DISCERNING THE MECHANISM OF γδ T CELL-MEDIATED DAMAGE IN MULTIPLE SCLEROSIS: THE POTENTIAL ROLE OF ANTIBODIES IN DISEASE PATHOGENESIS

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ABSTRACT

Background: Both the innate and adaptive immune systems contribute to autoimmune injury in multiple sclerosis (MS). We have been particularly interested in elucidating the role of the innate γδ T-cell population in MS pathogenesis. In particular, some γδ T-cells that express Fc receptors (FcR), such as CD16, that bind antibody are more prominent with MS disease progression and have been shown to exert cytolysis via antibody-dependent cellular cytotoxicity (ADCC). We postulated that if there were also relevant and detectable antibodies in MS patients that might engage these FcR-bearing γδ T-cells then this might be a purported mechanism of neuro-axonal injury. A search for antibodies specific to axonal elements in MS revealed the presence of antibodies to neurofascin (Nfasc).

Methods: Anti-Nfasc antibody titres, and concentrations of the light and heavy chains of neurofilament (NfL and NfH, respectively), markers of neuro-axonal injury, were measured in the sera and cerebrospinal fluid (CSF) of MS patients using enzyme-linked immunosorbent assays (ELISA), including those that underwent autologous hematopoietic stem cell transplantation (aHSCT), both prior to and yearly for 3 years thereafter. HeLa cells were transfected with the axonal variant of Nfasc, Nfasc-186, and were utilized as targets in ADCC assays involving γδ T-cells as the effectors, and anti-Nfasc antibodies that were enriched from MS patient sera.

Results: Positive anti-Nfasc antibody titres were detected in of 22% and 25% of MS patient sera and CSF, respectively. The most elevated serum titres were in secondary progressive MS (SPMS), and highest CSF titres in relapsing-remitting MS (RRMS) ($p<0.05$ and $p<0.0001$, respectively, vs. other neurological disease [OND] controls). Patient serum and CSF antibody titres correlated and, in the CSF, the titres correlated positively with the

concentration of NfL. Though NfL and NfH concentrations declined markedly following aHSCT in the CSF, anti-Nfasc antibody titres failed to decline. When co-cultured with $CD16^+$ γδ T-cells in the presence of MS patient-derived anti-Nfasc antibodies, the percent specific cytolysis of the Nfasc-transfected HeLa cells was significantly greater than that of the non-transfected control HeLa cells, at 18% and 1%, respectively, indicating cytolytic kill via ADCC.

Summary: Anti-Nfasc antibodies were detectable in the sera and CSF of MS patients, and rarely in OND controls, suggesting they are relevant to MS. Higher titres in the serum support peripheral synthesis, while higher CSF titres in the relapsing phase, that correlate with serum titres, imply that antibodies access the CNS during periods of active inflammation that are associated with disruption of the blood-CSF barrier. CSF anti-Nfasc antibody titres correlated strongly with the release of NfL, suggesting that axonal injury could be related to the presence of Nfasc-specific antibodies. Following aHSCT, CSF NfL and NfH release were reduced without concomitant CSF anti-Nfasc antibody reductions, suggesting that the presence alone of anti-Nfasc antibodies is not enough to cause axonal injury. Indeed, when co-cultured with $CD16^+$ γδ T-cells in the presence of MS patient-derived anti-Nfasc antibodies, the percent specific cytolysis of the Nfasc-transfected HeLa cells was significantly greater than that of the non-transfected control HeLa cells, proving that FcR-bearing γδ T-cells can cause axonal damage by lysing axonal membranes via ADCC, when armed with axon-specific antibodies such as anti-Nfasc. This is the first report of γδ T-cell-mediated cytolysis by ADCC using both γδ T-cells and antibodies derived from MS patients.

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LIST OF ABBREVIATIONS

ADCC: antibody-dependent cellular cytotoxicity
aHSCT: autologous hematopoietic stem cell transplantation
APC: antigen-presenting cell
BAFF: B-cell activating factor
BSA: bovine serum albumin
CAM: cell adhesion molecule
Caspr: contactin-associated Protein
CCPD: combined central and peripheral demyelination
CD16: complementarity-determining region 16
CIPD: chronic inflammatory demyelinating polyradiculoneuropathy
CNPase: 2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS: central nervous system
CRMP: collapsin response-mediator protein
cRPMI: complete Roswell Park Memorial Institute medium
CSF: cerebrospinal fluid
DMT: disease-modifying therapy
DNA: deoxyribonucleic acid
EAE: experimental autoimmune encephalomyelitis
EBV: Epstein Barr Virus
ECM: extracellular matrix
EDSS: Expanded Disability Status Scale
ELISA: enzyme-linked immunosorbent assay
E:T: effector-to-target ratio
FBS: fetal bovine serum
FcR: Fc receptor
FcγRIII: Fcγ receptor Type III
HeLa: **He**nrietta **La**cks cell line
HSC: hematopoietic stem cell
HSP: heat shock protein
IgG: immunoglobulin G
IL: interleukin
KIR: killer-cell immunoglobulin-like receptor
mAb: monoclonal antibody
MBP: myelin basic protein
MMP: matrix metalloproteinase
MRI: magnetic resonance imaging
MRS: magnetic resonance spectroscopy
MOG: myelin oligodendrocyte glycoprotein
MS: multiple sclerosis
Na_v: voltage-gated sodium channel
NAGM: normal-appearing grey matter
NAWM: normal-appearing white matter
Nfasc: neurofascin
NfH: neurofilament heavy chain
NfL: neurofilament light chain

NK cell: natural killer cell
OCB: oligoclonal bands
OD: optical density
OGD: oligodendrocyte
OND: other neurological disease
PBMC: peripheral blood mononuclear cell
PNS: peripheral nervous system
PPMS: primary progressive multiple sclerosis
PPP: pentose phosphate pathway
RAG: recombination activating gene
ROS: reactive oxygen species
RR-EAE: relapsing-remitting experimental autoimmune encephalomyelitis
RRMS: relapsing remitting multiple sclerosis
SLE: systemic lupus erythematosus
SPMS: secondary progressive multiple sclerosis
TCR: T-cell receptor
TNF: tumour necrosis factor

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CHAPTER 1. Introduction

Overview of thesis

The research focus was to investigate the role of anti-neurofascin antibodies in a γδ T-cell-mediated mechanism of antibody-dependent cellular cytotoxicity, to gain a better understanding of the mechanisms used to target neuro-axonal elements in the context of multiple sclerosis, and to correlate these findings with the degree of neurodegeneration, as evidenced by the release of neurofilaments. In the present Chapter, basic concepts relating to MS immunopathogenesis are reviewed, including identified autoantibodies and the role of γδ T-cells. This will be followed by an introduction to neurofascin and the role of neurofascin-specific antibodies in MS. Lastly, autologous hematopoietic stem cell transplantation will be addressed as a means to eliminate the cellular immune components that instigate central nervous system (CNS) inflammation in the context of MS. Neurofilaments will be described as quantitative indicators of neurodegenerative disease activity, or cessation thereof, occurring at the neuro-axonal level in the absence of inflammatory stimuli. Chapter 2 is a description of the materials and methods used to perform the studies described in Chapters 3-5.

Multiple Sclerosis

General Introduction and Clinical Features: MS is a chronic disease of the central nervous system that is characterized by co-existent inflammatory demyelination and neurodegeneration. It is the most common cause of neurological disability, non-traumatic in nature, in young adults in North America and Europe, with more than 2.5 million individuals affected (1-3). MS is three-to-four times more common in women than men (4), it shortens the average patient's lifespan by seven-to-eight years, some have difficulty performing

household and employment tasks satisfactorily by ten years from disease onset, and approximately half of patients are non-ambulatory by twenty-five years (5).

Genetic susceptibility: There is both genetic and environmental involvement in MS disease pathogenesis. It is most common in Caucasians, with the highest prevalence in northern Europe (6). The risk increases in individuals with a family history of MS, and ranges from 0.2% in the general European population to 2-4% in siblings of MS patients and 30% in the monozygotic twins of MS patients (7).

The human leukocyte antigen (HLA) was the first genetic locus to be associated with MS. Genes encoded in this region play a role in almost all immune-related disorders (8), and nearly half of the genes associated with an increased risk of MS are shared with other autoimmune diseases such as Crohn's disease, Type 1 diabetes and rheumatoid arthritis (9; 10). The strongest association of the HLA locus is the DRB1*1501 allele (11); its frequency is between 3-20% of the European population. The frequency of the gene increases as the population risk of MS increases from the South to North of Europe (12). Each copy of the allele increases the risk of MS 3-fold, and it is the strongest risk factor for developing MS (9). In addition, HLA-DRB1*0301 and *1303, HLA-A*0201 and the HLA-DPB1*0301 (13) are associated with MS, with changes in risk between 26% and 200% (9).

There are 57 non-HLA gene associations that increase the risk of MS, including the cytokine receptor genes for IL-2 (*IL2RA*) and IL-7 (*IL7R*) (14-16), cytokines (*IL12A, IL12B*), co-stimulatory molecules (*CD58, CD6, CD40, CD80, CD86*), and signal transducer molecules (*TYK2*, *STAT3*) (9). Together with the HLA variants, these genes explain 25% of sibling recurrence risk (9). The effects of less common or rare variants in the genome are less well understood but may still contribute to MS susceptibility. Examples include the *TNFRSF1A* gene, which doubles the risk of MS (17; 18) and 3 variants of the *CYP27B1* gene

that cause a lack of 1-alpha-hydroxylase, which prevents the conversion of 25-hydroxyvitamin D to its biologically active form 1,25-hydroxyvitamin D (19).

Very few candidate genes have an obvious neurological function; mutations in *MANBA* and *GALC* can result in myelination complications and, while variation in the *KIF21B* gene, a member of the kinesin family, is associated with MS, it also plays a role in other autoimmune diseases, suggesting that its association might be immunological rather than neurological (7; 20; 21). The clinical heterogeneity of MS has been difficult to explain with regards to genetic association, though there is a relationship between higher genetic risk and earlier age at onset; for each copy of HLA-DRB1*1501 an individual carries, the age at onset decreases by nearly one year (9).

Epidemiology: Geographically, the prevalence of MS varies greatly. Estimates as low as ≤ 20/100,000 have been reported for Malta (22), while in Scotland (23), Northern Ireland (24), and parts of Scandinavia, high incidence of ≤ 200/100,000 have been reported (25; 26). In Canada, the prevalence of MS ranges from <100/100,000 in Newfoundland and Labrador (27) to approximately 300/100,000 in Saskatchewan (28). In a study of MS parental ancestry, non-specific European ancestry positively correlated with MS while British ancestry was associated with less risk (29).

The mean age at onset is 30 years of age; 70% of patients present symptoms between the ages of 20 and 40 years (30), and onset after 55 years is rare. Since 1970, the MS prevalence ratio of women to men has increased prominently to 2.3-3.5:1, which demonstrates that MS prevalence is on the rise in women but not in men (31-34). The influence of gender on the clinical features of MS is not as evident as its effect on prevalence, however, women have a slightly lower prevalence of primary progressive MS

and a slightly younger mean age at onset. In addition, the female:male ratio decreases as the age at onset increases (35; 36). It is possible that external factors, such as environmental or hormonal stimuli, cause different epigenetic modifications of deoxyribonucleic acid (DNA) between men and women (4). The X chromosome has been implicated as having a direct role in autoimmunity (37). In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, susceptibility is increased by the presence of two X chromosomes, independently of hormones (38), however, there has been no confirmation that any MS susceptibility genes are located on the X chromosome in humans (4). Gender differences in the immune system may also play a role. Adoptive transfer of EAE caused stronger symptoms when the T-cells were derived from female mice (39), and female recipients were much more susceptible to developing EAE (40). Vitamin D-enriched diets ameliorated the EAE disease course for female mice but the protection was abrogated when ovarectomy was performed (41). In humans a protective role for estrogen hormones in conjunction with vitamin D has been shown with respect to T-cell reactivity and disease course (42).

MS susceptibility is also associated with month of birth, whereby birth in the spring is associated with increased risk in the northern hemisphere, and the same is observed for autumn births in the southern hemisphere (43). This indicates that seasonal environmental agents may have some bearing on the risk of future MS development, and both maternal exposure to ultraviolet radiation or viral infections during pregnancy have been proposed to have an effect (44; 45).

There are a number of environmental factors associated with MS risk; the best documented include Epstein-Barr virus (EBV) exposure, smoking, and, as briefly stated above, low vitamin D concentrations. Ninety-nine percent of patients display seropositivity for EBV, however, this finding is complicated by the concurrently high rates of infection in

the general population. Suggestions for EBV and MS associations include immortalization of autoreactive B-cells, molecular mimicry with the CNS as the target, and modification of the 'hygiene hypothesis' with respect to the fact that MS is rare amongst EBV-negative individuals, who would normally be considered at greater risk if the hypothesis formulation were true (46; 47). Smoking approximately doubles the risk of developing MS, and the growing number of women who partake may explain, in part, the increasing MS incidence observed in women (48; 49).

Lower serum concentrations of vitamin D have been associated with an increased risk for MS development and they correlate with disability and brain atrophy (50). It is thought that the latitudinal gradient observed in the natural history of MS may be the result of lower serum vitamin D concentrations with increased distance from the equator (51). Vitamin D is converted into its active form in the liver and the kidneys; it then binds the vitamin D receptor in a wide variety of tissues and, in cells of the immune system, downregulates Th1 responses and enhances regulatory T-cell activity (52).

Disease Subtypes: Approximately 85% of patients are diagnosed with a relapsing/remitting course of MS (RRMS), where neurological deficits develop and last for several days to months and gradually improve (5; 53). Two thirds of RRMS patients will ultimately transition into a second phase of disease, secondary progressive MS (SPMS), that is characterized by unremitting, irreversible neurological decline that is less associated with relapses. The remaining 15% of patients have a primary progressive (PPMS) course of disease that is characterized by a continuous worsening of neurological deficits in the absence of specific relapses (5; 54). The median age at onset of progression is 40 years of age, regardless of whether the course was progressive at onset or secondary to an earlier relapsing/remitting phase (55; 56).

In addition, there is subset of patients who experience a controversial course of disease, referred to as benign MS. Studies have demonstrated that it was present in 20-26% of patients, that were followed for up to 50 years (57). There is a lack of consensus with respect to what defines this course but it is most often considered present when the Expanded Disability Status Scale (EDSS) score of a patient remains ≤ 3.0 for at least 10 years after disease onset (58). McAlpine described these patients as "without restriction of activity for normal employment and domestic purpose but not necessarily symptom-free," after a mean disease duration of more than 18 years (57). Though its definition is largely based on physical disability, namely of ambulation, it can be associated with significant non-motor symptoms including cognitive impairment, fatigue and depression (58; 59).

Prognosis: The prognosis varies greatly from patient to patient. Older age at onset, long tract-related dysfunction, such as muscle spasticity or bladder involvement, progressive initial disease course, and male gender are associated with poor outcomes, while younger age at onset, optic neuritis as the initial symptom, initial relapsing/remitting disease course, and being female are associated with a much better prognosis (36).

Immunopathogenesis

Clinical features: Nerve impulse conduction of myelinated axons in the CNS is dependent upon the high-density clusters of voltage-gated sodium (Na_v) channels at the nodes of Ranvier (60) between the ensheathing glia. Both the paranodal junctions and nodes of Ranvier must be intact to support saltatory conduction (61). The reversible disability experienced in RRMS is caused by a breach in the blood-CSF barrier and resultant focal inflammatory demyelination and axonal loss, caused by infiltrating autoimmune components. This results in temporary conduction block at the nodes of Ranvier and appearance of subsequent neurological symptoms. Resolution of the inflammatory response, reorganization

of axonal Na_v channels and remyelination help restore transmission and contribute to clinical remission. The axonal transection that occurs early in RRMS does not always manifest clinically due to the ability of the CNS to compensate for neuronal loss (5).

Immune-mediated CNS tissue injury is considered the primary pathology of MS, and the major cause of progressive and lasting disability is axonal loss (62). But for many years myelin was thought to be the target of autoreactive T-cells in MS, and demyelination was the primary disability-causing result, with newly-demyelinated axons becoming susceptible to soluble or cellular immune components by virtue exposure (63). The axonal component of MS has, historically, received less attention but since the late 1990s research has refocused on the role of axonal loss and neurodegeneration as the primary cause of irreversible and permanent neurological disability in MS (5). Currently, MS pathobiology is described as a primary demyelinating disease with secondary degeneration of axons. Demyelination may be T-cell- or antibody-mediated, or due to the death of myelinating oligodendrocytes (OGD) (64).

Axonopathy: A reduction in axon density has been observed in active and chronic MS lesions as well as normal-appearing white matter (NAWM) (63; 65; 66). Markers for axonopathy are dephosphorylated neurofilaments, disturbances in axonal transport (β-amyloid precursor protein [APP]), expression of specific sodium channels, and transection of axons (63; 67-69). There is a strong correlation between the degree of inflammation and the frequency of transected axons (67; 68), and early transection might result from exposure to inflammatory mediators (5), such as reactive oxygen species (ROS), proteolytic enzymes, free radicals and cytokines (70), as well as $CD8^+$ T-cells (71-73). In addition, though they have not received as much attention until recently, it has been postulated that autoantibodies may play a role in MS immunopathogensis.

Breakdown of the blood-CSF barrier

The microvasculature of CNS capillaries and post-capillary venules is composed of blood-CSF barrier endothelial cells. They lack fenestrations, display low pinocytic activity and have high efflux transporter activity, which restricts transcellular diffusion (74-76). Additionally they express tight and adherens junction proteins that limit paracellular flow. Tight junctions are composed of members of 3 transmembrane protein families, the claudins, the occludins and junctional adhesion molecules. These protein complexes form lipid rafts and are anchored to actin filaments. Adherens junctions are composed of transmembrane proteins such as vascular endothelial-cadherin and are linked to the actin cytoskeleton by catenins α, β, and p120 (77-81). The blood-CSF barrier endothelium is supported by pericytes (82; 83) and two basement membranes. The vascular basement membrane consists of extracellular matrix (ECM) molecules including laminins 8 and 10, type IV collagen, and perlecan (84; 85). The parenchymal basement membrane is composed of laminins 1 and 2, and dystroglycan, and the ensheathing astrocytic endfeet, which cover almost the entire abluminal surface of the microvasculature (77; 86). The perivascular space, the small area between the basement membranes, is a key area for reactivation of lymphocytes after they cross the blood-CSF barrier endothelial cells (86). Furthermore, astrocytes and microglia are closely associated with the blood-CSF barrier, and influence maintenance, integrity and immune regulation, though both are implicated in blood-CSF barrier dysfunction through the secretion of inflammatory cytokines in neuropathological conditions such as MS (77; 78; 87-89).

Despite a highly regulated blood-CSF barrier, leukocyte entry into the CNS is an early event in MS. Resting T-cells are limited in their ability to access the CNS parenchyma yet freshly activated T-cells, even with irrelevant antigen specificity, can enter the CNS (90;

91). Leukocyte migration further permeabilizes the blood-CSF barrier, which permits the infiltration of additional leukocytes (92; 93). The entry of inflammatory immune cells into the CNS is most likely the first step in MS that induces neuroinflammation, blood-CSF barrier compromise and permits the formation of lesions (94; 95).

