by metabolically active cells and highly red fluorescent, was added into each well of plate by multi-drop liquid dispenser. Then fluorescence intensity was measured by Microtiter plate reader Perkin-Elmer Envision.

Propidium Iodide Uptake Assay - Cell membrane permeability was assessed by determining the ability of cells to exclude the DNA-binding fluorescent dye propidium iodide as previously described (McGahon, Martin et al. 1995). Cells were grown in 6 well culture plates and treated with ETX for indicated times. Then cells were immediately stained by propidium iodide of pSIVA™-IANBD Apoptosis/Viability Microscopy Set (IMGENEX) and imaged under fluorescent microscope.

Jurkat T cell culture and ETX treatment - Human T lymphoblastoid Jurkat cells (ATCC Cat#TIB-152) were grown in RPMI 1640 supplemented with 5% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50mg/ml streptomycin at 37°C in an atmosphere of 5% CO2/95% air, in T75 flasks until confluent. ETX (150nM) or PBS vehicle control was added to each flask and incubated for 37 degrees C for 8 hours.

Fluorescent labeling of toxins - His tagged ETX prototoxin was procured from BEI Resources. One mg was fluorescently labeled using Alexa Fluor 594 Protein Labeling Kit (Invitrogen) and 1 mg was labeled using Alexa Fluor 647 Protein Labeling Kit (Invitrogen) as per manufacturer's instructions. His tagged Shiga toxin 1β was procured for BEI Resources and fluorescently labeled using Alexa Fluor 488 Protein Labeling Kit (Invitrogen).

Flow Cytometry - Transfected cells were removed from the tissue culture substrate by incubating with citric saline (135μM potassium chloride, 15μM sodium citrate dissolved in sterile water) for 5 mins at 37 degrees C. Cells were

washed of the plate and triturated well to obtain single cell suspensions. Cells were washed 3 times with DMEM without phenol red (Invitrogen), containing 10% fetal calf serum, 50 U/ml penicillin, and 50mg/ml streptomycin. Cells were diluted to 5×10^5/ml and incubated with 20nM Alex 647 labeled ETX for 1 hour at 37 degrees C. Labeled cells were washed 3 times in DMEM lacking phenol red, and read on the AccuriC6 Flow Cytometer.

Fluorescence microscopy:

Cells - Stably transfected rat MAL (CHO-MAL) and GFP (CHO-GFP) were plated on glass bottom dishes (Mattek) at a density of 6×10^4 cells/ml, and allowed to grow overnight. Cells were subsequently washed and fixed with 4% PFA for 10 mins at room temp. Cells were washed and incubated with Alexa 594 labeled ETX (50nM) for 1 hour at room temp. After incubation, the cells were washed 3 times in PBS and Hoechst (1μg/ml) was added to visualize nuclei. Stained cells were visualized by confocal microscopy at The Rockefeller University Bio-Imaging Facility.

Brain Tissue - Fresh frozen tissue sections were fixed in 4% PFA (10 mins RT), permeabilized in a 1% sodium cholate, 1% BSA, 10% donkey serum, PBST solution overnight at 4 degrees C (Dorca-Arevalo, Martin-Satue et al. 2012). Sections were then incubated with rabbit anti-PLP (ThermoScientific) at 1:1000 overnight at 4 degrees C. Following three washes with PBS, sections were then incubated with Donkey anti-rabbit Alexa 488 (Jackson ImmunoResearch) at 1:1000, and Alexa 594 labeled His-tagged protoxin (50nM) for 2 hrs at RT. The stained tissue was washed 3X in PBS, mounted and imaged.

Retina/Cornea - Fresh frozen tissue sections were incubated with BSLI (Vector Labs) 1:200, and Alexa 594 labeled His-tagged protoxin (50nM) for 1 hr at RT. After three 5 minute washes in PBS, stained sections were post fixed in 4% PFA for 10 mins at RT. The stained tissue was washed 3X in PBS, mounted and imaged.

Kidney - Fresh frozen tissue sections were fixed in 4% PFA (10 mins RT), washed and incubated with Alexa 488 labeled His-tagged Shiga toxin 1β (200nM) and Alexa 594 labeled His-tagged protoxin (50nM) for 1hr RT. The stained tissue was washed 3X in PBS, mounted and imaged.

ETX toxicity in mice - All animal work was conducted under the WCMC animal protocol number 2012-0030. Each mouse was injected with ETX via intraperitoneal injection and the time-to-death determined.