Activated immune cells from MS patients directly enhance permeability of the blood-CSF barrier by expressing and secreting pro-inflammatory mediators such as cytokines and other soluble factors, ROS, and matrix metalloproteinases (MMPs) (88). Elevated tumour necrosis factor (TNF)-α in serum and peripheral blood mononuclear cells (PBMCs) correlates with disease activity and progression in MS (96; 97), and interferon (IFN)-γ tends to be elevated in MS, particularly during relapses (98; 99). These cytokines act synergistically to influence the expression of a variety of chemokines (88), such as CXCL9 and -10, CX3CL1, and CCL3, -4, and -5, which promotes adhesion and migration of leukocytes across the blood-CSF barrier endothelial cells (100-103). They also upregulate the expression of cell adhesion molecules (CAMs) that are important for capture and adhesion of T-cells to the CNS microvasculature (94; 104), such as ICAM-1, (105; 106) VCAM-1 (105-107), and the E- and P-selectins (108; 109). Furthermore, they upregulate members of the IL-6 family that can be involved in the generation of Th17-expressing cells (110). Th17 mRNA is elevated in the PBMCs of clinically isolated syndrome (CIS) and RRMS patients (98; 111), particularly during relapse (112; 113). The blood-CSF barrier endothelial cells of MS patients express the IL-17R, and engagement promotes down-regulation of occludin and causes endothelial cell dysregulation, which is thought to result in leukocyte migration (114; 115). Migrating leukocytes produce ROS, which disrupt junctional proteins (116). Upon CNS entry they continue to produce oxidative damage to astrocytes that further compromises

the blood-CSF barrier (117). Inflammatory cytokines lead to the leukocyte production of MMPs that are effectors of inflammation and degradation of ECM components that are important for migration (118). Disease activity in MS correlates with the potential of PBMCs to degrade ECM components (119) and MMP-8 and -9 are upregulated in MS sera (97; 120-122).

Autoantibody-induced Mechanisms of Pathogenesis

Demyelination: Antibodies are frequently observed in acute MS lesions, and they can induce demyelination by several mechanisms. Most commonly they are observed in conjunction with complement deposition. Complement components bind antibodies, leading to activation of the complement cascade, the assembly of the membrane attack complex and, finally, target destruction. Antibodies also opsonize target antigens for phagocytosis of the antigen-antibody complexes by macrophages (123) that produce TNF-α and nitric oxide, that have both been implicated in demyelination and oligodendrocyte (OGD) toxicity (124). Less commonly, autoantibodies bound to tissue have been shown to alter the migratory pathway of autoantigen-specific T-cells to the site, resulting in the infiltration of effector cells of the adaptive immune system and subsequent tissue destruction (125). Besides their role in the activation of immune effectors or the complement pathway, antibody deposition itself might induce demyelination. Pathogenic anti-myelin oligodendrocyte glycoprotein (MOG) antibodies have been shown to repartition MOG into lipid rafts, which induces both a stress-related pathway that upregulates heat shock protein (HSP)-70, and reduces cytoskeletal integrity through changes in surface phosphorylation that leads to a retraction of OGD processes and cell membranes (126; 127).

Rat spinal cord implantation of an immunoglobulin (Ig)M-antigalactocerebroside hybridoma caused focal demyelination and remyelination, along with the presence of axonal

and blood-CSF barrier damage. The lesions resembled those observed in MS and were probably the result of the interaction between intrathecal IgM and complement, and macrophages (128). A similar hybridoma secreting IgM-antisulfatide produced similar lesions and also prevents remyelination at sites where the hybridomas persist, due to ongoing damage to OGD precursor cells that express sulfatide (129).

Antibody-dependent cellular cytotoxicity: It is not known if there is a pathogenic component to the generation of autoantibodies in MS or whether their production is a secondary consequence of the hallmark immune-mediated tissue destruction that permits the exposure of otherwise sequestered antigens to immune effectors. ADCC is a lytic process whereby FcR-expressing effector cells, usually of the innate immune system, lyse target cells that are bound to specific immunoglobulin (Ig)G antibodies (123; 130). Lysis is carried out by the release of inflammatory mediators such as perforin or granzymes, by the interaction of FasL and TRAIL, or by the production of cytokines (131).

Autoantibodies in MS

There is a growing list of reactive Ig molecules, detected in the sera and CSF of MS patients, and they have been implicated as potential effectors in the pathogenesis of MS, though there is currently little evidence to substantiate this hypothesis *in vivo*. Intrathecal IgM antibodies are the first immune effectors present in the MS CNS, and their presence predicts disease course (132-135). Though antibody presence in MS is well documented, most target antigens have yet to be elucidated. The first CNS-specific antibodies identified in MS were OGD-specific and included antibodies that targeted intracellular MBP (136) and myelin-associated glycoprotein (MAG) (137). Anti-MOG antibodies were later discovered (138), followed by anti-proteolipid protein (PLP) antibodies in a small subset of patients (139). The majority of MS patients, however, do not exhibit reactivity to MOG (140), but

evidence supporting therapeutic plasma exchange in disease amelioration for a proportion of patients (141) suggests there are more to be discovered.

Oligodendroglial Antigens: Oligodendroglial antigens are plausible targets of autoimmunity due to OGD distribution and their large surface area in the CNS architecture. MS serum IgG has been shown to recognize tubulin β4, collapsin response mediator protein (CRMP)-5, CNPase I and transketolase. MS CSF IgG also recognized 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase) I and transketolase in addition to CRMP2 (142). CNPase 1 is involved in membrane biogenesis and maintenance in myelinating cells (143), while tubulin β4 plays a role in the OGD microtubule establishment for molecular transport during myelination (144). Transketolase was largely expressed by mature OGD but also in the OGD-precursor cells near lesion borders, probably because of its fundamental role in the pentose-phosphate pathway (PPP) (145), which is also involved in myelination. Antibodies to transaldolase were detected in the sera of approximately 30% of MS patients assayed but not in other autoimmune patients, and antibodies were also detected in the CSF of 76% of the seropositive patients (146; 147). Transaldolase is a rate-limiting enzyme of the PPP and is involved in the maintenance of cellular integrity by neutralizing reactive oxygen intermediates (145-147), and not surprisingly, transaldolase is lost at demyelinated sites. It is not yet known whether antibody production occurs early in the course of disease (147), or if production is ongoing in NAWM.

Several studies have indicated that antibody production may be MS subtype-specific. SPMS and PPMS patients exhibited an increase in CNS-reactive IgG antibodies to isoforms of CNP, MOG, OGD-specific protein and PLP, none of which were detected in RRMS patients (148). In addition, antibodies to galactocerebroside, which accounts for one third of

CNS myelin lipid content, were detected in the sera of MS patients, with significantly high titres in RRMS patients. In addition they were identified in the plasma in marmoset RR-EAE but with later appearance than other anti-lipid autoantibodies, anti-MOG and anti-MBP (149). Galactocerebroside-reactive antibodies have demyelinating properties both *in vitro* (150-152) and *in vivo* (128; 153; 154), though their pathogenic role in the context of MS remains elusive. In addition, IgM and IgG antibodies that target sulfatide, a myelin glycosphingolipid that plays a role in OGD differentiation, were detected in the CSF of 20% of MS patients; significantly more than in other neurological diseases (OND) and controls. Anti-sulfatide antibodies were detected in all subtypes of MS, though the frequency was higher in SPMS (30%) than RRMS (15%) and PPMS (14%) (155).

Neuro-axonal antigens: MS CSF IgG has been shown to recognize cytoskeletal molecules Sirtuin 2, Radixin and actin-interacting protein 1, preferentially in SPMS. MS serum IgG recognized cytoskeletal α-centractin, CRMP1, pyruvate kinase and syntaxin-binding protein 1 (142). The cytoplasmic subcellular localization of these molecules suggests that antibodies are produced following neuroaxonal injury. CRMP1 plays a role in neurite outgrowth and growth cone steering (156) while syntaxin-binding protein 1 participates in synaptic vesicle docking and fusion in neuronal cells (157).

Intrathecal production of antibodies to the light chain of the neuronal structural protein, neurofilament, has been observed in PPMS and SPMS. Titres correlated with disease duration and the EDSS, and the antibodies bound neuronal components in almost all patients assessed (158). A significant correlation was observed between antibodies specific to the light chain of neurofilament (NfL) and magnetic resonance imaging (MRI) markers of inflammation and tissue destruction, the strongest being for the parenchymal fraction, a

measure of atrophy. The correlations were especially strong for the RRMS phase of disease (159).

Ubiquitous antigens: Antigen microarray analysis identified serum autoantibody signatures that were associated with the clinical subtypes of MS. RRMS patients reacted more often with IgM reactivity to HSP60 and HSP70, but this was not the case for SPMS and PPMS patients (148). The proteasome is a ubiquitous complex responsible for non-lysosomal protein degradation in eukaryotes, and autoantibodies to proteasomal subunits have been identified in MS patients (160). IgM and IgG anti-proteasome antibodies were detected, respectively, in 66% and 61% of MS sera and CSF compared to no healthy controls and, of these patients, 67% were seropositive at the time of the first attack, suggesting that anti-proteasome antibodies are present very early in the course of disease. Interestingly, proteasome seropositivity was also reported in other inflammatory autoimmune diseases such as systemic lupus erythematosus (SLE) (161; 162) and Sjogren's syndrome (163), though the prevalence of anti-proteasome seroreactivity in MS remains significantly higher (160).

Antibodies to β-arrestin were detected in the sera of more than half of MS patients assayed, but not in the CSF or in OND. Serum titres were higher in patients in relapse compared to those in remission (164). In addition, anti-glycan IgG antibodies have been detected in the CSF (165) and sera (IgM) of RRMS patients (166; 167) and the presence of higher serum concentrations of at least one anti-glycan antibody (anti-GAGA2, -GAGA3, -GAGA4, and –GAGA6) at the time of first presentation predicted a shorter time to first relapse (167). Furthermore, a synthetic glycopeptide antigenic probe, termed CSF114(Glc), has been established as a tool to detect, isolate and characterize N-glucosylated autoantibodies as biomarkers of MS. An essential β-hairpin conformation exposes the

minimal epitope, Asn(Glc), to autoantibodies, recognizing them with high affinity and specificity. Using this method, high-titre IgM antibodies were detected in 21% of RRMS patients (the IgG response was unspecific) (168; 169), which supports the emphasis not only on IgG antibodies in MS (170) but that IgM binds different glycosylated epitopes (129).

Glycoprotein antigens: IgM and IgG_2 autoantibodies to peptide and glycosylated epitopes of glycoprotein contactin-2/TAG-1 were identified in the sera and CSF of MS patients. Contactin-2/TAG-1 is sequestered in the juxtaparanodal region of myelinated axons on both the axolemma and myelin sheath where it is involved in the clustering of voltage-gated potassium channels (K_v) (171; 172). While adoptive transfer of TAG-1-specific $CD4^+$ T-cells induced EAE with lesions in the cerebral cortex and spinal cord, co-transfer of TAG-1-specific monoclonal antibodies (mAb) did not alter the immunopathology observed, suggesting the TAG-1 antigen is not available for binding *in vivo* (173).

A proteomics-based approach, however, identified an additional glycoprotein, neurofascin (Nfasc), as a target of autoantibodies in MS (173; 174). Nfasc-specific antibodies were identified in the sera of MS patients; when immuno-affinity purified they recognized and bound to Nfasc186-transfected HeLa cells. Subsequent investigation into their functional role in the context of EAE demonstrated a rapid and considerable increase in initial disease severity. Histological examination resulted in no significant differences between anti-Nfasc antibody-treated subjects and their control counterparts in the degree of inflammation or demyelination observed, but disease exacerbation was associated with an increase in immunoreactivity for β-APP. Of pivotal importance, antibody binding was restricted to the nodes of Ranvier, where it colocalized with Na_v, suggesting its target is Nfasc-186, rather than the oligodendroglial Nfasc-155 isoform.

Gamma-delta (γδ) T-cells

The human γδ T-cell receptor (TCR) was first discovered in 1986 (175). It has since been shown that γδ T-cells are important effector elements of the immune system, unique in their ability to bridge the gap between what are known traditionally as the innate and adaptive immune systems. γδ T-cells have pluripotent abilities; they are capable of assessing the identity of invading organisms and lysing them directly, or initiating a secondary response from the adaptive immune system.

Like αβ T-cells, γδ T-cells develop in the thymus and generate their diverse TCR via recombinant activating gene (RAG)-mediated V(D)J recombination (176). Most circulating γδ T-cells lack CD4/CD8 (177; 178), though some γδ T-cells are weakly $CD8^+$ (179). In adults γδ T-cells comprise 0.5-5% of circulating $CD3^+$ T-cells, with $V\gamma2^+V\delta2^+$ T-cells being the most prevalent (180-182), and capable of rapid proliferation upon antigenic encounter (183). Effector $V\gamma2^+V\delta2^+$ subsets resemble cytotoxic natural killer (NK) cells with high levels of perforin and NK receptors (CD16, CD94, NKG2A, killer-cell Ig-like receptors [KIR] and others) and with low levels of chemokine receptors (184; 185).

Biological Function: Many functions of γδ T-cells have been described. They are involved in immune and tumour surveillance (186), they lyse specific target cells (187; 188) and can act as professional antigen-presenting cells (APC) to αβ T-cells *in vitro* (189). Human γδ T-cells are capable of phagocytosis, and subsequent antigen processing and presentation on the major histocompatibility complex (MHC)-II (183). When stimulated, they can produce and express *de novo* MHC-II molecules and express adhesion molecules and cluster with naïve αβ T-cells, inducing their proliferation and differentiation into T helper (Th)1/2 cells, as well as inducing the differentiation of $CD8^+$ naïve αβ T-cells into alloreactive cytotoxic T

lymphocytes (CTL)s (189). γδ T cells produce and release a full spectrum of cytokines and chemokines (190; 191), can exist phenotypically and functionally as distinct Th1/Th2 subsets (191-194) and can provide the necessary help for B-cells to make Ig (191; 195-198).

Antigen Recognition: What truly sets γδ T-cells apart from their αβ T-cell relatives is that they are largely unrestricted of MHC molecules (191; 199-201) and do not require the processing and subsequent presentation of antigen (202). The TCR of γδ T-cells is a specialized pattern recognition receptor, similar to that of an antibody, which gives this T-cell subset a unique and widespread capacity for reactivity to an array of largely non-peptide antigens. Examples include modified phospholipids, lipoproteins and oligonucleotides (203-205). They also recognize alkylamines (205), glycolipids (206; 207) and unprocessed proteins directly, such as viral proteins (205; 208) and HSP (209-212). They also express specialized receptors for non-classical stress-induced MHC class-I-like molecules such as MICA or MICB (213; 214) and glycolipids linked to CD1 (215). While many of these antigens are produced by pathogens, others are cellular manifestations of stress or transformations, (216; 217) suggesting that γδ T-cells are inherently self-reactive. There is a fine balance between self-immunotolerance and autoimmunity and it is, therefore, conceivable that a slight disturbance could shift the response towards self-reactivity (217).

γδ T cells in MS: γδ T-cells are more numerous in MS CSF than controls (218) and they are concentrated in plaque tissue compared to other organs (219-221). Brain-derived γδ T-cell receptors demonstrate limited heterogeneity, which is indicative of a localized clonal response to antigen (218; 222-224). γδ T-cells recognize HSP, which are expressed on OGD in MS (225-227) and they release cytokines that are directly cytotoxic to human OGD *in vitro* (228), possibly via a perforin-mediated mechanism (221), and perforin is increased in

the brains (229) and blood (113) of MS patients. Human OGD are also susceptible to γδ T-cell-mediated killing via NKG2D (230). Although OGD may be injured by a Fas (CD95)-mediated mechanism (231-233), and γδ T-cells are Fas-ligand positive (191), the majority of OGD death is probably via a lytic pathway and not by apoptosis (234).

γδ T cells can be responsible for the early damage and clinical symptoms of EAE induced by myelin-reactive αβ T cells (235) and may provide the necessary early signals for infiltrating autoreactive MBP cells (235; 236). They have a restricted pattern of δ chain gene rearrangement, demonstrate an activated immunophenotype, expressing CD25 (IL-2R) and CD56 (237) and MS disease progression is positively correlated with an increase in $CD16^{+}$ γδ T-cells (217) that are known to induce ADCC (130).

FcγRIII (CD16): CD16 is a low-affinity Type III activating receptor that binds the Fc portion of IgG. Two nearly identical CD16 transcripts exist, encoded by two homologous genes: *FcγRIIIa* and *FcγRIIIb*. CD16a is expressed by a number of leukocytes including macrophages, NK cells, granulocytes and γδ T-cells (238). Its cytoplasmic tail associates with an FcRγ chain, and engagement triggers immunoreceptor tyrosine-based activation motif (ITAM)-dependent activation that is initiated by SRC-family protein kinase-mediated phosphorylation of tyrosine residues at the FcRγ-ITAM motif. This recruits Syk kinase to interact with the phosphorylated ITAM, which activates PI-3K and PLCγ to mobilize Ca^{2+} and stimulate MAPK, reorganizing the cytoskeleton (239). In spite of weak binding affinity, immune-complexed IgG enables more than one low-affinity receptor to bind antibody concurrently; therefore, cells with multiple FcγRs have higher avidity for opsonized targets (240-242). On the contrary, CD16b expression is exclusive to neutrophils. It does not contain an intracytomplasmic tail, and functions as a decoy receptor (238).

Downstream effector functions of CD16-immune complex engagement include phagocytosis, cytokine release and ADCC (243). Activation of CD3 or CD16 on resting γδ T-cells results in a highly cytotoxic cell population that can release cytokines, such as TNF-α, upon CD16 engagement (185; 244). The existence of a population of CD16-expressing γδ T-cells suggests the possibility that these cells might have cytotoxic capabilities indirectly via ADCC through CD16 engagement and subsequent activation.

CD16-expressing γδ T-cells are decreased in number in Graves' Disease (Bossowski et al., 2003), SLE (245) and rheumatoid arthritis (246), whereas they are increased in the blood of patients affected by active inflammatory bowel disease (247) and Sjögren's syndrome (248). With respect to MS, CD16 is expressed in active lesions and NAWM, as well as on perivascular macrophages and endothelial cells in the MS brain (249). The percentage of circulating $CD16^+$ γδ T-cells is significantly increased in patients, particularly in those with a progressive disease course. The percentage of $CD16^+$ γδ T-cells correlated with both disease duration (years since disease onset) and EDSS. Control and MS patient-derived γδ T-cells were also shown to upregulate their expression of CD16 following exposure to proinflammatory cytokines, IL-2, IL-12 and IL-15 (217).

Antibody-dependent cellular cytotoxicity: ADCC is a mechanism of cell-mediated defense whereby an effector cell of the immune system lyses a target cell that is opsonized by specific antibodies; it has generally been described in the context of limiting viral infection and tumour cells. NK cells are thought to be the key mediators of classical ADCC since they do not co-express inhibitory FcR, but it can also be mediated by macrophages and granulocytes (131; 238). The effector cell possesses FcR that bind the Fc portion of specific antibodies bound to a target, usually a virus-infected cell. Upon binding, a strong activating

signal is elicited and the effector cell responds with the release of pro-inflammatory cytokines that destroy the target (250).

$CD16^+$ Vγ9Vδ2 T-cells recognized and exerted ADCC on $CD20^+$ B-cell targets in conjunction with rituximab, a genetically engineered chimeric murine/human mAb (251). Rituximab acts through several mechanisms but a homozygous valine at amino acid position 158 of CD16 is thought to result in a higher affinity for IgG that preferentially mediates ADCC, rather than complement-mediated cell lysis or induction of apoptosis (252). γδ T-cell-mediated ADCC has since been confirmed in the Freedman laboratory; $CD16^+$ γδ T-cells specifically lysed rituximab-coated target cells (Burkitt's B lymphoma, expressing CD20*) in vitro*. Lysis correlated with rituximab concentration, surface CD16 expression, and effector-to-target (E:T) ratio (130).

Neurofascin

Neurofascin was initially discovered in the chick (253), and is an ankyrin-binding CAM of the CNS (254) that is regulated by tyrosine phosphorylation (255). It belongs to the neuronal L1 subgroup of the Ig superfamily of glycoproteins that also includes L1, Neural cell adhesion molecule L1-like protein (CHL1) and neural cell adhesion molecule (NrCAM). Together these polypeptides regulate the development and maintenance of neuronal elements and their networks (256-258).

Structure: The Nfasc gene maps to human chromosome 1q31-q32 (259). It is subject to a great deal of alternative mRNA splicing and, in the chicken, is expressed temporally throughout development in at least fifty isoforms through the generation of ten alternatively spliced exons (260), suggesting a complex network of possible interactions in neural

regulation (261) from early development to structural maintenance in the mature nervous system.

Four major Nfasc variants, Nfasc-155, -160, -180 and -186, are expressed in the nervous system (Hassel et al., 1997), though expression of Nfasc-160 and -180 is thought to be limited to early development and they are not expressed in the mature CNS (262). The two most thoroughly-characterized Nfasc isoforms are Nfasc-155 and Nfasc-186, named for their molecular mass on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their expression is cell-type-specific with Nfasc-186 expressed by cells of neuronal lineage and Nfasc-155 expressed by myelinating Schwann cells or OGD (263; 264). They exhibit a high degree of sequence homology (265). The extracellular domain of all Nfasc variants contains six Ig-like domains. These are followed by four fibronectin (FN)-III repeats in the Nfasc-155 variant, while the third is absent from Nfasc-186. Additionally, the fourth FN-III domain is replaced by a mucin-like domain (rich in proline, alanine and threonine) and a fifth FN-III domain is present at the proximal end of the cytoplasmic portion of the protein. All Nfasc polypeptides contain a single transmembrane domain and a short cytoplasmic tail that binds to cytoskeletal elements such as ankyrin$_G$ (254) and ezrin, which allows for signal transduction (266). Lastly the N-terminal Ig1-Ig4 domains form a horseshoe-like structure that mediates homophilic interactions in *trans* between membranes (267; 268). This interaction is responsible for neurite transduction (267).

Localization and function: Nfasc is implicated in many physiological processes including the outgrowth of neurites, fasciculation, and interneuronal adhesion (265; 269-272). More importantly, it is necessary for the localization, assembly, and organization of axon initial segments and nodes of Ranvier in both the CNS and peripheral nervous system (PNS) (273-276).

Structural Variants

Neurofascin-155: Oligodendroglial Nfasc-155 expression begins at the onset of myelination (262; 264). It is targeted to the paranodal loops of the sheath where it associates with contactin and contactin-associated-protein (Caspr) on the axonal membrane to form a complex at the paranodal junction that flanks the node of Ranvier (264; 277; 278). Once axonal ensheathment is underway oligodendroglial mRNA transcripts decline abruptly, suggesting Nfasc may be, at least in part, responsible for mediating and signaling the recognition and subsequent interaction between axonal and glial counterparts at the onset of myelination (263; 264).

Neurofascin-186: Nfasc-186 is localized to the axonal membrane at the node of Ranvier in the mature nervous system where it associates mainly with ankyrin$_G$, NrCAM, and a high-density cluster of Na_v channels (275; 279; 280), suggesting its importance in the initiation and propagation of action potentials (281). It accumulates by an ankyrin-independent mechanism, but spatial maintenance is dependent upon ankyrin-binding (282).

Temporal Expression: Nfasc and its binding associates are detected along a very similar timeline temporally, though variation has been documented. In CNS spinal motor neurons, Na_v and ankyrin$_G$ precede (pan)Nfasc expression in the nodes of Ranvier (283); however, in the PNS, Nfasc-186 precedes the Na_v channel clustering and ankyrin$_G$ recruitment, and nearly always accumulates before Nfasc-155 (275; 284). The expression of the Na_v channel β1 subunit, specifically, is initialized in the rat sciatic nerve and in the brain along the same timeline as Nfasc (days 3 and 10, post-natally), and their interaction persists through adulthood, supporting the importance of Nfasc in node of Ranvier formation as well as in stabilizing the mature node. Interaction between the β1 and β2 Na_v channel subunits and

Nfasc occurs at the Ig1 and FN2 sites of Nfasc (275; 285). Interestingly, Na_v channel β subunits have CAM properties in that they interact with ECM and cytoskeletal elements, and they are involved in cell migration and aggregation (286; 287). In support of Nfasc-186 as a regulator of the mature nervous system, neurite outgrowth was inhibited by the expression of the fifth FN-III domain of adult Nfasc-186 (261; 274).

Disturbance: The importance of Nfasc is exhibited in subjects where it is absent or its expression disrupted. The interplay between nodal molecules is necessary for the correct assembly of Na_v channels, and Nfasc-186 appears to be pivotal in the process as clustering fails to occur in Nfasc-186-null animals (278). Nfasc-186-deficient mice also suffer from abnormal PNS node of Ranvier formation and disrupted localization of $ankyrin_G$ and Na_v channels (278), which are recruited to the node of Ranvier after Nfasc targeting (273). These mice also exhibit disorganization of the paranodal junctions, which reduces conduction velocity and results in muscle paresis and tremors (278; 288; 289). Ablation of the Ig5 and Ig6 domains of mouse Nfasc-155 abolishes functionality; septate junctions are absent with Caspr and contactin diffusing from the paranodes and juxtaparanodal potassium channels redistributing in the direction of the nodes. Additionally, contactin fails to associate with Nfasc-155. Ablation of Nfasc-186 Ig5 and Ig6, however, is not functionally catastrophic (290).

Neurofascin alteration in MS: Neurofascin was first implicated as a target of the immune system in MS when it was observed that Nfasc-155^+ paranodal structures were altered both within and adjacent to actively demyelinating white matter lesions, post-mortem, and that it was associated with damaged axons, prior to demyelination (291).

Neurofascin antibodies in MS: Anti-Nfasc antibodies have been detected in the sera of as many as 30% of MS patients (174; 292; 293) and, functionally, they recognize and bind to

Nfasc-186-expressing cells *in vitro* (174). In the context of EAE, co-transferred pan-Nfasc antibodies colocalized with Na_v channels at the nodes of Ranvier, and were shown to enhance initial disease severity that likely resulted from acute axonal injury (174). An axonopathic autoantibody response was again responsible for increased disease severity when animals were primed with Nfasc-186 prior to EAE induction (294). These reports provide evidence that the CNS target is Nfasc-186, rather than the oligodendroglial Nfasc-155 isoform (174).

Commercial pan-anti-Nfasc antibodies bound to the axonal surface of myelinating cultures generated from embryonic rat spinal cord and resulted in complement-dependent loss of axons and secondary complete demyelination (292). Additionally, when one individual patient-derived IgG fraction was depleted of anti-Nfasc-155, the resultant IgG fraction was unable to mediate demyelination and axonal loss *in vitro*, confirming that Nfasc can, indeed, be a target of an injurious response in MS and also suggests that pathogenic mechanisms differ between patients (292). In a subsequent report, one third of MS patient-derived anti-Nfasc antibodies mediated complement-dependent demyelination of terminally-differentiated OGD in a myelinating culture system. Autoantibodies capable of demyelination were detected more frequently in RRMS patients than PPMS (292).

Neurofascin in the peripheral nervous system: Inflammatory demyelinating diseases also occur in the PNS. Nfasc density was diminished at the nodes of Ranvier prior to the onset of clinical symptoms in a model of Guillain Barré Syndrome (acute inflammatory demyelinating polyneuropathy [AIDP]) in the Lewis rat, induced by immunization against peripheral myelin. Subsequently, nodes lacking Na_v channels and $ankyrin_G$ were observed, and correlated with increasing demyelination. This suggests that adhesive molecules of the nodes are disrupted or lost first, and that diffusion of Na_v channels is dependent upon paranodal disruption. Conduction deficits were also observed, antibodies directed at both

Nfasc-155 and -186 were detected in the sera of these animals and antibody deposition at the nodes of Ranvier was independent of complement (295).

Autologous hematopoietic stem cell transplantation

History and therapeutic benefits in EAE and MS: Conventional disease modifying therapies (DMT) for MS largely target cellular components of the immune system and the blood-CSF barrier, and have proven efficacious with regards to reducing relapse rates (2; 296), slowing clinical disease progression (2), and reducing the number of new lesions (297; 298); though during the latter progressive phase, efficacy declines as the neurodegenerative processes seem to be habitually independent of immune-mediated inflammation (299-301).

The therapeutic benefits of autologous hematopoietic stem cell transplantation (aHSCT) for the treatment of severe autoimmune disease in animals and humans are well documented. Since 1996, it has become a promising treatment for systemic sclerosis (302), SLE (303-305), juvenile idiopathic arthritis (306), hematologic immune cytopenia (307), and MS (297; 298; 308-316). The rationale for its use in the treatment of MS has come from studies in EAE that demonstrated reversal of clinical symptoms and prevention of relapses following allogeneic transplant (317-319). Others demonstrated suppression of chronic EAE and the induction of tolerance to the immunizing antigens with high-dose cyclophosphamide treatment for the elimination of immunocompetent lymphocytes and recovery by syngeneic bone marrow transplantation (320-322).

Procedure: Hematopoietic stem cells (HSC) are derived from the bone marrow, and typically express CD34, CD133, CD45. They give rise to blood cells including red blood cells, T- and B-lymphocytes, NK cells, macrophages, granulocytes and monocytes (323; 324). HSCs are harvested from bone marrow by aspiration from the ileac crest or they are mobilized into the peripheral blood by use of cyclophosphamide (1.5-4.0 g/m^2 over 1-2 days, followed by G-

CSF (5-12 μg/kg/day) until the harvest is complete. HSCs are identified by CD34 expression, collected by leukapheresis, depleted of T-cells, and cryopreserved until engraftment. A minimum dose of $3x10^6$ $CD34^+$ cells/kg bodyweight is generally administered. In addition, a potent immune-ablative conditioning regimen (Busulphan 9.6 mg/kg, Cyclophosphamide 200 mg/kg, rabbit Anti-thymocyte globulin (ATG) 5 mg/kg) is undertaken within 1-2 months of stem cell collection to eliminate autoreactive clones (lymphocytes and hematopoietic cells) from the peripheral blood, bone marrow, lymphoid tissue and the CNS. The graft is then thawed and infused through a central venous catheter; recovery of cell counts occurs 10-20 days thereafter. Autoreactive T-cells that survive the conditioning regimen or that are reinfused with the graft are depleted with polyclonal anti-thymocyte globulin or thymocyte-specific mAbs (314).

Clinical outcomes: Three-year follow-up studies have reported that most patients did not progress clinically (as assessed by EDSS) (298; 325), most had fewer relapses (309), contrast-enhancing MRI activity was suppressed (297; 298; 308-310; 326; 327) and brain atrophy declined (328; 329).

Neurofilaments as indicators of neurodegeneration in MS

Structure and clinical features: Neurofilaments are classified as Type IV intermediate filaments (330), and are the most abundant structural proteins in neurons. They are primarily localized in the axons and dendrites, and consist of filaments composed of heteropolymers of 4 subunits, each 10 nm in diameter (331). The light (61 kDa) and medium (NfM; 102.5 kDa) chains are coded on chromosome 8p21 and consist of 543 and 916 amino acids, respectively, and the heavy (NfH; 111 kDa) chain is coded on chromosome 22q12.2 and consists of 1020 amino acids (332). The fourth subunit is α-internexin in the CNS or peripherin in the PNS

(330; 331). Neurofilament proteins are released into the CSF in episodes of acute CNS destruction such as cerebral infarction (333; 334) and subarachnoid hemorrhage (334), and are also predominant in neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, (335) and MS.

Neurofilament heavy chain: NfH exerts regulatory influence on axonal transport and cell structure homeostasis (332). Its C-terminal domain, consisting of 42 lysine-serine-proline repeats (336-338), is the most extensively phosphorylated protein in the human brain (332; 338-340) and is highly resistant to protease degradation (332; 340-343). It has been associated with chronic CNS injury and predominates in the CSF of PPMS and SPMS patients (344), which likely indicates accumulation of axonal damage. Importantly, it has been consistently related to disease progression as measured by the EDSS (332; 345-347), though it has been correlated with relapse activity as well (345; 347).

Neurofilament light chain: Due to its relative abundance and the existence of specific detection assays, neurofilament release associated with MS has focused largely on NfL. It is the most abundant of the neurofilaments, and plays an essential role as the backbone of the fibre, to which NfM and NfH co-polymerize (348). NfL is considered a marker of acute inflammatory axonal damage; in MS it is more concentrated during relapse than remission, it correlates with contrast-enhancing lesions, its levels are elevated in patients with OCB, and it relates to CSF cell count (347; 349-352). NfL is enriched in RRMS CSF compared to controls (219; 333; 349; 350; 353; 354) and it is decreased following treatment in RRMS and SPMS (355; 356). In addition, NfL has been indicated as a good predictor of conversion to clinically-definite MS (357) and, when compared to NfH as a therapeutic biomarker following natalizumab treatment, NfL emerged as superior with changes being more pronounced than with NfH, though the latter demonstrated improved correlation in patients

experiencing a relapse. It has not, however, definitively been shown to correlate with disease progression (356; 358; 359).

It has been proposed that the presence of neurofilaments in the CSF may be an indicator of ongoing neurodegeneration in MS (345; 355; 360-362). As such, neurofilament has naturally emerged as biomarker for disease activity, a measure of clinical disability (344; 347; 349-351; 356; 359; 362) and as a potential prognostic indicator (352; 363).

Summary

MS is an autoimmune demyelinating neurodegenerative disease of the CNS characterized by a multitude of mechanisms with involvement from both the innate and adaptive immune systems. γδ T-cells are thought to bridge this gap through their unique approach to antigen recognition, and more recently the gap was further reduced when, in their role as effectors, γδ T-cells specifically lysed target cells via ADCC (130; 251). In parallel, antibodies targeting the axonal glycoprotein Nfasc were identified in a proportion of MS sera. Harvested antibodies bound to Nfasc-186-expressing targets, and were pathogenic both *in vitro* and *in vivo* by demyelination and impairment of axonal transport, respectively (174), but the effector mechanisms involved and the extent to which antibodies specific to self-antigens are pathogenic remain unknown. Autoantibody-mediated targeting of the CNS could, thus, provide an alternative mechanism whereby γδ T-cells are pathogenic in the context of immune dysregulation in MS. Furthermore, immune dysregulation in MS is supported by patient improvement following the elimination of cellular immune components and autologous hematologic reconstitution. Quantification of neurodegenerative markers as a means of assessing neuronal integrity in both the presence and absence of inflammatory stimuli might provide evidence that neurodegeneration in MS is at least in part immune-

mediated, and that patient improvement is the result of a cessation of neurodegenerative activity.

RESEARCH OVERVIEW

Hypotheses

(i) MS patients produce antibodies to neurofascin; production is subtype-specific and correlates with neurodegeneration; (ii) In the absence of CNS inflammation following autologous hematopoietic stem cell transplantation, neuro-axonal elements are preserved; (iii) γδ T-cells induce ADCC by binding MS patient-derived anti-neurofascin antibodies.

Research Questions

1. *Are anti-Nfasc antibodies detectable in the sera and CSF of MS patients, and do they correlate with neurodegeneration? [Chapter 3]*

2. *In the absence of inflammation following aHSCT, is neurofilament release reduced [Chapter 4]*

3. *Do anti-neurofascin antibodies interact with γδ T-cells to lyse Nfasc-expressing target cells?[Chapter 5]*

CHAPTER 2. Materials and methods

2.1 Patients and sample collection

2.1.1 Anti-neurofascin antibody study

Sera and CSF samples were obtained from MS patients and OND controls at the time of diagnostic assessment at the MS Centre of the Ottawa Hospital General Campus. Samples were chosen retrospectively based on MS subtype, according to the "revised McDonald criteria" (364).

2.1.2 Autologous Hematopoietic Stem Cell (aHSCT) study

Sera and CSF were obtained at 4 time points from MS patients enrolled in the Canadian MS aHSCT study. Briefly, the subjects were between the ages of 18 and 50 years and had a diagnosis of active MS with relapses or progression, and sustained impairment. They were considered at high risk for progression and experienced disease activity such as deterioration in the EDSS or significant relapses in the previous 2 years.

2.1.3 Blood and cerebrospinal fluid collection

Blood was collected by venipuncture into a vacutainer containing no anticoagulant and allowed to rest for 30-45 min to permit clotting, after which time the tube was centrifuged for 10 min at 2400 rpm in an IEC Centra GP8R centrifuge (Thermo Fisher Scientific, Burlington, ON) with the brake turned off. Sera were removed to 5 ml polypropylene tubes. CSF was obtained by lumbar puncture, centrifuged at 2400 rpm for 10 min to remove cells and removed to a fresh 5 ml polypropylene tube. Sera and CSF were immediately stored at -80°C.

2.2 Anti-neurofascin antibody detection, purification, and characterization

2.2.1 Anti-Nfasc antibody Enzyme-linked immunosorbent assay (ELISA)

Wells of a 96-well microplate (Costar, Cole-Parmer Canada Inc., Montreal, QC) were coated with 100 μl of 5 μg/ml recombinant rat Nfasc-155 (R&D Systems, Cedarlane Laboratories, Burlington, ON) and incubated at 4°C overnight. The contents were removed and the wells were briefly washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Extraneous binding sites were blocked with 200 μl of blocking buffer (5% human serum albumin [Alburex HS-25, CSL Behring AG] in PBS) for 1 h with gentle agitation. Sera were diluted 100-fold in sample buffer (blocking buffer containing 0.05% Tween-20) and 100 μl were plated, in duplicate, and incubated for 1 h at room temperature (for CSF, 250 μl of undiluted CSF were added). A commercial goat anti-human neurofascin antibody (P-19) (Santa Cruz Biotechnology Inc., Santa Cruz, California) was used as a positive control. The plates were extensively washed, and 200 μl of 10^{-3} goat anti-human IgG-horseradish peroxidase (HRP) in sample buffer (or 10^{-4} rabbit anti-goat IgG-HRP for the positive control) were added to the sample wells and incubated at 37°C with 5% CO_2 for 1 h. Next, 150 μl of 3,3',5,5' tetramethylbenzidine (TMB) substrate reagent (BD Biosciences Pharmingen, Mississauga, ON) were added to each well and incubated in the dark for 5 min. The reaction was stopped with the addition of 100 μl of 1 M H_2SO_4. The absorbance was measured at λ450 nm using a microplate photometer (Multiskan Ascent 354; Thermo Fisher Scientific).

2.2.2 Western Blots

0.5 μg (5 μl of 0.1 μg/μl) of recombinant rat Nfasc-155 was mixed with 10 μl of 4x LDS sample buffer (Life Technologies, Burlington, ON) and heated at 70°C for 10 min. The

protein standard consisted of 4 μl of SeeBlue® Pre-stained protein standard (Life Technologies) and 4 μl of MagicMark XP Western Protein Standard (Life Technologies). Samples were electrophoresed in 4-12% NuPAGE® Bis-Tris gels (Life Technologies) at 200 V for 45 min in NuPAGE® MES running buffer (Life Technologies). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Thermo Scientific, Burlington, ON) in transfer buffer (20% methanol, 25 mM Tris-HCl in ddH_2O) at 30 V for 1 h. The membrane was blocked in 5% skim milk in Tris-buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5) for 1 h at room temperature or overnight at 4°C. The PVDF membrane was probed with high or low (anti-Nfasc antibody)-titre serum diluted 100-fold or CSF diluted 2-fold (or a commercial goat anti-human Nfasc antibody diluted 1:500 was used as a positive control) in 5% skim milk overnight at 4°C. The membrane was extensively washed with washing buffer (0.1% Tween-20 in PBS). The membrane was then probed with 1:14,000 goat anti-human IgG- HRP (or a rabbit anti-goat IgG-HRP for the positive control) in 5% skim milk for a minimum of 1 h at room temperature. The membrane was washed extensively, and during that time the enhanced electrochemiluminescence (ECL) solution was prepared by combining 400 μl of each of the following stock solutions:

Solution 1: 1 M Tris/HCl, 90 mM *p*-Coumaric acid, 250 mM Luminol in DMSO

Solution 2: 1 M Tris/HCl, 30% H_2O_2 in dH_2O

In a dark room, the membrane was placed in a propylene sheet protector and 800 μl of ECL solution were added. A sheet of CL-XPosure film (Thermo Scientific) was placed on top and the film was developed using a Kodak M35A X-ray film processor.