DISCUSSION

Although confident that MAL is a functional ETX receptor, we realized one discrepancy that had to be addressed. Previous investigators have postulated that the ETX receptor would be a glycoprotein because the elimination of carbohydrate moieties by neuraminidase treatment (Nagahama and Sakurai 1992) and by beta-elimination (Dorca-Arevalo, Martin-Satue et al. 2012) mitigate ETX toxicity. While MAL is not a glycoprotein, it does reside in a glycolipid rich environment, the lipid raft. We speculated that neighboring glycolipids may act as ETX co-receptors or may modulate the ETX/MAL

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interaction. While it has been recognized that glycosphingolipids can affect ETX toxicity, experimental data contradicts our hypothesis. Inhibitors of sphingolipid or glycosphingolipid synthesis increase cell susceptibility to ETX, whereas inhibition of sphingomyelin synthesis or addition of GM1 dramatically decreases ETX binding and subsequent heptamerization (Shimamoto, Tamai et al. 2005). Importantly, Shimamoto et al. suggest that the GM1 inhibition is not due to competitive inhibition by free GM1 in the culture media. They also find that lipid raft GM3 inhibits ETX binding. Finally, the fact that C. *perfringens* secretes a neuraminidase of its own that reportedly increases ETX toxicity (Li, Sayeed et al. 2011) suggests that carbohydrate moieties may inhibit ETX binding. As many conflicting data regarding the role of carbohydrates in ETX binding/toxicity exist, we wished to determine if glycolipids were necessary for ETX binding and toxicity.

We procured the glycosphingolipid deficient GM-95 cell line, which lacks the enzymes necessary for glycolipid synthesis from Dr. Deborah Brown (Stony Brook). We also procured the MEB-4 melanoma cell line, which is the parental cell line for the GM-95 mutant, to use as an appropriate glycosphingolipid expressing positive control. We found that both cell lines were susceptible to ETX toxicity after transient transfection with MAL (data not shown), thus glycosphingolipids are not necessary for ETX/MAL binding or ETX pore formation.

It also seems that a rigid 3 dimensional structure of the MAL extracellular loops (ECLs) may be necessary for ETX binding. We generated short peptides of both MAL ECLs and pre-incubated ETX with each ECL alone and with the two loops combined, and found that no competitive inhibition occurred (data not

shown). These data suggest that ETX may only be able to recognize MAL in a particular conformation that is stabilized by the rigidity of the surrounding membrane.

MAL in the context of Multiple Sclerosis: In Table 8, it is clear that many different cell types (mostly epithelial cells) in many different tissues express MAL. If ETX triggers MS and ETX binds to MAL, wouldn't one observe tissue damage in these various sites? One must realize that the cells that express MAL typically form lumens. If ETX is being absorbed via the intestine, it will have access to the gut lumen, the lumen of vascular system and after disrupting the BBB, the specialized membranes of MAL expressing oligodendrocytes. Other MAL expressing lumenal tissues, which have no contact with blood such as stomach epithelium, thyroid epithelium and thymus epithelium will remain unexposed to ETX during hematogenous dissemination.

Since human T lymphocytes express MAL and circulate in the blood, would one not observe T cell abnormalities during an MS exacerbation? Provocatively, this is exactly what has been observed. A 1981 study by Dr. Byron Waksman (The National MS Society) found that during, or up to a week prior to an acute MS attack, T cell abnormalities could be observed. The number of circulating T lymphocytes dropped, and up to 20% of the T cells still in circulation displayed an enlarged morphology (Figure 57) (Waksman 1981).
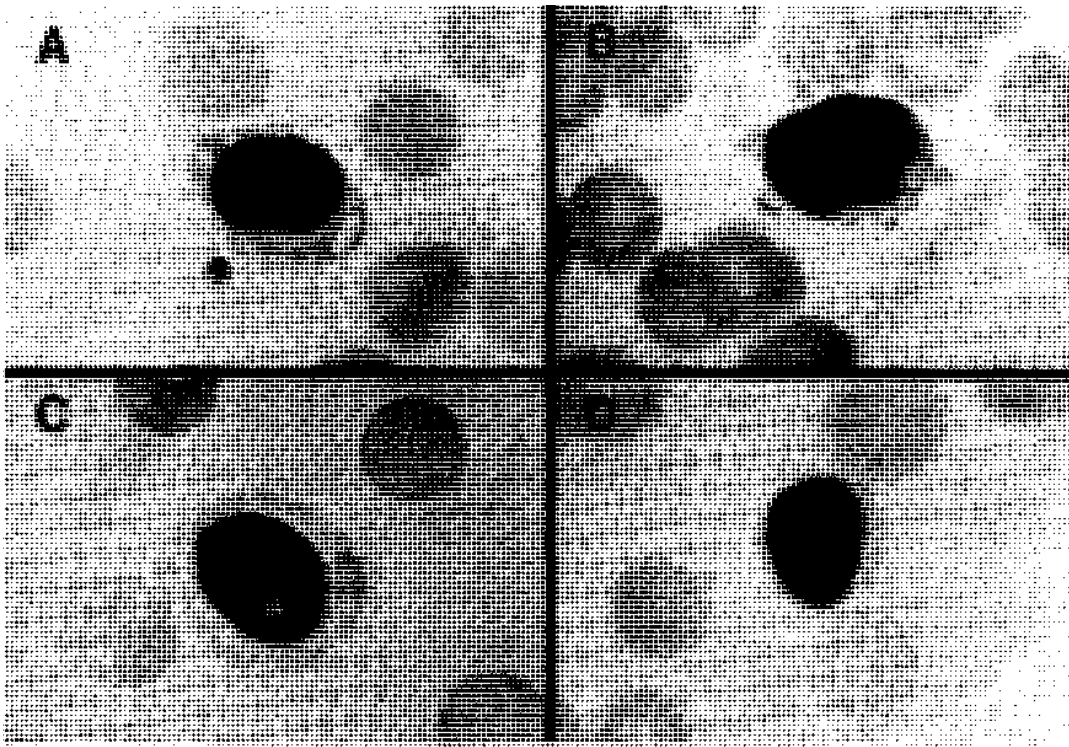
Figure 57. Enlarged Lymphocytes During Acute MS. Lymphocytes from patients with active multiple sclerosis (A, B, C) contrasted with a lymphocyte from a normal donor obtained under identical conditions (D). Blast-like cells similar to those illustrated here may account for 20% of the white cells in the circulation of MS patients. Wrights stain (l000X). (Waksman 1981)