2.2.3 Purification of serum-derived anti-Nfasc antibodies

The NHS HP SpinTrap kit (GE Healthcare, Mississauga, ON) was utilized in conjunction with the NHS HP SpinTrap Buffer kit according to the manufacturer's instructions to purify anti-Nfasc antibodies from MS patient sera. Except where stated, all washes and equilibration steps involved the addition 400 μl of reagent, each step was repeated 3 times, and all centrifuge steps were performed at 150xg using an Eppendorf 5417R centrifuge (Thermo Fisher Scientific). Briefly, 400 μl of 50% gel slurry containing sepharose beads was placed into a SpinTrap column, which was placed into a 2 ml microcentrifuge tube and centrifuged to remove the storage solution before being equilibrated with ice-cold 1 mM HCl. Next, 450 μg of recombinant rat Nfasc-155 was suspended in 300 μl of coupling buffer (0.75 M triethanolamine; 2.5 M NaCl, pH 8.3) and added to the column and mixed end-over-end for 30 min at room temperature. The tube was centrifuged to remove unbound protein. The column was then blocked with a series of incubations alternating between high and low pH (2.5 M ethanolamine/2.5 M NaCl [pH 8.3] and 0.5 M NaAc/2.5 M NaCl [pH 4.0]) and equilibrated with binding buffer (0.5 M Tris; 1.5 M NaCl, pH 7.5). Next, 300 μl of high-titre patient serum was mixed with 100 μl of binding buffer and added to the column, mixed end-over-end for 1 h, and then repeated to maximize the anti-Nfasc antibody yield. The column was then washed with wash buffer (0.5 M Tris; 1.5 M NaCl; 2 M urea, pH 7.5). Anti-Nfasc antibodies were eluted with the addition of 200 μl of elution buffer (1 M glycine-HCl, pH 2.9) and by centrifuging for 1 min at 1000xg. Eluted antibodies were immediately neutralized with 45 μl 1 M Tris (pH 9) and stored at -80°C until use.

2.2.4 Quantification of MS patient-derived anti-Nfasc antibodies

Wells of a 96-well microplate were coated with 100 μl of 5 μg/ml recombinant rat Nfasc-155 and incubated at 4°C overnight. The contents were removed and the wells were briefly washed 3 times with PBS containing 0.05% Tween-20. Extraneous binding sites were blocked with 200 μl of 5% HSA in PBS (blocking buffer) for 1 h with gentle agitation. A 6 point standard curve was generated by making serial dilutions of a commercial goat anti-human Nfasc antibody. One hundred μl of each standard and a 10-fold dilution of harvested anti-Nfasc antibodies were added to appropriate wells and incubated for 1 h at room temperature with gentle agitation. The contents of the wells were removed and the plate was washed extensively. 100 μl of rabbit anti-goat IgG-HRP diluted 10^{-4} and goat anti-human IgG-HRP diluted 5×10^{-3} were added to appropriate wells, and the plate was incubated for 1 h at 37°C. The plate was again washed extensively and 150 μl of TMB were added to each well and incubated for 5 min in the dark. The reaction was stopped with the addition of 100 μl of 1 M H_2SO_4. The absorbance was measured at λ450 using a microplate photometer and a standard curve was generated using the OD values and known concentrations of the standard curve.

2.2.5 Anti-Neurofascin IgG Profiling

The Human IgG Subclass Profile kit (Invitrogen, Burlington, ON) was utilized according to the manufacturer's instructions and all steps were performed at room temperature. The lyophilized human serum control and human IgG subclass standard were reconstituted with 1 ml diluent buffer. A 6-point standard curve of the human IgG subclass standard was then generated with serial dilutions. Fifty μl of each human subclass-specific antibody (IgG_1, IgG_2, IgG_3, IgG_4) were added to appropriate IgG antibody pre-coated wells,

except for the zero wells. Then 50 µl of 1:5 diluted MS patient-derived anti-Nfasc antibody, standards, or the human serum control were added to respective wells and incubated for 30 min. The contents were removed and the wells washed 3 times with wash buffer. Next, 100 µl of diluted peroxidase anti-human IgG conjugate solution was added to each well and incubated for 30 min. The washes were repeated and 100 µl of TMB were added to each well and incubated for 10 min in the dark. Quickly, 100 µl of stop solution were added to each well and the plate tapped gently to mix. The absorbance was measured at λ450 using a microplate photometer and a standard curve was generated using the OD values and known concentrations of the standard curve.

2.3 Stable Transfection of HeLa cells

HeLa cells (CCL-2; originally obtained from ATCC) kindly provided by Dr. Kathryn Wright (Dept. of BMI, University of Ottawa) were seeded at 2 x 10^5 cells per well of a 24-well plate (Costar) and cultured in complete Roswell Park Memorial Instituate medium (cRPMI) overnight or until reaching 80% confluency. The full-length turbo-green fluorescent protein (tGFP)-tagged human neurofascin-186 cDNA clone in a pCMV6-AC-GFP vector was obtained from Origene (Rockville, Maryland, USA). The NCBI accession number is NM_001005388.2. The HeLa cell culture medium was replaced with 500 µl of opti-MEM, reduced-serum medium (Life Technologies) and transfection was carried out with the addition of 0.5 µg of the tGFP-tagged human neurofacin-186 cDNA clone and 0.75 µl of lipofectamine (Life Technologies). The cells were incubated for 6 hours at 37°C, then the media was replaced with cRPMI. The following day the medium was replaced with selection medium; cRPMI supplemented with 700 µg/ml geneticin (G418; Invitrogen, Burlington, ON), and it was replaced every other day thereafter. Flow cytometry was used to assess the

transfection efficiency, and performed using a Coulter FC500 flow cytometer (Beckman Coulter) equipped with one Argon laser. A tube containing control, untransfected ($tGFP^-$) HeLa cells was used to set up the voltage and compensation values and the emission of tGFP was redirected through the 500 nm specific band-pass optical filter and detected through FL1. Ten thousand events were acquired.

The transfection efficiency as measured by the percentage of $tGFP^+$ cells was <10%, thus the $tGFP^+$ cells were separated by fluorescence-activated cell sorting (FACS; StemCore Flow Cytometry Facility, Ottawa Hospital Research Institute) and re-seeded in selection medium with replacement every other day until use.

2.4 Immunohistochemistry

Control and Nfasc-transfected HeLa cells were seeded at 6 x 10^5 cells/well of an 8-well Lab-Tek II chamber slide (Cole Parmer Canada Inc.) and incubated overnight at 37°C to promote adherence. The following day, the medium was removed, and the chambers were washed with PBS. The chamber was removed and the slide was fixed in ice-cold methanol for 10 min. Fifty µl of universal protein block (Dako, Burlington, ON) was applied to each section and allowed to incubate for 30 min at room temperature.

A rabbit anti-human Nfasc antibody raised against an intracellular domain of Nfasc, recognizing Nfasc-155 and Nfasc-186, was kindly provided by Dr. Peter Brophy at the Centre for Neuroregeneration at the University of Edinburgh and was used as a positive control. It was diluted 1:1000 and MS patient-derived anti-Nfasc antibodies were diluted 1:2 in blocking buffer (1:5 universal protein block in 5% bovine serum albumin [BSA] buffer [5 g BSA in 100 ml 0.01 M PBS; pH 7.6]) and 30 µl were carefully added to the appropriate chambers and incubated for overnight at 4°C in the dark. Fifty µl of wash buffer (0.01 M

Tween-20 in PBS) were applied to each section and allowed to incubate for 5 min at room temperature. The buffer was pipetted off and the step repeated. Secondary antibodies, donkey anti-human-cy 3 and donkey anti-rabbit-cy 3, (Jackson ImmunResearch, West Grove, Pennsylvania, USA) were diluted 1:600 in 0.01 M PBS and 30 μl were added to appropriate sections and incubated for 30 min at room temperature in the dark. Washes were repeated before the sections were counter-stained with 1:200 Hoechst for 10 min in the dark. Washes were repeated and cover slips were applied. Immunofluorescence was performed using a Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss Canada, Mississauga, Canada).

2.5 Blood samples and γδ T-cells

2.5.1 Peripheral blood mononuclear cell isolation from whole blood

Fresh blood samples were obtained by venipuncture from MS patients, using heparin as an anticoagulant. PBMCs were isolated by density gradient centrifugation according to standard procedures. In a 50 ml conical tube, 15 ml of whole blood was washed with 30 ml of PBS. Fifteen ml of Ficoll-Hypaqe (Pharmacia Biotech AB, Uppsala, Sweden) was underlayered carefully and the tube centrifuged at 300xg for 30 min at room temperature in an IEC Centra GP8R centrifuge, with the brake turned off. The buffy coat, enriched in PBMCs, was transferred to a new 50 ml conical tube and washed twice with 45 ml of PBS and resuspended at 1 x 10^6 cells/ml in cRPMI.

2.5.2 γδ T-cell Expansion

γδ T-cells were expanded as previously described (221). Wells of a 24-well culture plate were treated sequentially with 300 μl (5 μg/ml) of sheep anti-mouse IgG (The Binding Site, Birmingham, UK) in PBS for 1 h at 37°C, 300 μl cRPMI for 5 min at room

temperature, and a 10^{-6} monoclonal anti-TCRγδ antibody (courtesy of Dr. Mike Brenner, Harvard University, titrated to yield maximum stimulation) for 30 min at 37°C. PBMCs isolated as described above were seeded in treated wells at 3 x 10^6 cells/well in a total of 3 ml of cRPMI. Cells were incubated at 37°C in a humidified atmosphere supplied with 5% CO_2. Fifty U/ml IL-2 (a gift from Hoffmann-La Roche, Nutley, NJ) was added the following day. On the fifth day, the medium was replaced with serum-free AIM V medium (Life Technologies) and cultured for an additional 3 to 5 days with the addition of fresh IL-2.

2.5.3 Purification of γδ T-cells by complement lysis

γδ T-cell cultures were further purified by the elimination of the αβ T-cells using the complement lysis procedure. Cultures were collected following expansion, washed, and suspended in 100 μl of PBS. The number of contaminating αβ T-cells was calculated by multiplying the total number of collected cells (enumerated with a haemacytometer) with the percentage of this population as determined by flow cytometry. For every 1 x10^6 αβ T-cells, 4 μg (4 μl from stock) of each anti-CD4 and anti-CD8 mAbs were added and incubated at 4°C for 30 min. Cells were washed with ice-cold PBS and resuspended in PBS at room temperature. Two-to-three hundred μl of sterile baby rabbit complement (C') was added to the cells that were incubated in a water bath at 37°C for 30 min with occasional agitation. The cells were washed and suspended in 3 to 5 ml cRPMI, dependent on the determination of the final cell count. Cytolysed cellular debris was removed by passing the cell suspension through a 40 μm sterile nylon strainer (Falcon, Thermo Fisher Scientific). Purified γδ T-cells were seeded and cultured for future use under the same conditions as described above, or suspended in 5% DMSO in FBS and cryopreserved in liquid nitrogen.

2.5.4 Extracellular staining of γδ T-cells by flow cytometry

Extracellular staining with mAbs and flow cytometry were used to investigate the percentage of γδ T-cells in cultures, in addition to the percentage of γδ T-cells that expressed CD16. Freshly expanded or previously-frozen γδ T-cells were washed with flow cytometry buffer (5% BSA in PBS with 0.1% [w/v] sodium azide) and 10^5 cells were suspended in 100 μl of flow cytometry buffer in each of 4 microcentrifuge tubes. Cells in the first tube were not stained and were used as an autofluorescence control. Cells in the additional tubes were stained with 2 μl FITC-TCRγδ (BD Biosciences, Mississauga, ON), 2 μl phycoerythrin (PE)-CD16 (Beckman Coulter, Mississauga, ON) and a combination of the two mAbs. The tubes were incubated at 4°C in the dark for 20 min before they were washed with 1 ml of flow cytometry three times, and finally suspended in 400 μl flow cytometry buffer for analysis. Flow cytometry was performed using a Coulter FC500 flow cytometer. Ten thousand events were acquired for all tubes.

2.5.5 Anti-Nfasc antibody binding to γδ T-cells

10^5 γδ T-cells were seeded in wells of a 96-well round-bottom microplate (Costar) in cRPMI. One μg of MS patient-derived anti-Nfasc antibody was added and the microplate was incubated for 1 h at 37°C. The cells were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 2500 rpm for 4 min. The medium was removed and the cells washed three times with 1 ml of cold flow cytometry buffer. All but 100 μl of flow cytometry buffer was removed and the 4 μl of PE-conjugated mouse monoclonal anti-human IgG(Fab) (ExBio Antibodies, Cedarlane Laboratories) was added, and the tubes incubated for 30 min at 4°C in the dark. The cells were washed three times with 1 ml of flow cytometry buffer and

resuspended in 350 μl in flow cytometry buffer for analysis. Flow cytometry was performed using a Coulter FC500 flow cytometer. Ten thousand events were acquired for all tubes.

2.6 γδ T-cell ADCC Killing Assay

2.6.1 Co-culture of γδ T-cells and HeLa cells in the presence of anti-Nfasc antibodies

Control and $tGFP^+$ HeLa cells were seeded at 10^5 cells/well of a 24-well flat-bottom plate (Costar) in cRPMI. $tGFP^+$ HeLa cell medium was supplemented with 800 μg/ml geneticin. The cells were cultured overnight to promote adherence. The following day, the medium in each well was replaced with 1 ml of cRPMI in the absence of geneticin. To appropriate wells, 1 μg of MS patient-derived anti-Nfasc antibody (derived from patient 1304 and 1023), or IgG isotype control antibody (Sigma-Aldrich, Burlington, ON) was added and incubated for 1 h at 37°C. MS patient-derived γδ T-cells with high CD16 expression were added in a 4:1 ratio with the HeLa cells (4×10^5) and incubated for 4 h at 37°C.

2.6.2 Flow Cytometric analysis of ADCC

After co-culturing, the medium from each well containing the γδ T-cells and detached HeLa cells was transferred into 1.5 ml microcentrifuge tubes. The tubes were centrifuged at 2500 rpm for 3 min and the supernatant discarded. The adherent HeLa cells that remained were detached with the addition of 200 μl of 1 mM ethylenediaminetetraacetic acid (EDTA) (in PBS) to each well, and incubating the plate at 37°C for 10 min. The plate was gently tapped, 500 μl of flow cytometry buffer was added to each well, and the cells were resuspended and added to the appropriate 1.5 ml microcentrifuge tubes with the previously collected cells. The tubes were centrifuged for 3 min at 2500 rpm and the supernatant discarded. Three μl of 1.5 mM propidium iodide (PI) was added to each suspension and

incubated at 4°C in the dark for 20 min. The cells were washed 3 times with 1 ml flow cytometry buffer, then resuspended in 350 µl and placed into a flow tube for immediate flow cytometric analysis. Acquisition was performed on the FC500 flow cytometer. The emissions of tGFP and PI were redirected through specific band-pass optical filters - 500 nm for tGFP and 575 nm for PI - and detected through FL1 and FL2, respectively. Compensations were adjusted by running control and transfected ($tGFP^+$) HeLa cells separately. Cell mixtures were then assessed and 10,000 events from each tube were acquired. Percent specific lysis was defined as:

% specific lysis =% total lysis – (% spontaneous lysis +% γδ T-cell-mediated lysis) x100

2.7 Detection of CSF Neurofilaments

2.7.1 Quantification of CSF phosphorylated neurofilament-heavy chain

The Human Phosphorylated Neurofilament H ELISA was obtained from Biovendor Research and Diagnostic Products (Candler, NC, USA). The sandwich enzyme immunoassay permits the quantitative measurement of the human phosphorylated NfH from serum, plasma and CSF. All steps were performed at room temperature, according to the manufacturer's instructions. Briefly, previously cryopreserved CSF samples were diluted 1:3 in dilution buffer and 100 µl were incubated, in duplicate, in microplate wells pre-coated with chicken polyclonal anti-pNFH antibody for 60 min, followed by thorough washing. Captured NfH was detected upon incubation for 60 min with 100 µl of a rabbit polyclonal anti-pNfH detection antibody. After washing, 100 µl of HRP-conjugated anti-rabbit antibody was added for 60 min. After an additional washing step, 100 µl of TMB was added and incubated for 15 min. The reaction was stopped with the addition of 100 µl of stop solution. The absorbance,

measured using a microplate photometer, at λ650 nm was subtracted from the absorbance at λ450 nm. A standard curve was generated by plotting the mean absorbance of the standards against the known concentration of the standards in logarithmic scale.

2.7.2 Quantification of CSF Neurofilament-light chain

The NF-light® (Neurofilament light) ELISA was obtained from UmanDiagnostics (Umea, Sweden). The solid-phase sandwich ELISA permits the quantitative determination of human NfL in CSF. All steps were performed at room temperature, according to the manufacturer's instructions; all wash steps consisted of 3 washes with 300 µl of wash solution, and all incubations were performed with agitation on an orbital shaker (The Belly Dancer, Storvall Life Science Inc., Greensboro, USA) at 700 rpm. Briefly, CSF samples were diluted 1:1 with sample dilution buffer to a final volume of 220 µl. The lyophilized bovine NfL standard was reconstituted and diluted according to the standard dilution table provided. Wells of the pre-coated anti-NfL microplate were washed, 100 µl of each standard or sample were added, in duplicate, and the plate was incubated for 1 h. The contents were removed, the plate washed, and 100 µl of biotin anti-NF mAb were added to each well and incubated for 45 min. The plate was washed and 100 µl of HRP conjugate were added to each well and incubated for 30 min. The plate was washed and 100 µl of TMB were added to each well and incubated for 15 min. The reaction was stopped with the addition of 50 µl of 8% v/v H_2SO_4. The absorbance was measured at λ450 nm using a microplate photometer. The concentration was then calculated by plotting the mean absorbance against the known standard concentrations in a linear scale.

2.8 Statistics

Comparisons between multiple groups were evaluated with the nonparametric Kruskal-Wallis one-way Analysis of Variance (ANOVA). When statistical significance was noted, Dunn's post-hoc test was applied for determination of significant differences between the multiple groups. Comparisons between 2 groups were evaluated with the Mann-Whitney U Test. The Wilcoxon signed rank test was used to compare repeated measurements in individuals over time. For correlation analyses, the Shapiro-Wilk test determined that the data were not normally distributed; therefore, R coefficients were obtained using the Spearman's rank correlation coefficient; p values obtained by linear regression. p-values < 0.05 were considered statistically significant.

Statistical analyses were performed using Statistica v6.0 (Statsoft, Inc., Tulsa, USA) and GraphPad Prism v4.02 (GraphPad Software, Inc., La Jolla, USA).

CHAPTER 3. Anti-neurofascin antibodies access the CNS during periods of active inflammation and are associated with neuro-axonal injury.

ABSTRACT

Background: γδ T-cells have been shown to express FcR and they are capable of binding antibody. These FcR-bearing cells correlate with disease progression in MS and have been shown to exert ADCC. This led us to wonder if there were relevant and detectable antibodies that might engage γδ T-cell FcR in the context of contributing to the immunopathogenesis of MS. A search for antibodies specific to axonal elements in MS revealed the presence of antibodies to neurofascin, and previously reported findings suggested these might be good candidates.

Methods: Sera and CSF were obtained for diagnostic purposes. A direct ELISA was developed to measure anti-Nfasc antibody titres in the sera and CSF of clinically confirmed MS patients and OND controls. A commercial ELISA was used to quantify the concentrations of NfL, a marker of acute axonal injury.

Results: Positive anti-Nfasc antibody titres were detected in of 22% and 25% of MS patient sera and CSF, respectively. Stratification according to MS disease subtype indicated that this might be a factor in anti-Nfasc antibody production. In the sera, the mean SPMS titre was the most elevated ($p<0.05$ vs. OND controls), while in the CSF the mean RRMS titre was the highest ($p<0.0001$ vs. OND controls). MS patient serum and CSF anti-Nfasc antibody titres correlated ($p=0.001$), and this was not observed for controls ($p=0.65$). Anti-Nfasc antibody titres failed to correlate directly with disease duration or patient age, though CSF anti-Nfasc

antibody titres were significantly reduced in patients over 50 years of age. CSF anti-Nfasc antibody titres correlated positively with NfL (p=0.0003).

Summary: Anti-Nfasc antibodies were present and detectable in both the sera and CSF of MS patients, and rarely in OND controls, suggesting they are relevant to MS. Higher titres in the serum support peripheral synthesis, while higher CSF titres in the relapsing phase imply that antibodies access the CNS during periods of active inflammation that are associated with disruption of the blood-CSF barrier. CSF anti-Nfasc antibody titres correlated strongly with the release of NfL, suggesting that axonal injury could be an immune-mediated pathology, and that Nfasc-specific antibodies might be involved.