Dr. Waksman described these cells as "blast-like" due to their enlarged cytoplasm, however, one would not expect T cell blasts to be present in the circulation in cases other than leukemia. We propose that this enlargement of the cytoplasmic space may be due to ETX exposure, pore formation and cellular swelling, reminiscent of what was observed in Figure 51 when we treated the Jurkat T cell line with ETX. The fact that T cell counts also decreased during and immediately before MS exacerbations corroborates this idea of T cell cytotoxicity.

Interestingly, a decrease in T cell membrane potential has also been observed (Bauer, McFarlin et al. 1982) further supporting this notion.

Future Directions

Using T cells to probe for ETX during acute MS attacks: T cell susceptibility may provide the perfect opportunity to identify ETX in MS patients. Because nascent MS lesion brain specimens will be very rare and difficult to procure, circulating T cells harvested from symptomatic MS patients may serve as an easily accessible tissue that may be ETX positive. Indeed, we show that one can detect the ETX pore in T cells that have been isolated from blood incubated with ETX via a Western blot assay (Figure 58).
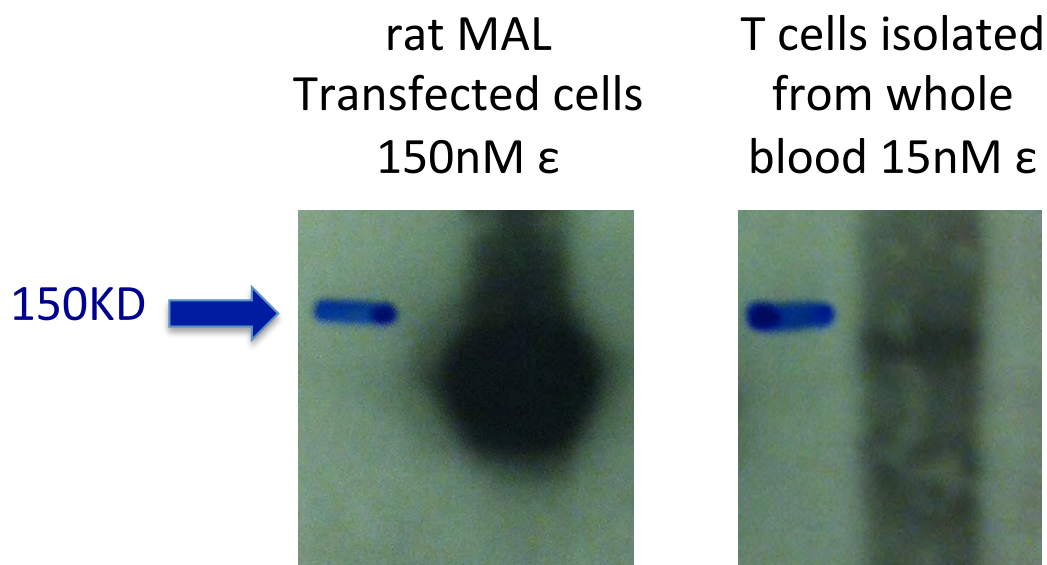
Figure 58. Detection of The ETX Pore in Human T Cells. ETX forms pores in human T cells that can be detected by Western blot. (Left) membranes from ETX treated (150nM) rat MAL transfected CHO cells are strongly positive for the ETX complex (approx. 145kD). (Right) T cell membranes isolated from ETX treated (15nM) human blood are positive for the ETX complex.

MATERIALS AND METHODS

T cell isolation - We drew two full lavender tops of human blood and spiked

one tube with ETX (15nM). The blood was incubated on a shaker at RT for 1 hr.

After an hour we added an equivalent volume of PBS 2%FBS to each sample and

mixed gently. We layered the diluted blood on the appropriate volume of ficoll

and centrifuged for 20mins, 1200g at 25C. We collected and centrifuged cells

from all layers above RBC layer at max speed for 1 min and resuspended the

cells in PBS and washed 2X. We eliminated contaminating RBCs by

resuspending the cells in RBC lysis buffer and incubating for 5 mins on a shaker

at RT. The cells were then resuspended in PBS and washed 2X.

Membrane isolation and Western blot analysis - The isolated T cells were

resuspended in transmembrane exaction buffer (starting blood volume/50) and

incubated on ice for 1hr (vortexed for 30secs every 15 mins). We added 4x

sample buffer and ran on SDS page. We then transferred to a PVDF membrane,

blocked with 2% milk for 1hr RT and incubated with rabbit anti-ETX peptide

KASYDNVDTLIEKGR (a custom antibody made by Pacific Immunology) 1:1000

at 4 C ON. The following day, we incubated with goat anti-rabbit F(ab')2

1:10,000 and developed and imaged.

While an important preliminary experiment, harvesting T cell membranes in

aggregate and screening by Western blot may not be the most sensitive

approach. If we successfully develop an aptamer that can access the ETX pore,

as discussed in Chapter 2, we may be able to use a fluorescently tagged aptamer

to probe individual T cells by FACS analysis (Figure 59). This may increase our

sensitivity for detecting ETX on the T cell membrane many fold.
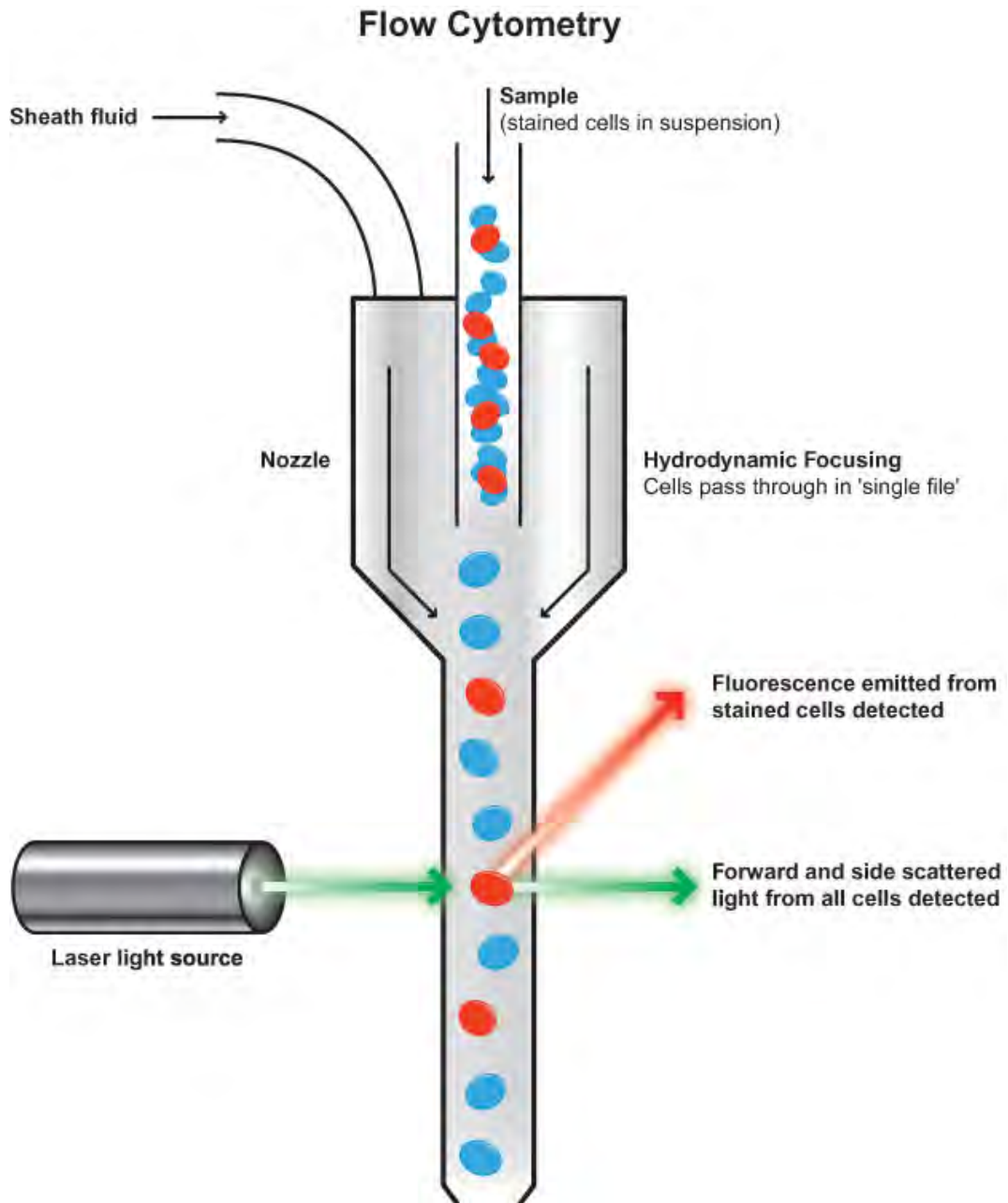
Figure 59. FACS Analysis of T Cells from Symptomatic MS Patients.
Fluorescently labeled DNA aptamers that have been generated against the ETX
pore will be used to probe MS T cells, cell by cell. (adapted from Abcam)