INTRODUCTION

Multiple sclerosis is an inflammatory autoimmune disease of the CNS that is characterized by demyelination and axonopathy. Though the exact patho-immunological mechanisms that produce the damage underlying the disease are incompletely understood, there are many suspected processes, including mechanisms mediated by autoantibodies that target CNS myelin and neuro-axonal elements. The first CNS-specific autoantibodies identified in MS were OGD-specific and included antibodies that targeted intracellular MBP (136) and myelin-associated glycoprotein (MAG) (137). Anti-MOG antibodies were later discovered (138), followed by anti-PLP antibodies in a small subset of patients (139).

Nerve impulse conduction of myelinated axons in the CNS is dependent upon high-density clusters of Na_v channels at the nodes of Ranvier (60). Both the paranodal junctions and nodes of Ranvier must be intact to support saltatory conduction (61), and antibodies that target this CNS architecture have recently acquired attention, such as those specific to Nfasc.

Antibodies to Nfasc, a protein expressed at the paranodes and the nodes of Ranvier, were detected in the sera of 30% of MS patients, which recognized and bound to Nfasc-186-

expressing cells *in vitro* (174). The role of these MS patient-derived antibodies was not assessed but, in the context of EAE, co-transferred pan-Nfasc antibodies colocalized with Na_v channels at the nodes of Ranvier and were shown to enhance initial disease severity that likely resulted from acute axonal injury (174). The pan-Nfasc antibodies also bound the axonal surface of myelinating cultures, where they mediated complement-dependent injury to axons that led to secondary demyelination, while depletion of anti-Nfasc-155 antibodies abrogated the entire effect on axonal injury. These findings suggest that targeting Nfasc with complement-fixing antibodies might be a mechanism of axonal injury in MS (292). When rats were primed with Nfasc-186 (but not Nfasc-155) prior to EAE induction, subsequent disease severity was increased as the result of an axonopathic autoantibody response (294).

RESEARCH OVERVIEW

Rationale and Hypothesis

As MS progresses evidence of axonal injury mounts. Progressive phases of disease are characterized by an increase in the presence of antibody-forming follicle-like structures in the meninges and the presence of a greater B-cell component and antibody. Some of this antibody might be targeting axonal structures and, thus, mediating axonal injury. This role might be fulfilled by anti-Nfasc antibody and if so, it should be present in higher titres and in greater proportions of patients entering the progressive phase of disease.

Therefore, I hypothesize that anti-Nfasc antibodies are present in the serum and CSF of MS patients. Anti-Nfasc antibody titres are elevated in progressive forms of disease in comparison to relapsing disease and OND controls, and are associated with disease duration and neuro-axonal injury.

Research Questions

1. *Are anti-Nfasc antibodies detected in the serum of MS patients?*

2. *Are anti-Nfasc antibodies detected in the CSF of MS patients?*

3. *Is there a relationship between anti-Nfasc antibody titres and MS disease course?*

4. *Is there a relationship between anti-Nfasc antibody titres and neuro-axonal damage in MS, as indicated by the release of neurofilament proteins into the CSF?*

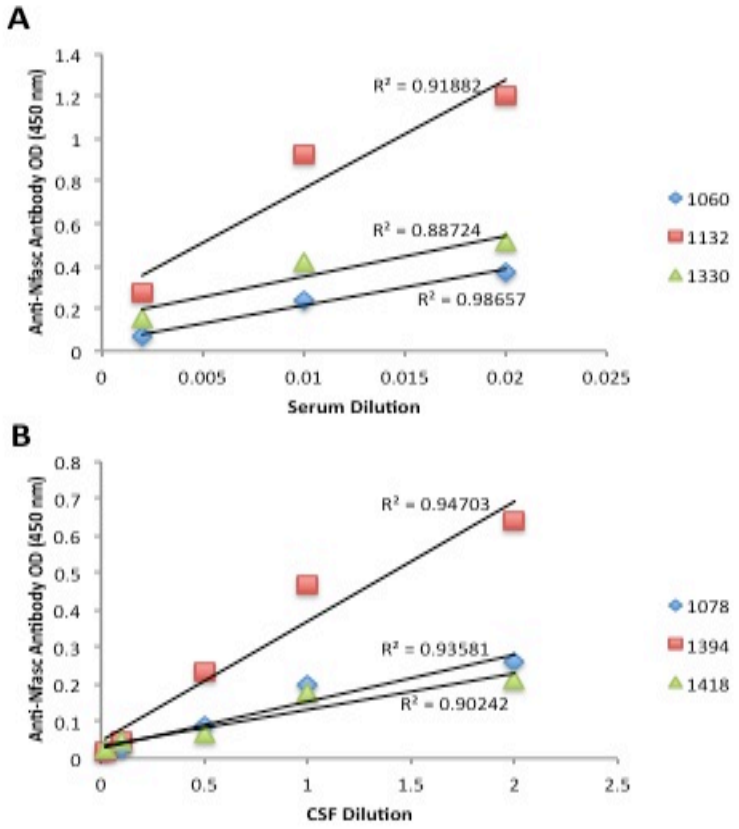
RESULTS

Titration of anti-Nfasc antibodies

Sera were titrated to establish the linear range of the ELISA in order to make determinations of antibody titre in samples of unknown concentration. Sera were titrated to 10^{-3}, and ELISA was performed. Antibody detection was linear for dilutions from 1:50 to 1:500 for the three sera assessed, with R^2 values of 0.9865, 0.9188 and 0.8872 (**Fig. 3.1A**). This established a linear curve, and subsequent ELISAs were performed with sera diluted 100-fold. The same assay was conducted for MS patient CSF with 2-, 10- and 50-fold dilutions of the neat samples. Antibody detection was linear for the neat samples through to 50-fold dilutions, with R^2 values of 0.947, 0.9358 and 0.9024 (**Fig. 3.1B**).

Figure 3.1 Detecting the linear range of anti-Nfasc antibodies in sera and CSF.

Dilution series of MS sera and CSF were prepared and assessed for the presence of anti-Nfasc antibodies using ELISA. (**A**), Serum antibody detection was linear for the range of dilutions between 1:50 and 1:500 with R^2 values of 0.9188 (PPMS, ■ red squares), 0.8872 (SPMS, ▲ green triangles) and 0.9865 (SPMS, ◆ blue diamonds) for the three sera assessed. (**B**), CSF antibody detection was linear for the range of dilutions from neat to 1:50 with R^2 values of 0.9358 (RRMS, ◆ blue diamonds), 0.9024 (SPMS, ▲ green triangles) and 0.947 (RRMS, ■ red squares) for the 3 CSF assessed.


A
Anti-Nfasc Antibody OD (450 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.005
0.01
0.015
0.02
0.025
Serum Dilution
R² = 0.91882
R² = 0.88724
R² = 0.98657
1060
1132
1330
B
Anti-Nfasc Antibody OD (450 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
CSF Dilution
R² = 0.94703
R² = 0.93581
R² = 0.90242
1078
1394
1418

Proportions of positive anti-Nfasc antibody titres in sera and CSF

Sera from 183 individuals were evaluated using direct ELISA for the presence of antibodies specific for Nfasc-155, including 55 RRMS, 42 PPMS, 29 SPMS patients and 19 patients who possibly had MS, as these individuals probably represent the earliest of the disease cohorts. Thirty-eight individuals with OND such as stroke or migraine were examined as controls. Titres derived from 100-fold dilutions that were greater than 2 standard deviations from the mean of the OND controls were considered positive, and anti-Nfasc antibodies were detected in 22% of MS patient sera (15.1% of RRMS, 27.5% of PPMS and 28% of SPMS) (**Fig. 3.2**) compared to 9.1% and 7.5% of patients who possibly had MS and OND controls, respectively.

CSF from 126 individuals were assessed using indirect ELISA for the presence of antibodies specific for Nfasc-155 including 38 RRMS, 21 PPMS, 24 SPMS patients, 14 patients who possibly had MS, and 29 OND controls. Anti-Nfasc antibodies were detected in the CSF of 25.3% of the MS patients (36.1% of RRMS, 9.5% of PPMS and 22% of SPMS) assessed (**Fig 3.3**) in comparison to 25% and 3.3% of patients who possibly had MS and OND controls, respectively.

Figure 3.2 Detection of anti-Nfasc antibodies in MS patient sera.

The presence of anti-Nfasc antibodies was assessed in the sera of MS patients by ELISA. An OD greater than 2xSD of the mean of the OND controls was considered positive (denoted by large horizontal bar). Positive titres were detected in 22% of MS patient sera in comparison to 7.5% of OND controls. When stratified by disease course positive titres were detected in the sera of 28% of SPMS (median OD 0.325), 27.5% PPMS (median OD 0.201), 15.1% RRMS patients (median OD 0.162), and 9.1% of patients who possibly had MS (median OD 0.151). The most elevated serum antibody titres were observed in SPMS (mean OD 0.423) and PPMS (mean OD 0.367) followed by RRMS patients (mean OD 0.275), OND controls (mean OD 0.221), and patients who possibly had MS (mean OD 0.173). The mean for each group is denoted with a short horizontal bar. n=126 MS patients (55 RRMS, 42 PPMS and 29 SPMS), 19 patients who possibly had MS and 38 OND controls. *p*-values obtained by Kruskal-Wallis ANOVA and Dunn's post-hoc test.

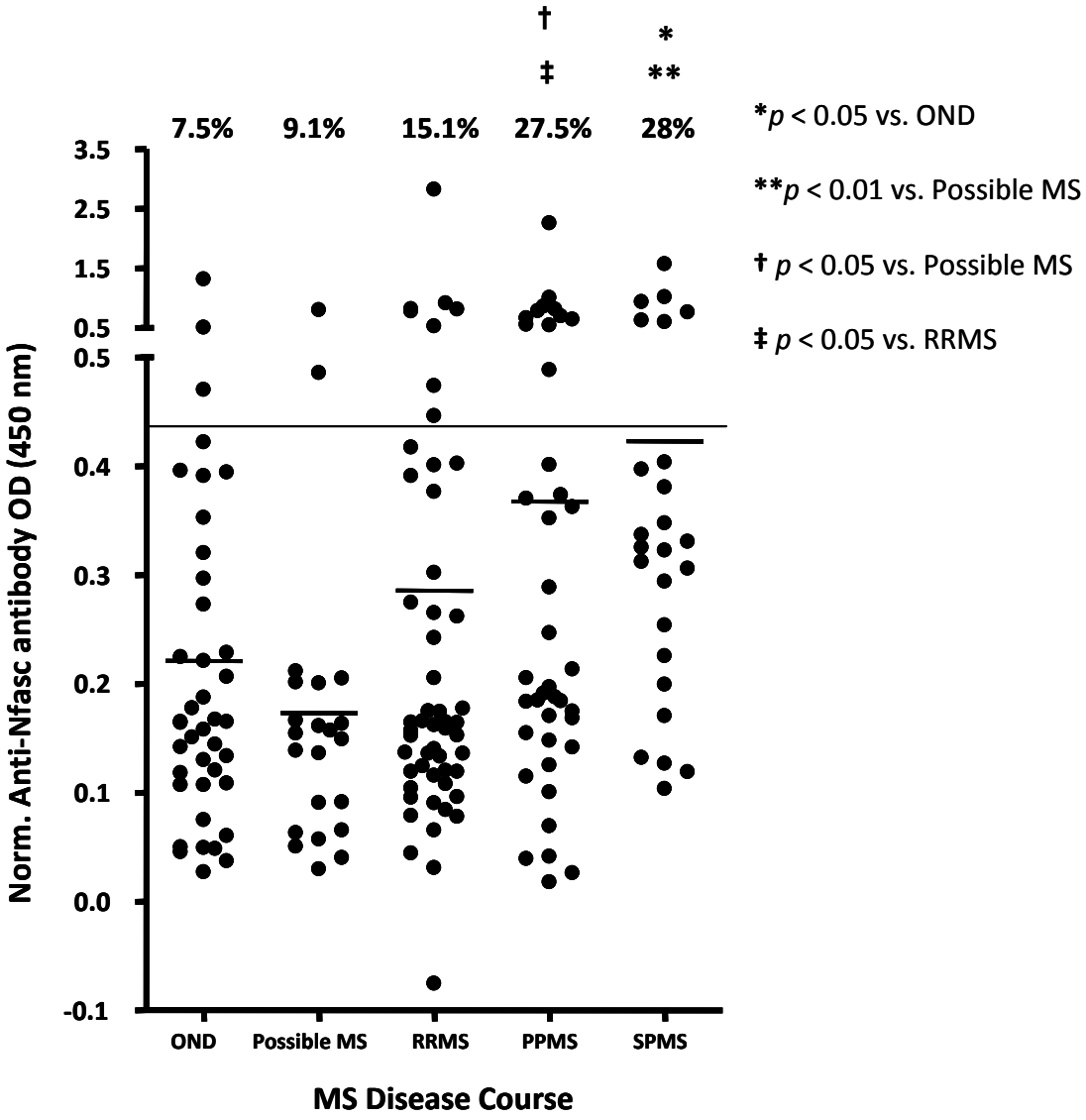
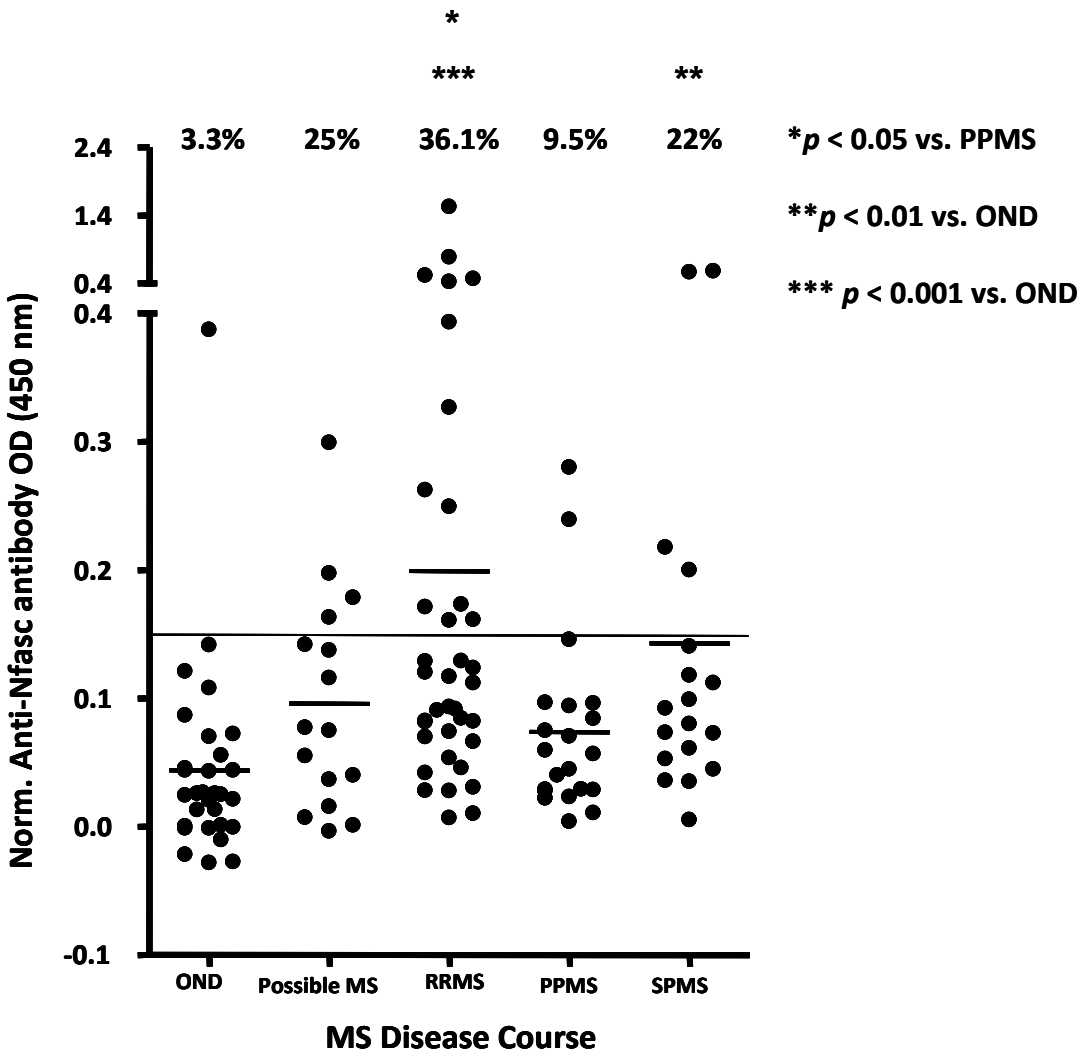

†
‡
*
**
7.5%
9.1%
15.1%
27.5%
28%
3.5
2.5
1.5
0.5
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Norm. Anti-Nfasc antibody OD (450 nm)
OND
Possible MS
RRMS
PPMS
SPMS
MS Disease Course
*p < 0.05 vs. OND
**p < 0.01 vs. Possible MS
† p < 0.05 vs. Possible MS
‡ p < 0.05 vs. RRMS

Figure 3.3 Detection of anti-Nfasc antibodies in MS patient CSF.

The presence of anti-Nfasc antibodies was assessed in the CSF of MS patients by ELISA. An OD greater than 2xSD of the mean of the OND controls was considered positive (denoted by large horizontal bar). Positive titres were detected in 25.3% of MS patient CSF in comparison to 3.3% of OND controls. Positive anti-Nfasc antibody titres were detected in 36.1% of the RRMS patients (median OD 0.1116) compared with 25% of patients who possibly had MS (median OD 0.0758), 22.2% of SPMS (median OD 0.0859), and 9.5% of PPMS patients (median OD 0.0563). The most elevated titres were detected in RRMS (mean OD 0.199), followed by SPMS (mean OD 0.143) and PPMS patients (mean OD 0.074). The mean OD for the individuals who possibly had MS and OND controls were 0.076 and 0.044, respectively. The mean for each group is denoted with a short horizontal bar. n=83 MS patients (38 RRMS, 21 PPMS and 24 SPMS), 14 patients who possibly had MS and 29 OND controls. *p*-values obtained by Kruskal-Wallis ANOVA and Dunn's post-hoc test.


*
***
**
3.3%
25%
36.1%
9.5%
22%
*p < 0.05 vs. PPMS
**p < 0.01 vs. OND
*** p < 0.001 vs. OND
Norm. Anti-Nfasc antibody OD (450 nm)
2.4
1.4
0.4
0.4
0.3
0.2
0.1
0.0
-0.1
OND
Possible MS
RRMS
PPMS
SPMS
MS Disease Course

Disease Duration

Axonal disease tends to increase with disease duration; therefore, disease duration was examined in the context of its relationship to anti-Nfasc antibody titres. Patient history was available for only a subset of the cohort (126/183 and 71/126, respectively, for patients evaluated for serum and CSF anti-Nfasc antibody titres), thus disease duration was calculated for this limited group of subjects.

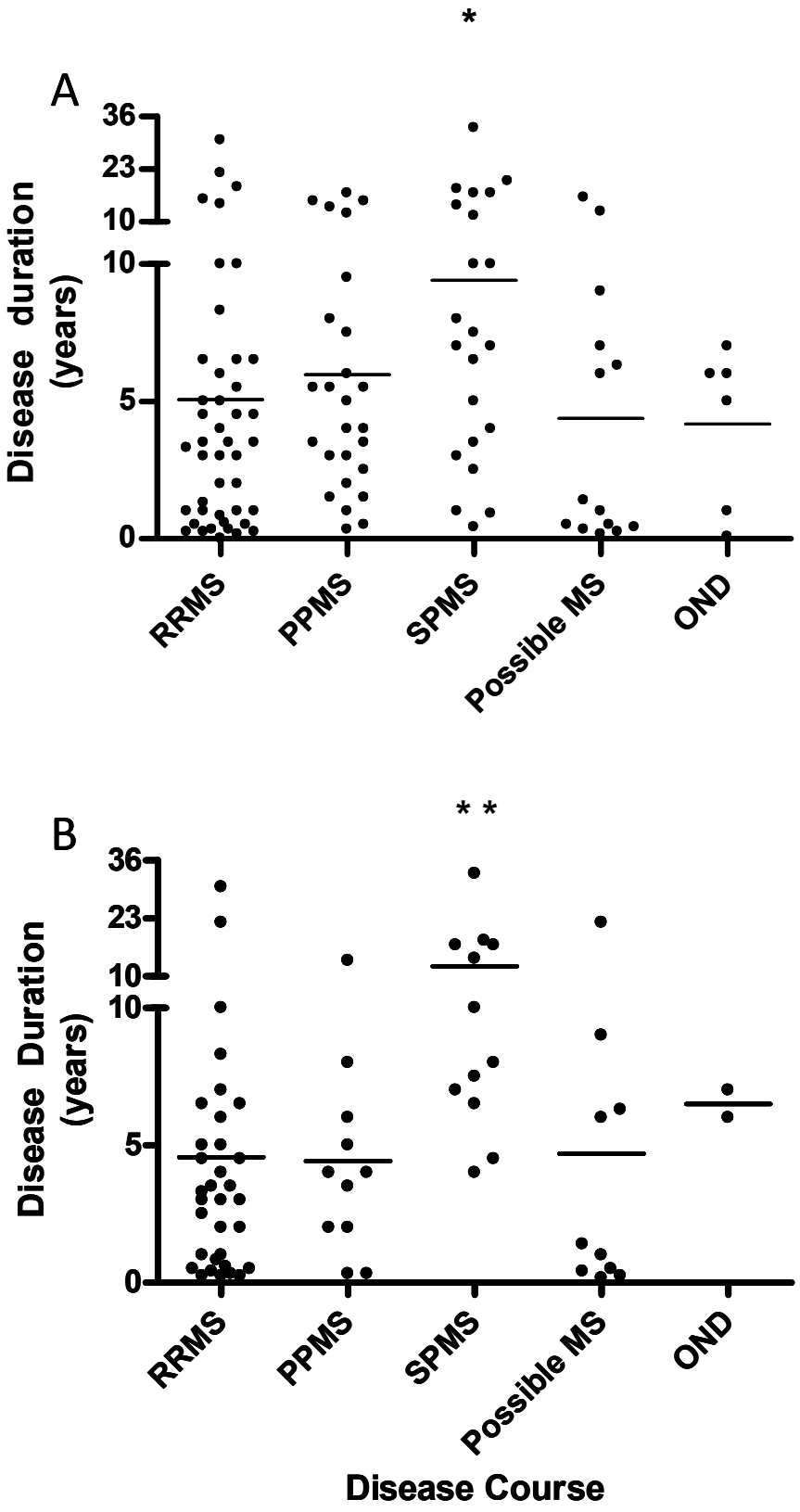
The mean disease duration for patients who were evaluated for the presence of serum anti-Nfasc antibodies was 5.1 years (median 3.4 years), 5.9 years (median 4.5 years), and 9.4 years (median 7.25) for RRMS, PPMS, and SPMS, respectively. It was 4.4 (median 1.2 years) and 4.2 years (median 5.5 years) for the patients who possibly had MS and OND controls, respectively.

The mean disease duration for the patients who were assessed for the presence of CSF anti-Nfasc antibodies was 4.6 years (median 3 years), 5.9 years (median 4.5 years), and 12.2 years (median 9 years) for RRMS, PPMS, and SPMS, respectively. It was 4.7 years (median 1.2 years) and 6.5 years (median 6.5) for patients who possibly had MS and OND controls, respectively.

As expected, the mean disease duration was significantly greater for SPMS patients than for RRMS patients in the analyses of both serum and CSF ($p<0.05$ and $p<0.01$, respectively) (**Fig 3.4A and Fig 3.4B**, respectively). No correlations were observed, however, between the respective mean serum or CSF anti-Nfasc antibody titre and duration of disease for any MS subtype ($p=0.9358$ and 0.3902 for RRMS; $p=0.7994$ and 0.4941 for PPMS; and $p=0.7502$ and 0.5747 for SPMS). The mean and median disease duration data for each group is shown in **Table A1** of APPENDIX II.

Figure 3.4 Disease duration is longer in SPMS than RRMS.

When patient history was available, the disease duration (the time from the onset of clinical symptoms to sample collection) was calculated for 126/183 and 71/126 of the individuals assessed for serum and CSF anti-Nfasc antibodies, respectively. For the **(A)** serum and **(B)** CSF analyses, the disease duration was significantly longer for SPMS compared to RRMS patients. Data represent the disease duration in years for individual patients. The mean disease duration for each group is denoted with a short horizontal bar. *$p<0.05$ and **$p<0.01$ vs. RRMS, obtained by Kruskal-Wallis ANOVA and Dunn's post-hoc test.


A
*
36
23
10
10
5
0
Disease duration (years)
RRMS
PPMS
SPMS
Possible MS
OND
B
* *
36
23
10
10
5
0
Disease Duration (years)
RRMS
PPMS
SPMS
Possible MS
OND
Disease Course

Patient Age at sample collection

Axonal disease also tends to increase with age, thus age was examined in the context of its relationship to anti-Nfasc antibody titres. With respect to the serum analysis, the mean age of the subjects at sample collection was 38.2, 47.6 and 38.4 years for RRMS, PPMS, and SPMS patients, respectively. It was 40.5 and 43.6 for the patients who possibly had MS and the OND controls. The PPMS cohort was significantly older than RRMS and SPMS patients ($p<0.001$ and $p<0.01$, respectively).

For the CSF analysis, the mean age of the subjects was 36.1, 46.2, and 38.5 for RRMS, PPMS, and SPMS, respectively. It was 40 and 42.6 years, respectively, for the patients who possibly had MS and the OND controls. The age at sample collection was significantly greater for PPMS than RRMS patients ($p<0.01$).

When linear regression analysis was applied to anti-Nfasc antibody titres in the serum and CSF, respectively, in the context of patient age, no significant correlations were observed in any of the MS subtypes ($p=0.8491$ and 0.7785 for RRMS; $p=0.9071$ and 0.3746 for PPMS; and $p=0.8734$ and 0.3546 for SPMS, respectively). The mean and median age at sample collection for each group is shown in **Table A1** of APPENDIX II.

Stratification of MS patients by age

Recently, it was suggested that inflammation and neurodegeneration are reduced in older MS patients, and that the boundary approaches 54 years of age (365). To investigate whether a similar phenomenon could be observed in this MS cohort, patients were stratified by age, over or under the age of 50 years. There were no significant age differences calculated between any subgroups in the patients under or over 50 years of age. With respect to patients under the age of 50, SPMS patients assessed for serum anti-Nfasc antibodies were significantly older than patients who possibly had MS ($p<0.001$), but no other age

differences were observed for patients who were assessed for serum or CSF anti-Nfasc antibody titres.

When comparing patients under the age of 50 to those over, CSF anti-Nfasc antibody titres were significantly greater in MS patients under the age of 50 than patients over the age of 50 (p=0.001), while there was no difference in serum anti-Nfasc antibody titres for the same group (p=0.516) (**Fig 3.5**). However, RRMS serum from subjects over 50 contained significantly reduced anti-Nfasc titres than did SPMS patients less than 50 years of age (p=0.016) (**Fig 3.6**).

Figure 3.5 CSF anti-Nfasc antibody titres decrease with age in MS.

The MS patient cohort for whom disease duration data were available was stratified into groups older and younger than 50 years of age. The CSF anti-Nfasc antibody titres were elevated in MS patients under 50 years of age compared to patients over 50 years of age. Data represent the anti-Nfasc antibody titres for individual patients. The mean anti-Nfasc antibody titre for each group is denoted with a short horizontal bar. ***p=0.001 obtained by Mann-Whitney U test.

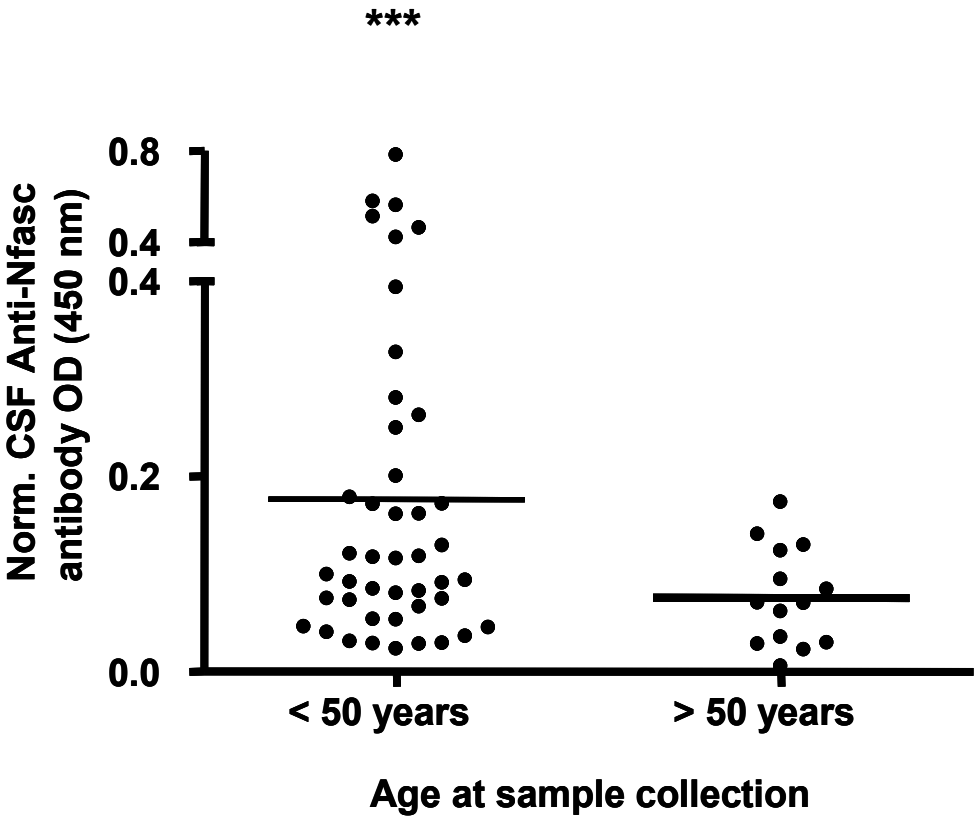
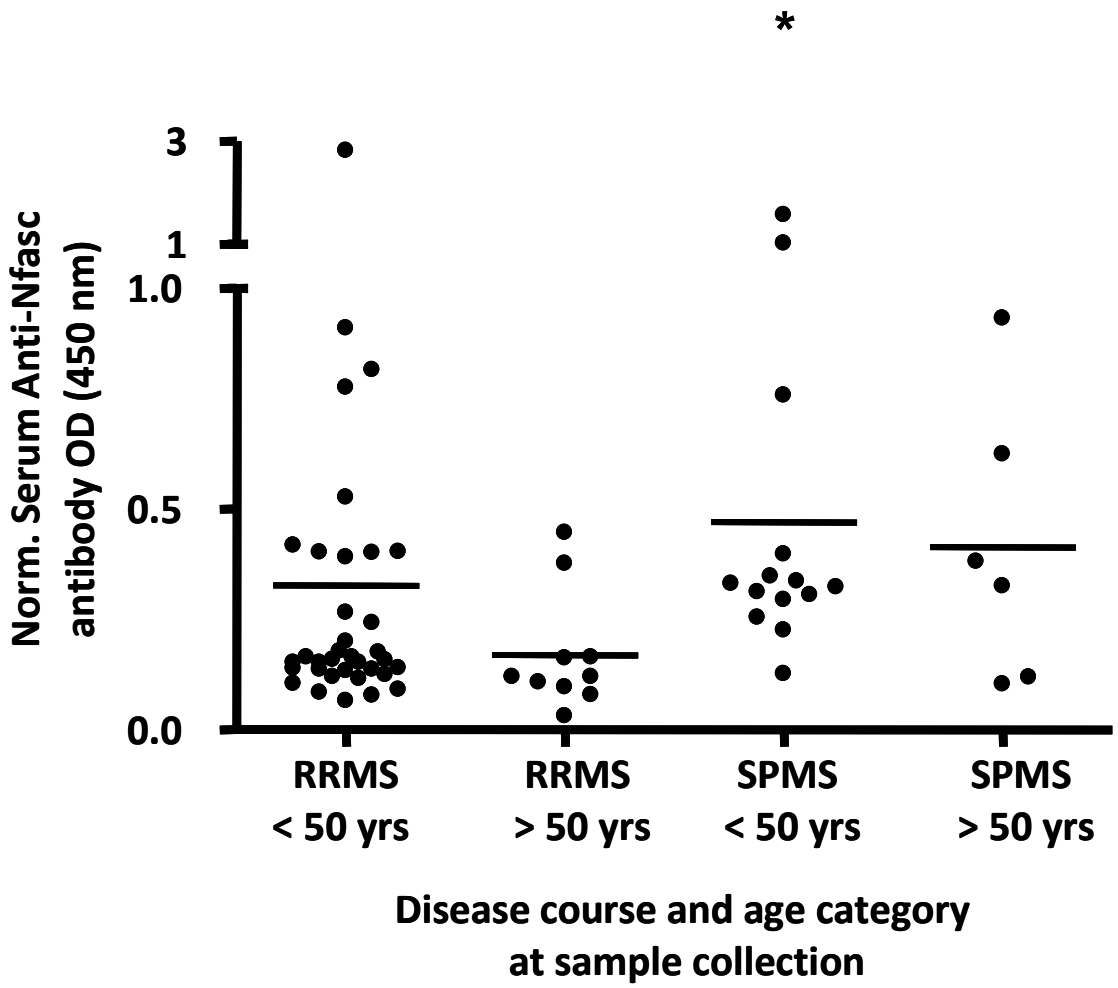

***
Norm. CSF Anti-Nfasc antibody OD (450 nm)
0.8
0.4
0.4
0.2
0.0
< 50 years
> 50 years
Age at sample collection

Figure 3.6 Serum anti-Nfasc antibody titres are reduced in older RRMS patients.

The serum anti-Nfasc antibody titres from MS patients for whom disease duration data was available were stratified according to disease course (RRMS or SPMS) and age. The serum anti-Nfasc antibody titres were reduced in RRMS patients older than 50 years compared to SPMS patients under 50 years of age. Data represent the serum anti-Nfasc antibody titres for individual MS patients. The mean for each group is denoted with a short horizontal bar. **p*=0.016, compared to RRMS patients >50 years, obtained by *t* test.


*
3
1
1.0
0.5
0.0
Norm. Serum Anti-Nfasc antibody OD (450 nm)
RRMS < 50 yrs
RRMS > 50 yrs
SPMS < 50 yrs
SPMS > 50 yrs
Disease course and age category at sample collection

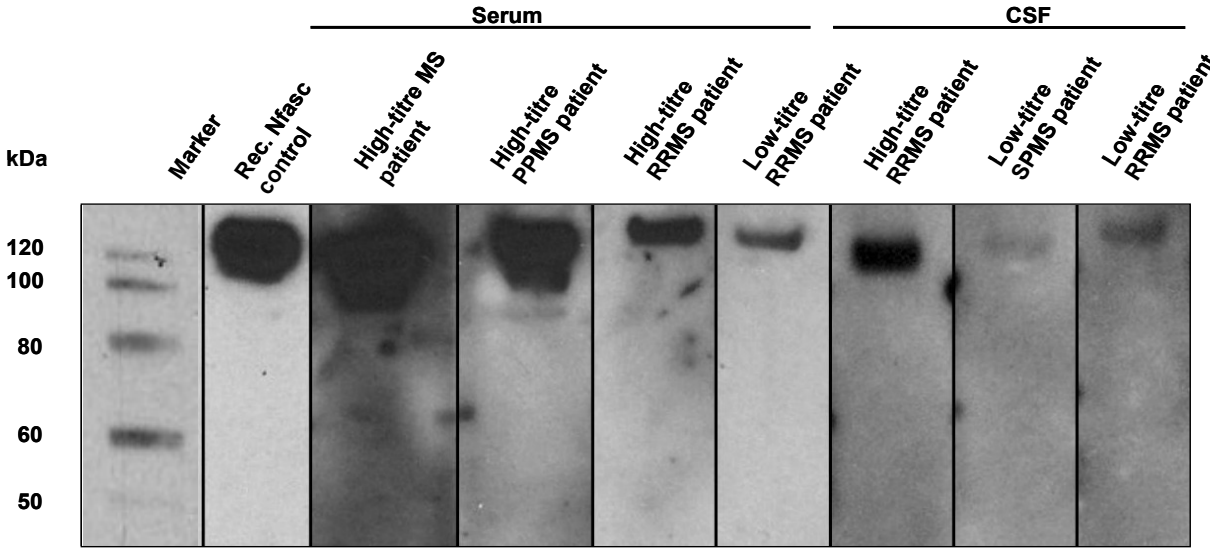
Stratification of serum anti-Nfasc antibody titres by MS disease course

Stratification according to MS subtype indicated that this might be a factor in anti-Nfasc antibody production. Antibodies were detected in the sera of 28% of SPMS patients (median OD 0.325), 27.5% PPMS (median OD 0.201), 15.1% RRMS (median OD 0.162), and only 9.1% of patients who possibly had MS (median OD 0.151) (**Fig 3.2**).

The highest serum antibody titres were observed in SPMS (mean OD 0.423; $p< 0.05$ and $p< 0.01$ vs. OND controls and possible-MS, respectively) and PPMS (mean OD 0.367; $p< 0.05$ vs. possible-MS) followed by RRMS patients (mean OD 0.275), which were not significantly different from the titres of the OND controls (mean OD 0.221) or the patients who possibly had MS (mean OD 0.173). Additionally, a statistically significant difference was observed between the anti-Nfasc antibody titres of SPMS and RRMS ($p< 0.05$) (**Fig 3.2**). To support these results, Western blots were performed with a subset of the sera, consisting of low and high anti-Nfasc antibody titres. Bands were observed in the 150 kD range, consistent with the molecular weight of Nfasc-155 (**Fig 3.7**).

Figure 3.7 MS serum and CSF anti-Nfasc antibody detection by Western blot.

Western blots were performed using high and low (anti-Nfasc antibody) titre sera and CSF, as determined by ELISA. Bands representing anti-Nfasc antibodies were observed in the 150 kD range, which is consistent with the molecular weight of Nfasc-155. Three high-titre and 1 low-titre sera and 1 high-titre and 2 low-titre CSF were assessed.


Serum
CSF
kDa
Marker
Rec. Nfasc control
High-titre MS patient
High-titre PPMS patient
High-titre RRMS patient
Low-titre RRMS patient
High-titre RRMS patient
Low-titre SPMS patient
Low-titre RRMS patient
120
100
80
60
50

Stratification of CSF anti-Nfasc antibody titres by MS disease course

When antibody titres were examined according to MS disease course the profile of titres differed to what was observed in the sera. Positive anti-Nfasc antibody titres were detected in 36.1% of the RRMS patients (median OD 0.1116) compared with 22.2% of SPMS (median OD 0.0859), 9.5% of PPMS patients (median OD 0.0563). Furthermore, anti-Nfasc antibodies were also identified in 25% (median OD 0.0758) of patients who possibly had MS (**Fig 3.3**).

The highest titres were detected in RRMS (mean OD 0.199; $p< 0.001$ vs. OND controls), followed by SPMS (mean OD 0.143; $p< 0.01$ vs. OND controls) and PPMS patients (mean OD 0.074; $p> 0.05$, ns). The mean OD for the individuals who possibly had MS and OND controls were 0.076 and 0.044, respectively. A statistically significant difference was also observed between RRMS and PPMS patients ($p<0.05$) (**Fig 3.3**). As was performed with the sera, these observations were supported by Western blot analysis with a subset of low and high titre CSF samples (**Fig. 3.7**). Bands in the 150 kD range were, again, observed, which is consistent with the molecular weight of Nfasc-155.