Prove that a physical interaction between MAL and ETX exists: While we have determined that MAL is both necessary and sufficient for ETX binding we have not proved that a physical interaction between the two molecules exists. Because the extracellular surface of a living cell is rich with a variety of molecules and epitopes, we wish to simplify the surface with which ETX interacts to determine if it indeed binds to MAL. This can be achieved by making artificial vesicles that contain purified MAL and no other proteins. We have established a collaboration with the Dr. Brian Kloss at the New York Consortium of Membrane Protein Structure (NYCOMPS) and have begun to synthesize and purify different versions of the MAL molecule which we will then incorporate into unilamellar vesicles. Once incorporated into vesicles, we will utilize techniques such surface plasmon resonance (SPR) to determine the ETX dissociation constants for each MAL species.

Determine the species differences in ETX/MAL binding: One surprising finding was that rat MAL displayed a 10-fold increase in ETX binding affinity when compared to human MAL, and that zebra fish completely failed to bind ETX (Figure 47). We hypothesize that the varying number of aromatic residues in the second extracellular loop of MAL may account for this; rat (6), human (5), zebra fish (3). Interestingly, MAL expressing MDCK cells, which are of canine origin are also susceptible to ETX; canine (4). By our hypothesis, canine MAL should bind ETX with a lower affinity that human MAL, which has 5 aromatic residues in ECL2. When considering MAL from other species e.g. ruminant animals, there are differences in both the order and identity of aromatic residues, e.g. histidine may be replaced by tyrosine. We wish to clone as many different

MAL orthologues as possible into our expression vector and determine the relative binding affinities. Below is a sequence comparison of MAL ECL2 from different species (Figure 60):

Sheep MAL: TIELQDGFFYKYYHENISAVV   (6)
Rat MAL: TITMFDGFTYRHYHENIAAVV   (6)
Mouse MAL: TISMFDGFTYKHYHENIAAVV   (6)
Human MAL: TITMQDGFTYRHYHENIAAVV   (5)
Bovine MAL: AIQLQDGFLYKYYHENISAVV   (5)
Hamster MAL: TIMMYEGYTYKQYHENISAVV   (5)
Canine MAL: TIGMQEGYTYKQYHENISAVV   (4)
Zebra fish MAL: TINMKVFSAVNNYYQIDISAVV (3)
Xenopus MAL: TIALSNVTIYQLYQENIAAVV   (2)

Figure 60. Sequence Comparison of the MAL ECL2. Aromatic residues are colored in red and the number of aromatics in each sequence is tallied on the right side.