Correlation of anti-Nfasc antibody titres in serum and CSF

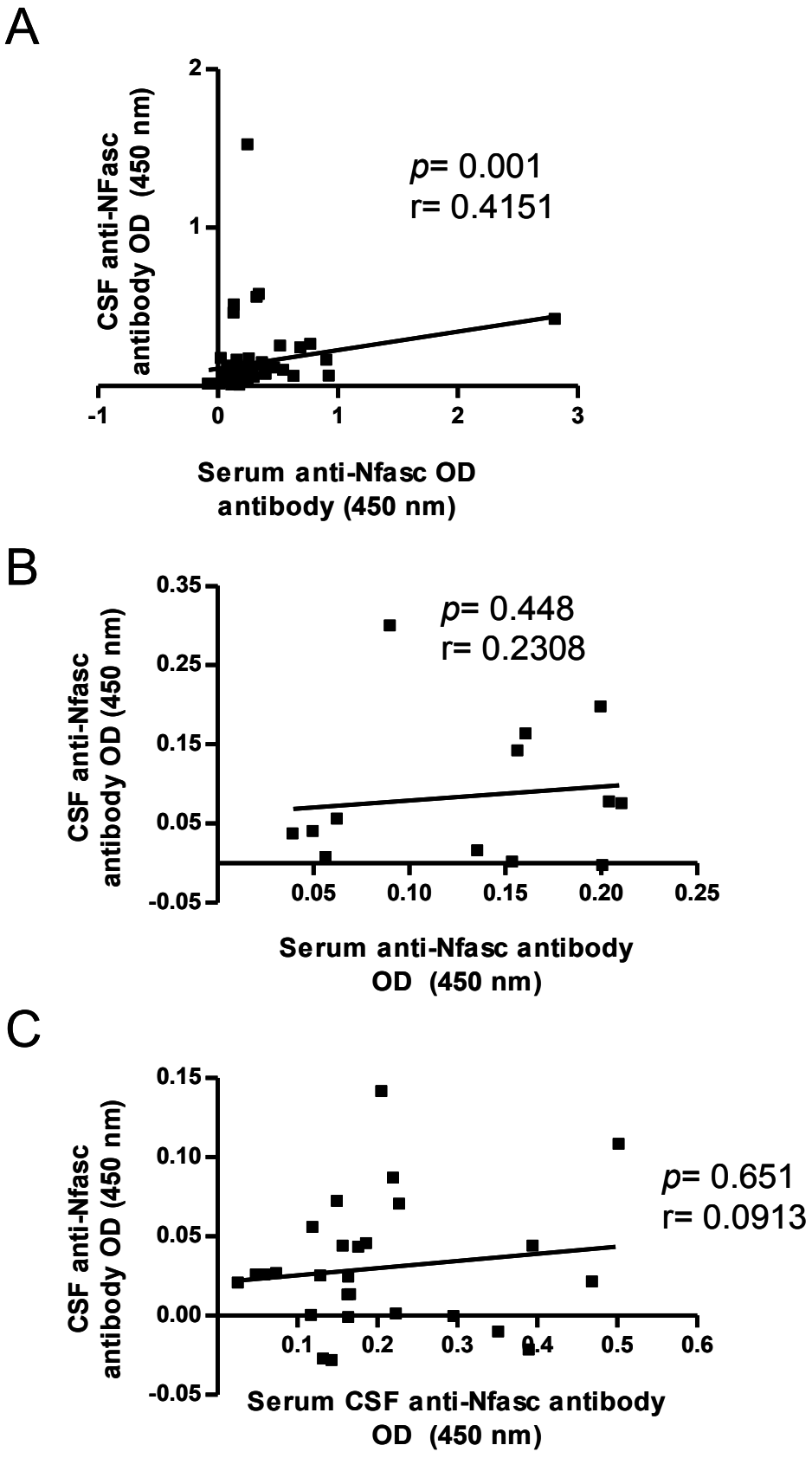
A correlation between the sera and the CSF would suggest that peripheral antibody enters the CNS, likely during break-down of the blood-CSF barrier, which is more likely to occur in the inflammatory stage of disease, RRMS. Correlation analyses were performed for 100 individuals whose serum and CSF were both available in the sample bank. In the 27 OND controls and the 13 individuals who possibly had MS, there was no correlation between serum and CSF titres ($p=0.651$ and 0.448, respectively), whereas a positive correlation was observed for the 60 MS patients ($p=0.001$) (**Fig 3.8**). When the MS patient data were stratified according to MS disease subtype statistically significant correlations were observed

in the RRMS and PPMS patient groups (p=0.011 and 0.017, respectively) and, while there was a trend towards a correlation for the SPMS cohort, it was not statistically significant (p=0.079).

No correlation, however, was observed between either CSF (p=0.8031) or serum (p=0.8278) and the EDSS, which suggests that antibody titres are not predictive of disease progression, as measured by that scale.

Figure 3.8 Anti-Nfasc antibody titres correlate in MS sera and CSF.

(A) Positive correlations were observed between the serum and CSF anti-Nfasc antibody titres for the MS patients (p=0.001). A lack of significant correlation was observed for the (**B**) individuals who possibly had MS (p=0.448) and the (**C**) OND controls (p=0.651). Data represent the serum and CSF anti-Nfasc antibody OD (450 nm). n= 60 MS patients, 27 OND controls, and 13 individuals who possibly had MS. Correlation coefficients obtained using Spearman's rank correlation coefficient; p values obtained by linear regression analysis.

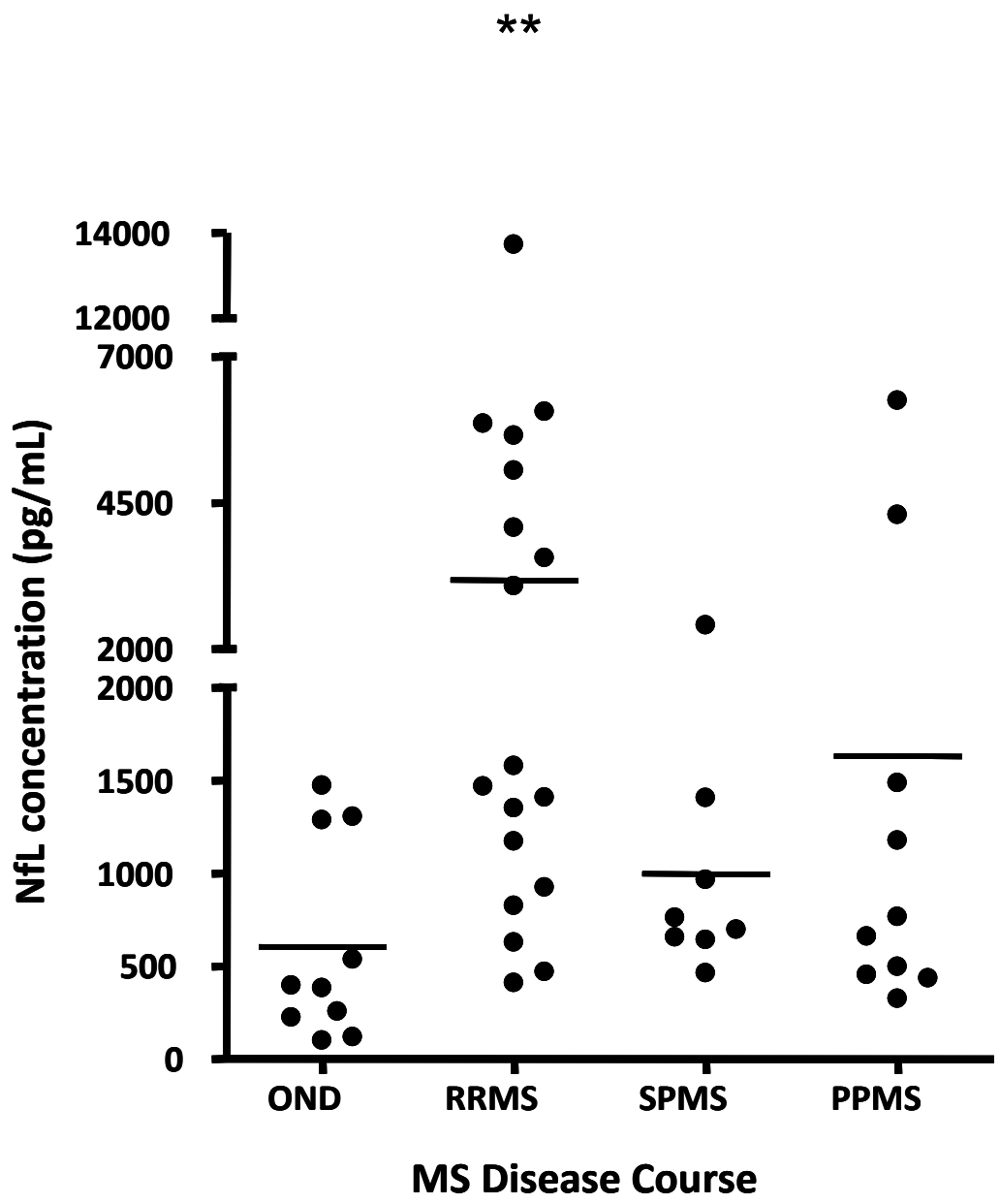

A
CSF anti-NFasc antibody OD (450 nm)
p= 0.001
r= 0.4151
Serum anti-Nfasc OD antibody (450 nm)
B
CSF anti-Nfasc antibody OD (450 nm)
p= 0.448
r= 0.2308
Serum anti-Nfasc antibody OD (450 nm)
C
CSF anti-Nfasc antibody OD (450 nm)
p= 0.651
r= 0.0913
Serum CSF anti-Nfasc antibody OD (450 nm)

Measurement of NfL in the CSF of MS patients

Levels of NfL in the CSF tend to correlate with acute axonal damage. To investigate neuro-axonal injury in a subset of the greater MS cohort, NfL concentrations were measured in the CSF of 36 randomly chosen MS patients, consisting of 18 RRMS, 8 PPMS and 10 SPMS patients, as well as in 10 OND controls that were previously evaluated for the presence of anti-Nfasc antibodies. The highest mean NfL concentration was measured in the RRMS cohort (mean 3176.3 pg/ml, $p<0.01$ vs. OND controls) followed by PPMS (1630.6 pg/ml, $p>0.05$, ns) and SPMS (mean 995.6 pg/ml, $p>0.05$, ns). The mean NfL concentration for the OND controls was 604.7 pg/ml (**Fig 3.9**).

Figure 3.9 NfL is enriched in RRMS CSF.

The CSF NfL concentration was measured in a cohort of MS patients by commercial ELISA. CSF specimens were diluted 1:1 with sample buffer and assayed in duplicate according to the manufacturer's instructions. The mean NfL concentration was 3176.3 pg/ml ($p<0.01$ vs. OND controls) for the RRMS patient cohort, 1630.6 pg/ml ($p>0.05$, ns) for the PPMS patient cohort, and 995.6 pg/ml ($p>0.05$, ns) for the SPMS patient cohort. The mean NfL concentration for the OND controls was 604.7 pg/ml. Data represent CSF NfL concentrations for individual patients. The mean of each patient group is denoted by a short horizontal bar. n= 36 MS patients (18 RRMS, 8 PPMS, 10 SPMS) and 10 OND controls. *p* values obtained by Kruskal-Wallis ANOVA and Dunn's post-hoc test.


**
14000
12000
7000
4500
2000
2000
1500
1000
500
0
NfL concentration (pg/mL)
OND
RRMS
SPMS
PPMS
MS Disease Course

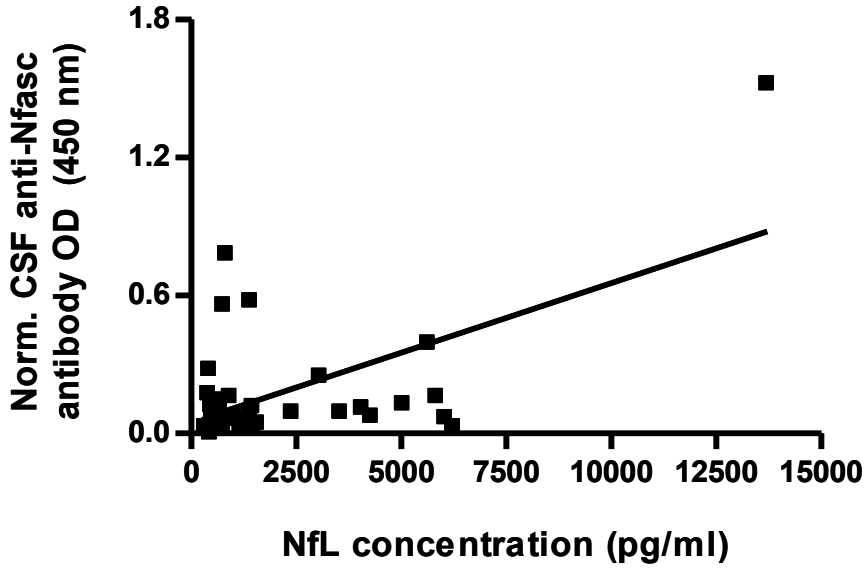
Correlation of anti-Nfasc antibody titres with acute axonal degeneration

Linear regression analysis was performed using the measured CSF anti-Nfasc antibody titres and calculated NfL concentrations. A strong positive correlation was observed for the MS patients (p=0.0003), and was not observed in the OND controls (p=0.883) (**Fig 3.10**). The positive correlation was driven by the RRMS subgroup (p=0.0001), as the correlations were not statistically significant for either the PPMS (p=0.3533) or SPMS (p=0.5331) subgroups.

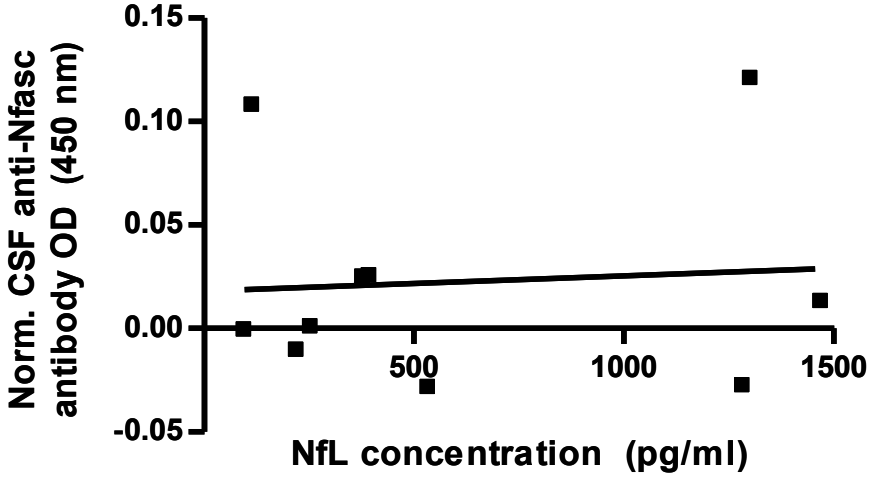
Fig 3.10 CSF anti-Nfasc antibody titres correlate with acute axonal injury in MS.

A positive correlation was observed between the anti-Nfasc antibody titres and the NfL concentrations for (**A**) the MS patients (p=0.003). A lack of significant correlation was observed for the (**B**) OND controls (p=0.883). Data represent the anti-Nfasc antibody titre and the NfL concentration for individual patients. n=36 MS patients and 10 OND controls. Correlation coefficients obtained using Spearman's rank correlation coefficient; p values obtained by linear regression analysis.

A


Norm. CSF anti-Nfasc antibody OD (450 nm)
1.8
1.2
0.6
0.0
0
2500
5000
7500
10000
12500
15000
NfL concentration (pg/ml)

B


Norm. CSF anti-Nfasc antibody OD (450 nm)
0.15
0.10
0.05
0.00
-0.05
500
1000
1500
NfL concentration (pg/ml)

DISCUSSION

There is a wealth of knowledge supporting a role for γδ T-cells in the pathogenesis of multiple sclerosis. They are generally involved in immune surveillance and have multipotential capabilities for target elimination, both directly through specific target lysis and by functioning as APCs for additional immune effectors. Some γδ T-cells express FcR, and they are capable of binding antibody and eliciting an ADCC response. This begs the question of whether there are relevant, detectable antibodies that might engage γδ T-cell FcR in the context of contributing to the immunopathogenesis of MS. Of particular interest would be antibodies directed to neuro-axonal elements as these might contribute to the irreversible loss observed pathologically.

A search for antibodies specific to axonal elements revealed descriptions of antibodies to neurofascin, a bi-variant glycoprotein expressed on both myelinating OGD at the paranode and on the axonal surface at the nodes of Ranvier. Reports suggested these antibodies might play a pathogenic role, thus an investigation was undertaken to determine if the potential pathogenicity might be a consequence of an interaction with FcR-bearing γδ T-cells that gain entry into the CNS in the context of MS.

Relevance to MS

The first phase of the study was to, indeed, determine if the antibodies were present, detectable and specific to MS, thus an ELISA was developed. In the serum, positive anti-Nfasc antibody titres were detected in 22% MS patients, compared with 7.5% of OND controls. This is consistent with a previous study that reported the detectable presence of anti-Nfasc antibodies in 20 to 30% of MS patients, and the occasional OND control (174). In the CSF, positive titres were detected in nearly 28% of MS patients and merely 3% of OND

controls. These observations suggested that the presence of antibodies to Nfasc was relevant to MS and that, perhaps, antibody production was associated with inflammation.

With respect to the detection of antibodies in the occasional OND subjects, it might have been expected that these individuals had experienced some other event that led to the exposure of neuro-axonal elements to the immune system. Interestingly, the only subject with a positive anti-Nfasc antibody titre in the CSF was diagnosed with macular edema, an inflammatory condition of the retina that results in break-down of the blood-retinal barrier. Of the 3 OND controls with positive serum titres, 2 experienced spinal stenosis and cervical spine compression. The third was diagnosed with recurrent ovarian carcinoma and concomitant Celiac disease, another autoimmune disease that is associated not only with the production of autoantibodies (366) but also shares genetic risk loci with MS (9).

However, a lack of definitive disease specificity was not unanticipated, as there is a precedent for autoimmune antibody responses in non-MS controls. Antibodies specific to MOG and Nogo-A, a neurite outgrowth inhibitor, have been detected previously in the CSF of controls (174; 367).

Compartmentalization

The blood-CSF barrier plays a central role in restricting the movement of soluble and cellular components from the periphery to the CNS, but despite a tightly controlled system, blood-CSF barrier dysfunction is one of the earliest pathological mechanisms in MS (88). An examination of anti-Nfasc antibody titres demonstrated that antibody concentrations were augmented in the serum when compared to the CSF, which suggests that they are synthesized and compartmentalized in the periphery. Peripheral antibodies to additional neuro-axonal elements have previously been identified in MS, including antibodies to cytoskeletal molecules α-centractin, CRMP1, pyruvate kinase and syntaxin-binding protein 1 (142) and

juxtaparanodal contactin-2/TAG-1 (173), and supports antibody synthesis to neuro-axonal elements as a potential mode of pathogenesis.

In the CSF, anti-Nfasc antibody titres were greatest in the RRMS subjects, suggesting that peripheral antibody is gaining entry to the CNS, and driving up titres during periods of active inflammation that are associated with increasing disruption of the blood-CSF barrier. Albeit lower than those measured for the relapsing subjects, the SPMS titres were also significantly greater than the OND controls, suggesting that peripheral antibody continues to diffuse across the blood-CSF barrier and into the CNS. SPMS is characterized by fewer relapses than RRMS patients but these individuals do continue to relapse and have contrast-enhancing lesions. Furthermore, endothelial abnormality continues in inactive SPMS lesions, NAWM and cortical grey matter (368), thus it is not surprising that CSF anti-Nfasc antibody titres are lower than those patients with active disease but nevertheless augmented when compared to OND controls.

Augmented peripheral antibody production was similar between the non-relapsing cohorts, yet little anti-Nfasc antibody was detected in the CSF of the PPMS non-relapsing sub-group; here the CSF titre was not significantly different than the OND controls. This points to blood-CSF barrier integrity as a potential reason for the disparity. While some degree of CNS inflammation is present in PPMS, it is reduced comparatively, with RRMS and SPMS, and this has been previously reported (369). PPMS patients have fewer and smaller cerebral abnormalities (370), including fewer focal brain lesions (370; 371) and contrast-enhancing lesions (372; 373), which might suggest that break-down of the blood-CSF barrier is a less prominent feature of this course. Secondly, there is less parenchymal inflammatory cell infiltration associated with PPMS (369), thus if anti-Nfasc antibodies enter

the CNS via γδ T-cell FcR engagement then intrathecal concentrations could be reduced if activated peripheral T-cells were prevented entry.