Develop a small molecule ETX inhibitor: Lewis et al. have used MDCK cells in a high throughput cell survival assay to screen for compounds that inhibit ETX toxicity (Lewis, Weaver et al. 2010). They have identified 3 compounds, which modestly decreased ETX toxicity (Figure 61). We wish to improve upon this work by using our stably MAL transfected CHO cells to probe for small molecule inhibitors. Our MAL transfected cells are approximately 1000X more sensitive to ETX than MDCK cells, thus less toxin must be used. Using a lower concentration of toxin in a more sensitive system may increase the sensitivity of the assay and increase the number of candidate molecules identified.
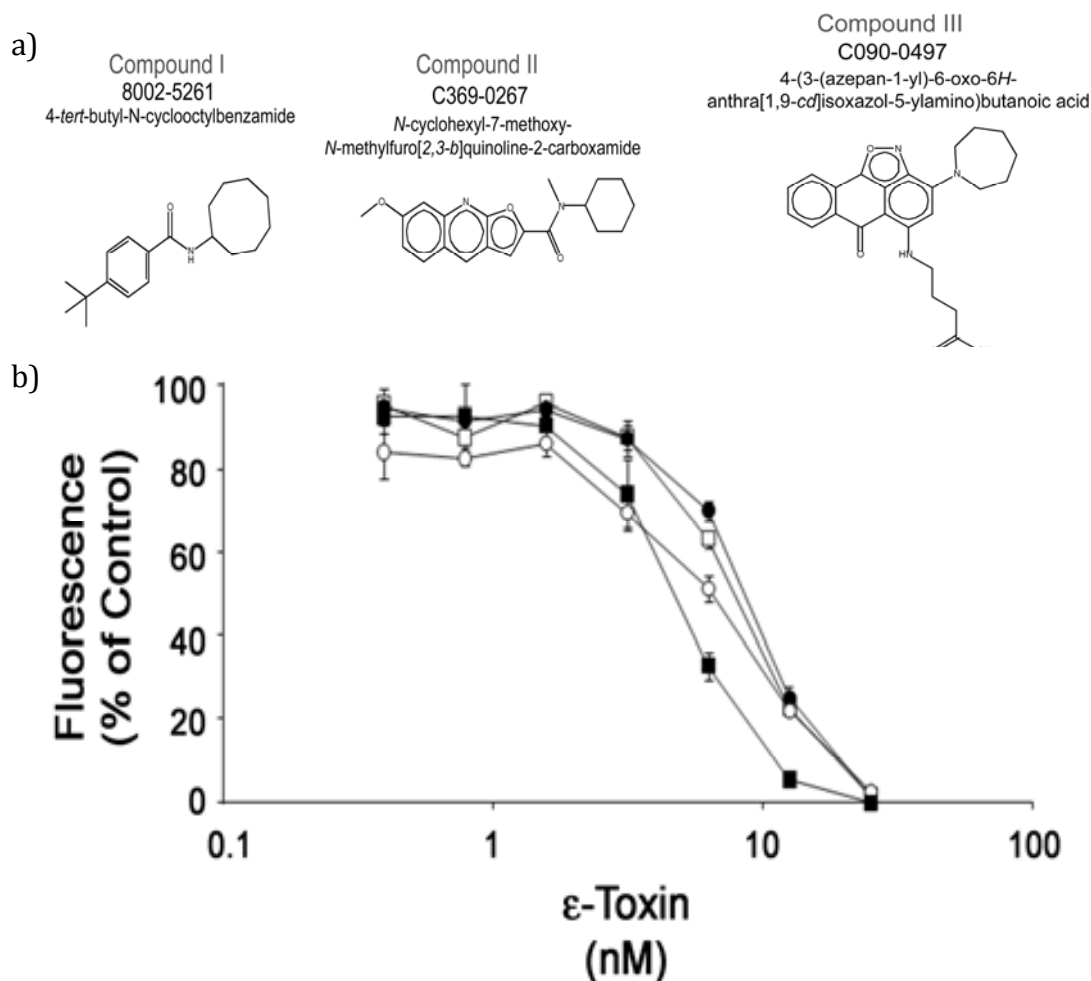
Figure 61. Identification of Candidate ETX Inhibitors. (**a**) The ChemDiv library compound numbers, names, and structures of three confirmed hits are shown (**b**) Serial dilutions of ε-toxin (25 to 0.39 nM) were added to MDCK cell monolayers in a 384-well plate, in the presence of 50 μM inhibitory compounds in 0.4% DMSO (□: compound I; ●: compound II; ○: compound III) or 0.4% DMSO as a control (■). After 16 hours incubation, CellTiter Blue (Promega) was added. Results were normalized to the fluorescent signal from MDCK cells that received compounds but no ε-toxin (100%) and cells treated with compounds and 0.1% Triton (0%). The CT_{50} values (4.7 nM, DMSO control; 7.3 nM, compound I; 6.5 nM, compound II; and 6.7 nM, compound III) were calculated by non-linear regression analysis. CT_{50} values for cells treated with compounds were significantly increased compared to the CT_{50} value of cells treated with the DMSO control (ANOVA followed by Dunnett's post hoc test, $P < 0.05$). (Lewis, Weaver et al. 2010)

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We have performed preliminary experiments using a LOPAC library of 1280 compounds and have identified a few compounds that seem to increase cell survival from 0% to approx. 30% (Figure 62). We will continue by screening a library with a much larger catalogue of compounds in hopes of identifying more ETX inhibitors.
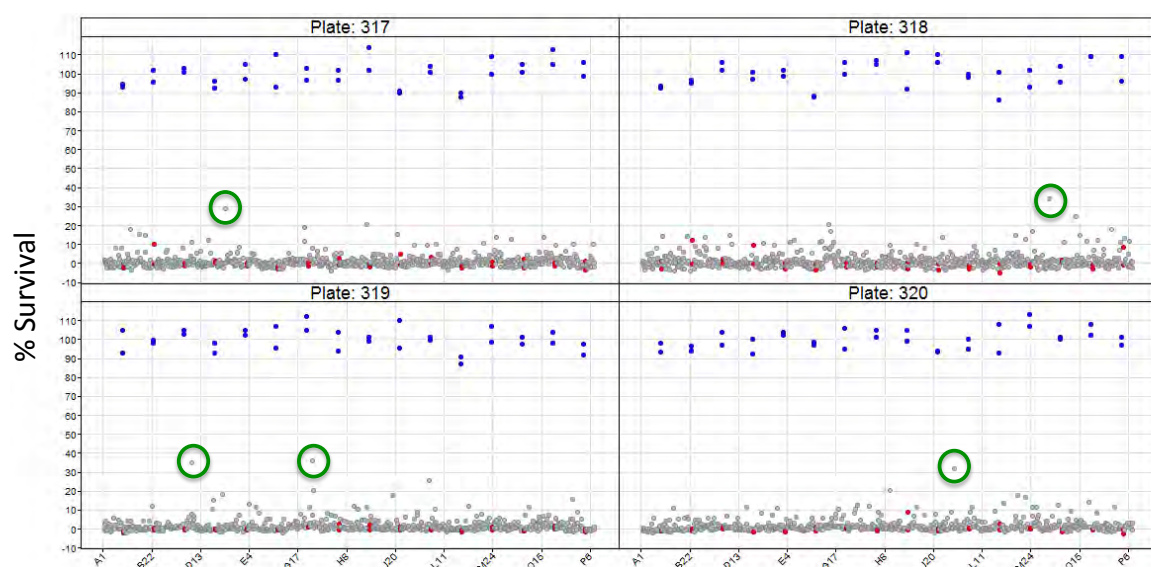
Figure 62. Candidate ETX Inhibitors from The LOPAC Library (1280 compounds). Blue dots signify cells that have not been treated with ETX, representing maximum survival. Red dots signify ETX treated cells that have not been treated with a test compound. Gray dots signify ETX treated cells that have also been treated with a test compound. Gray dots in green circles signify cells that have been treated with a potential ETX inhibitor.

CONCLUDING REMARKS

The ETX/MS Hypothesis Summarized

In summary, we propose that ETX secreted by Clostridium *perfringens* type B/D, or perhaps a bacterium that has acquired the ETX gene by horizontal gene transfer, may be sufficient to trigger MS. ETX targets the two tissues that are affected during each MS relapse, the BBB and the myelin sheath. Furthermore, ETX targets the retina, which is devoid of myelin and yet shows unexplained signs of vascular damage in MS patients. We have identified a 10-fold increase in anti-ETX seroreactivity in MS patients when compared to healthy controls and have found that MS patients are less likely to carry C. *perfringens* type A, a non-ETX producing competitor of toxinotypes B and D. We have described the first human to carry C. *perfringens* type B and this individual has been recently diagnosed with MS. We have identified MAL as a functional ETX receptor and have shown that it is both necessary and sufficient for ETX binding and toxicity. The fact that MAL is a myelin protein, and we have shown that it is also expressed at the BRB and BBB, supports the ETX/MS theory. Below we have illustrated and summarized a proposed disease mechanism for Multiple Sclerosis in which C. *perfringens* epsilon toxin is the triggering agent:
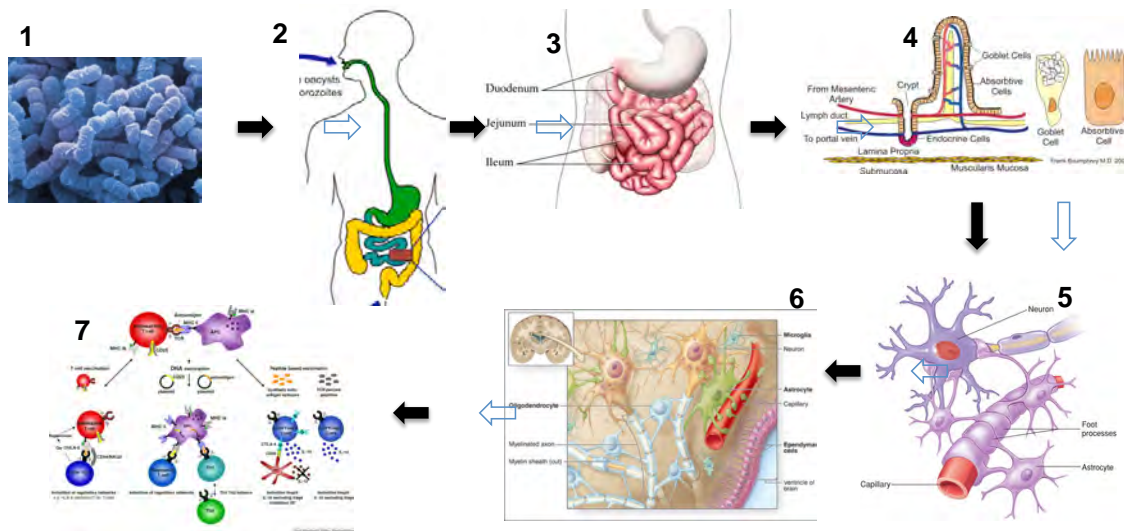
Figure 63. A Schematic of the ETX/MS Hypothesis

1. Clostridium *perfringens* exists in the environment (e.g. soil, marine sediment, food). C. *perfringens* type A outcompetes types B and D making them less prevalent in certain environs. Endospores are exceedingly stable in the environment and in animals. Thus once the organism enters an environmental niche it is likely there "forever".

2. Susceptible individuals become colonized by C. *perfringens* type B or D sometime during life. Colonization may be determined by host factors, including host genetics, the composition of resident microbiota and microbial exposure. Since C. *perfringens* type A, which does not carry ETX, competes with other toxinotypes for space within a niche, we predict that C. *perfringens* type A will be less prevalent in the MS population.

3. In colonized individuals, C. *perfringens* type B or D resides in low abundance within the upper gastro-intestinal (GI) tract, rendering detection difficult since endospores are highly resistant to degradation techniques used to isolate RNA/DNA. An environmental change, perhaps diet, free carbohydrate load or a currently unknown factor triggers a growth phase in C. *perfringens* type B or D. With log-phase growth comes commensurate transcription of the ETX gene,

translation and secretion of ETX. Unlike ruminant animals, humans (and rodents) do not support substantial C. *perfringens* type B or D bacterial loads, thus the amount of growth as well as toxin production is relatively small. This may explain why humans and rodents do not suffer as severe a disease course as ruminants.

4. Relatively small amounts of toxin enter the bloodstream through the small intestine. The small intestine epithelium has ETX binding sites, leading to subtle intestinal injury, barrier permeabilization, and leak of toxin from the intestine into the blood. Seroconversion to ETX antibody positive status happens in a fraction of patients and may be short lived due to the small quantity of toxin antigen, and the difficulties that mammals have maintaining humoral immunity against ETX.

5. Toxin binds to endothelial cells in the CNS leading to disruption of BBB function, a characteristic feature of the earliest MS lesions. Remarkably, prior studies in rodents show that ETX exposure results in ovoid lesions characteristic of MS Dawson's fingers.

6. Where the BBB has been disrupted, ETX enters the parenchyma and binds to oligodendrocytes, forms pores and leads to oligodendrocyte death in the absence of an inflammatory infiltrate, a characteristic feature of the earliest MS lesions.

7. Following the formation of the initial lesion, tissue injury leads to antigen presentation, epitope spreading and adaptive immune responses that can propagate damage to CNS.

Caveats to the ETX/MS Hypothesis

While a provocative hypothesis for initial lesion formation in MS, there are inconsistencies in the ETX/MS hypothesis that must be considered. 1) ETX reproducibly causes symmetrical brain lesions in affected animals, while MS lesions can be, but are not always symmetrical. 2) ETX enterotoxaemia is often lethal in ruminant animals, while MS exacerbations rarely result in death. 3) Natalizumab (Tysabri), an effective MS treatment, prevents the entry of immune cells into the CNS. Therefore, it is clear that the immune system plays an essential role in MS pathophysiology. In summary, while ETX offers mechanistic plausibility for early lesion formation, questions remain regarding its alleged role in Multiple Sclerosis.

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