Disease Duration and Age

It is known that neurodegenerative processes tend to increase with age and duration of MS, and that immunological abnormalities are associated with disease progression (374). Altered cytokine profiles have been associated with MS disease progression, including the enhanced production of pro-inflammatory IL-12 and its β subunit, IL12p40, in SPMS compared to RRMS with concomitant reduction in IL-10 (375; 376), which might be due to a reduction in IL-10-producing $CD5^+$ B-cells in SPMS (377). Interestingly, $CD4^+CCR5^+$ T-cells are resistant to apoptosis in progressive MS (378), and the sustained populations of T-cells might influence the longevity and function of B-cells. With respect to MS-specific antibodies, little is currently known about how antibody production, the potential for injury or inhibition of repair are associated with disease progression. Progressive MS patients exhibited an increase in CNS-reactive antibodies to isoforms of CNP, MOG, OGD-specific protein and PLP (148), and NfL-specific antibodies are enriched in the CSF of progressive MS patients compared to RRMS (158), which is in contrast to a wealth of literature that suggests NfL is released upon acute axonal injury and is associated more strongly with relapsing disease (347; 349-352). Fewer specific antibodies to NfL might, then, be expected in progressive MS, though there is the possibility that they were synthesized during the relapsing phase and persisted.

To investigate these concepts further in the context of anti-Nfasc antibody synthesis, the symptomatic disease duration was calculated for each subject. This assessment was limited by the fact that sera and CSF are obtained at the time of diagnosis, which tends to be

in younger individuals, thus patient numbers are limited across some age groups. Furthermore, symptomatic history was not available for all of the samples evaluated, therefore, disease duration was calculated and assessed in a smaller cohort within the larger, complete group of subjects, when it was possible to determine the disease duration at the time of sample collection. First, the expectation that the mean disease duration would have been longer for the SPMS cohort in comparison to the RRMS cohort was confirmed statistically. Anti-Nfasc titres, however, did not correlate with disease duration in either the sera or the CSF, which suggests that the augmented antibody titres in progressive MS might relate to something else, such as phenotypically distinct B-cell subsets or dissimilar regulatory mechanisms.

Next, anti-Nfasc antibody titres were assessed in the context of age at sample collection. The PPMS subjects were older than the RRMS subjects, which was expected given that PPMS is typically associated with an older age at onset than RRMS, approximately 10 years later (average age is approximately 39) (379). There was, however, no correlation observed between age and CSF or serum anti-Nfasc antibody titres.

The most prominent distinction between progressive patients and relapsing or patients who possibly have MS is that those with progressive disease have endured its pathological mechanisms longer. While anti-Nfasc antibody synthesis is apparently a common phenomenon in MS, the lack of a direct association between disease duration or patient age and anti-Nfasc antibody titres suggests that differences between these patients might pertain more to changes of intrinsic immune function or deregulation, as these occurrences indeed correlate with disease duration and age (380).

It has been suggested that the peripheral immune system becomes more dysregulated as patients transition from relapsing to progressive MS (380). This notion suggests that

antigen-specific adaptive immunity targeting myelinated elements of the CNS is predominantly involved in RRMS. In contrast, dysfunction of specific innate immunity may prevail in progressive disease as chronic inflammation is related to the activation of peripheral dendritic cells. This, in turn, leads to continued activation of CNS microglia and diffuse inflammation that injures axons and is exhibited clinically as progressive accumulation of disability (380; 381). However, contrary to that line of thought, systemic B-cells and plasmablasts were significantly increased in SPMS and they correlated with disease progression (382). Functionally, however, what role these cells play in pathogenesis remains unclear. In this investigation the highest serum anti-Nfasc antibody titres were detected in progressive MS and, while B-cell numbers were not investigated, it is possible that the increased titres observed in SPMS pertain to changes in B-cell regulation, such as dysfunction in B-cell longevity. B-cell-activating factor (BAFF), a member of the TNF family, is essential for B-cell development and survival (383-385). Its most critical signal transduction receptor is BAFF-R, which is expressed mainly on B-cells (386). T-cell BAFF mRNA levels were significantly higher in MS compared to controls, and were significantly higher in SPMS patients. Similarly, BAFF-R mRNA expression was significantly greater in MS patient-derived B-cells compared to controls, and was increased in SPMS and PPMS (387). These findings suggest that B-cell homeostasis could be altered in progressive courses of MS, such that B-cell survival and longevity might be extended. In the case of antibody-secreting cells, such a mechanism could result in the continued secretion of antibody and, therefore, augmented titres compared to subjects that do not overexpress mRNA for BAFF and its receptor.

Furthermore, it was recently shown that inflammation and neurodegeneration subside with older age in MS; CXCL13, MMP-9, and NfL were all reduced in the CSF of patients

over the age of 54 years (365). To determine if a similar phenomenon was evident in the older subjects in this cohort, the anti-Nfasc antibody titres were stratified into two groups according to patient age, those older and younger than 50 years of age at sample collection. CSF anti-Nfasc antibody titres were significantly reduced in patients over 50 years of age when compared to those under 50 years, while there was no parallel difference observed in the serum. This suggests that the pathological mechanisms involved do not reflect changes in antibody synthesis, but that blood-CSF barrier compromise might be reduced with increasing age. Blood-CSF barrier involvement is, in fact, supported by the findings of Khademi et al. where reductions in CXCL13, MMP-9 and NfL were reported. In MS, MMPs are thought to facilitate immune cell entry into the CNS (388) and CXCL13 is a B-cell chemo-attractant (389), with augmented concentrations in MS CSF (390-392). CXCL13 is associated with relapse as it is correlates with disease exacerbation, and high concentrations are thought to predict conversion to clinically-definite MS (365; 393). Reductions in these factors might facilitate inhibition of immune cell entry into the CNS. With respect to the current investigation, this might lessen further blood-CSF barrier injury, thus inhibiting the diffusion of serum components and, therefore, anti-Nfasc antibody into the CNS. Furthermore, if anti-Nfasc antibody enters the CNS via engagement of γδ T-cell FcR, then antibody titres in that compartment would be reduced if the T-cells were restricted to the periphery.

Analysis of patients under the age of 50 in the present study demonstrated higher titres of serum anti-Nfasc antibody in SPMS patients compared to those who possibly had MS, but all other significant differences that were observed between subgroups initially, when age was not considered, were lost in this subsequent analysis. However, in both analyses, serum anti-Nfasc antibody titres were significantly higher in SPMS than RRMS, thus additional examination of these 2 groups was performed. A comparison of RRMS and

SPMS showed no statistical differences between serum anti-Nfasc titres in younger RRMS patients and older SPMS patients; however, there was significantly more anti-Nfasc antibody detected in the serum of the younger SPMS patients when compared to the older RRMS patients. This indicates that, in the serum, the lower titres measured in RRMS patients over the age of 50 years is what drives the statistical significance between the SPMS and RRMS patients overall. And while the difference in anti-Nfasc antibody titres between RRMS patients younger and older than 50 years of age was not statistically significant ($p<0.09$), there was a pattern of lower titres in the older subjects. Taken together, and keeping in mind that the findings cited by Khademi et al. were derived from studies of CSF markers as opposed to serum markers, these findings might reflect a mechanistic difference in antibody synthesis and persistence between RRMS and SPMS that could also be age-dependent, and might support a decline in inflammatory activity with increasing age in RRMS.

Disease course

In MS, the disease course is defined on the basis of clinical phenotype, though distinguishing the disease course on the basis of pathogenesis is much more difficult as there are shared elements across all disease courses. Additionally, by definition, all SPMS patients derive from RRMS, though precisely when that occurs is unknown, and distinguishing them is nearly impossible during the transition. The most prominent distinguishing feature of relapsing disease is the presence of inflammatory signals.

RRMS is characterized by more frequent active and detectable inflammation marked by clinical relapses and the presence of new CNS lesions that enhance with contrast due to the increasing immune-mediated permeability of the blood-CSF barrier, and correlate with perivascular inflammation (394). Relapses are associated with more numerous myelin-reactive T-cells in the circulation (395), proinflammatory cytokine expression in peripheral

T- and B-cells (396) a decreased peripheral Treg/Th17 ratio (397), greater numbers of γδ T-cells in the CSF (218), as well as proinflammatory cytokine-secreting B-cells (396). Demyelinating lesions characteristically consist of areas of primary demyelination, some degree of axonal transection, and inflammatory infiltrates that contain T- and B-cells, macrophages and activated microglia (389).

On the other hand, PPMS and SPMS are characterized by progressive clinical deterioration in the absence of relapses. These courses are characterized by diffuse inflammation in the white matter, fewer inflammatory processes in white matter lesions, demyelination of the cerebral cortex (398), and yet an increased proportion of Ig-secreting cells in chronic lesions (399).

When anti-Nfasc antibody titres were examined according to disease course the most augmented titres were measured in the sera of the SPMS cohort, while in the CSF the greatest titres were measured in the RRMS subjects. The disparity in antibody titres between RRMS and both PPMS and SPMS suggests that in the absence of active inflammation in the progressive courses, anti-Nfasc antibodies are confined to the periphery, and that increased antibody entry into the CNS is probably a consequence of active inflammation in RRMS.

Contribution to axonal loss

The presence of MS-relevant antibodies that gain entry into the CNS prompted an inquiry into how this might cause injury and whether they were contributing to axonal loss. Currently one of the most reliable assessments of axonal loss is the measurement of NfL in the CSF as this polypeptide has been established as a marker for acute neurodegeneration due to its release into the extracellular space and the CSF upon axonal injury or loss. The increased mean NfL concentration in the CSF of RRMS patients is supported by previous reports stating that NfL is enriched in RRMS CSF compared to controls (333; 349; 350; 353;

400), and suggests that immune-mediated axonal transection occurs primarily in the relapsing phase of MS, which is in agreement with the seminal finding that active lesions contain the greatest degree of transected axons (68). Furthermore, in MS patients, anti-Nfasc antibody titres correlated strongly with NfL release and were driven by the RRMS cohort, which provides additional support for NfL as a marker of acute axonal injury in the relapsing phase of MS, and also suggests that antibodies to Nfasc might be involved. Acute axonal injury has been previously associated with inflammation in MS, where the most extensive axonal injury has been observed in the active lesions of relapsing subjects, compared to slowly-expanding and inactive lesions and NAWM, though this is likely due to the enrichment of active lesions in the relapsing cohort compared to the progressive (401), an occurrence that is probably a factor in the cohorts examined in this investigation as well.

The absence of a correlation between anti-Nfasc antibody titres and NfL in the progressive cohorts might be due to the use of NfL as an indicator of neurodegeneration as opposed to the heavy chain of neurofilament (NfH), which is the preferred marker for chronic accumulating axonal damage and has been associated with injury in both PPMS and SPMS (332; 356; 359). It could also suggest that neurodegeneration occurs independently of inflammatory processes during this phase of disease, a notion that has received a great deal of attention in recent years. MRI and MRS studies demonstrate only moderate correlations between contrast-enhancement and brain and spinal cord atrophy (402-405), profound axonal loss in NAWM has been shown to develop independently of axonal injury in demyelinated lesions (406-408), and ongoing myelin degradation has been observed in the cerebral cortex in the absence of parenchymal lymphocytic infiltration (409; 410).

Caveats

It is important to accept these interpretations with caution as these analyses reflect antibody titres at a single point in time and, might not be demonstrative of true physiological levels, or at least what would be expected during a period of stability. This pertains especially to the RRMS subjects who were at greater risk of experiencing heightened inflammatory activity in the form of relapses. Sample collection surrounding relapse activity may bias the results in favour of increased titres that might be short-lived, particularly in the CNS compartment. Furthermore, it is possible that the OD values obtained from the immunoassay do not reflect the true antibody titre. The assay binds antibody that is specific to Nfasc-155 and, while the two variants share a high degree of homology, it is possible that there are peripheral antibodies that target Nfasc-186-specific antigens only.

The MS patients in this study were defined on the basis of disease course, which largely describes the clinical phenotype. Distinguishing the disease course on the basis of pathogenesis is much more challenging as there are shared elements across all disease courses. While contrast-enhancing lesions, for example, are rare in PPMS and these individuals do not experience relapses, secondary progressive MS patients indeed experience occasional relapses with the presence of contrast-enhancing lesions, which might make them more similar to RRMS, from which they derive, than PPMS patients. There also may have been RRMS patients in transition to SPMS at the time of sample collection, which further confounds the distinction. It is, therefore, difficult to state with certainty that the patients are categorized into the groups in which they belong from the perspective of pathogenesis.

It cannot be ignored that anti-Nfasc antibodies are not exclusive to MS with respect to their potential involvement in disease pathogenesis. They have additionally been associated with the peripheral demyelinating disease Guillain Barré Syndrome (411; 412) and, most

recently, they have been identified in the serum and CSF patients presenting with combined central and peripheral demyelination (CCPD). Patients with positive titres responded to plasma exchange, suggesting that the antibodies had pathogenic potential, and they recognized mainly Nfasc-155, though binding to Nfasc-186 was observed in some patients (293), which is similar to what has been observed in MS (174; 292). In the CCPD patients CNS involvement was partly typical for MS, and included the presence of contrast-enhancing lesions, but the presence of OCB was rare, suggesting that these patients have distinct CNS involvement different than that observed in MS. It is, therefore, unlikely that anti-Nfasc antibody-positive CCPD is a coincidence of MS and Guillain Barré Syndrome but probably a unique condition due to a common immunopathogenic mechanism between CNS and PNS demyelination. Epitope spreading is a distinct possibility to explain the central and peripheral lesions, however, they often occurred almost simultaneously, which is contrary to that suggestion (293).

Summary and Future Directions

The presence of anti-Nfasc antibodies in such a great proportion of MS patients compared to OND controls indicates that these antibodies are relevant to MS. Higher titres in the serum suggest that they are synthesized in the periphery, while higher CSF titres in the relapsing phase suggests that they access the CNS during periods of active inflammation that are associated with disruption of the blood-CSF barrier. Anti-Nfasc antibody titres failed to correlate directly with disease duration or patient age, though CSF anti-Nfasc antibody titres were significantly reduced in patients over 50 years of age with no parallel discrepancy observed in the serum, suggesting that blood-CSF barrier compromise might be reduced with increasing age, rather than alterations in antibody synthesis. Finally, in MS, CSF anti-Nfasc

antibody titres correlated strongly with the release of NfL, suggesting that axonal injury is an immune-mediated pathology, and that Nfasc-specific antibodies might be involved.

It was hypothesized that anti-Nfasc antibodies might mediate neuro-axonal injury in the presence of γδ T-cells in the context of MS pathogenesis. The CSF titres were elevated in RRMS patients, suggesting that antibodies might gain access to the CNS by diffusion through an inflamed blood-CSF barrier, or by engagement of FcR-expressing T-cells. If antibodies enter the CNS under inflammatory conditions, then titres should be reduced in the absence of inflammation. To test this hypothesis, CSF derived from a cohort of MS patients undergoing aHSCT for the treatment of aggressive MS will be evaluated for anti-Nfasc antibody titres and potential axonal injury, as measured by the release of neurofilaments. With this cohort of patients, it is possible to evaluate the concentrations of antibodies and neurofilaments before the procedure, when the patients were plagued by active disease, and afterwards, in a state that is essentially free of immune-mediated disease activity.

CHAPTER 4. Reduction in measures of axonal injury by autologous hematopoietic stem cell transplantation in MS patients.

ABSTRACT

Background aHSCT has emerged as a promising treatment for severe autoimmune diseases, such as MS, that are uncontrolled with current DMT (302). A cohort of MS patients that were plagued with aggressive disease including multiple relapses, deterioration in the EDSS score, and worsening MRI underwent aHSCT. This procedure definitively eliminates inflammation, which provides an opportunity to investigate CNS tissue responses in MS, separated from the potent ongoing inflammatory activity. It has always been presumed, but never proven, that inflammation in MS contributes to ongoing axonal injury. One of the more promising biomarkers for axonal disease is neurofilament, a structural protein exclusive to neurons and axons, which is released into the extracellular space and CSF upon neuro-axonal injury. Neurofilament release has been measured in patients with MS, indicative of ongoing axonal injury. We were, therefore, interested in knowing whether this highly effective treatment for MS inflammation would change the ultimate release of neurofilament, thus showing indirectly that inflammation was responsible for neuro-axonal injury.

Methods: A cohort of 19 MS patients with an aggressive course underwent aHSCT, which consisted of HSC mobilization and harvest, immune-ablation, and reconstitution with an autologous stem cell graft of $CD34^+$ cells, depleted of T lymphocytes. Patients were followed-up for a minimum of 3 years, and clinical (neurological) assessment and laboratory tests (collection of blood [serum] and CSF) were conducted at 1, 2, and 3 years post-aHSCT. MRI was used to assess the volume of contrast-enhancing lesions and the T1- and T2-weighted (T1W and T2W, respectively) lesion volumes, and MRS was used to evaluate the

ratio of *N*AA/Cr. Anti-Nfasc antibody titres were measured by an ELISA developed in-house, and commercial ELISAs were obtained to measure patient CSF NfH and NfL concentrations at each of the 4 time points.

Results: In this cohort of MS patients that underwent aHSCT for the treatment of an aggressive course of MS, the mean anti-Nfasc antibody titre was not significantly reduced in the CNS compartment following the procedure (p=0.1514 at 3 years post-aHSCT vs. baseline), but was significantly reduced in the sera (p=0.0024 at 3 years post-aHSCT vs. baseline). The concentration of both CSF neurofilament variants, initially measured to be quite high, decreased in the 3 years following aHSCT. The reduction was most pronounced for the NfL variant with a statistically significant decrease immediately at the 1 year post-aHSCT time point (p=0.002 vs. baseline). More time was required to reach a statistically significant reduction in the concentration of NfH; it was achieved at the 2 year time point (p=0.003 vs. baseline). The significant reductions were maintained for both variants for the duration of the follow-up period. The concentration of NfH and NfL correlated with MR spectroscopy metrics of neuronal integrity (*N*AA/Cr), as well as with contrast-enhancing, T1W and T2W lesion volumes, depending on the disease course, but not with the EDSS.

Summary: aHSCT definitively shuts down inflammation in this aggressive disease cohort, and provides an opportunity to investigate CNS tissue responses in MS in the absence of inflammation. Anti-Nfasc antibody titres were significantly reduced in the sera with concomitant persistence in the CSF, which might suggest that, though antibody is being cleared in the periphery, it may have become trapped behind a repaired blood-CSF barrier. In spite of anti-Nfasc antibody persistence, however, NfL and NfH concentrations were reduced, which could suggest that antibody-mediated injury was abrogated in the absence of FcR-bearing γδ T-cells. The concentrations of measured NfH and NfL were also associated

with other measures suggestive of reduced neuro-axonal damage such as a decrease in brain atrophy, fewer new T1W lesions and overall progression-free survival. These data support the notion that inflammation is driving axonal injury in MS and once it is eliminated there is no longer evidence of neuro-axonal damage.

INTRODUCTION

MS is a chronic disease of the CNS that is characterized by co-existent inflammatory demyelination and neurodegeneration. Conventional immunomodulatory therapies largely target cellular components of the immune system and the blood-CSF barrier, and have proven efficacious with regards to reducing clinical relapse rates, slowing clinical disease progression, and reducing the number of new lesions (413; 414), but they are still not curative. Their efficacy seems greatest in the relapsing/remitting phase, but they have shown a limited capacity for preventing or mending the neurodegenerative process that dominates more the secondary progressive phase of disease where the role of the immune system is less prominent (299-301).

The use of aHSCT is becoming increasingly prevalent for the treatment of severe autoimmune diseases (302-307), including MS (297; 298; 308-316). The rationale is based on the ability to eliminate the autoreactive immune cells by intense immunosuppression followed by complete immune reconstitution through the engraftment of previously harvested aHSCs. Autologous HSCT has been shown to elicit profound qualitative changes in the immunological cell repertoire, which suggests that further to its immunosuppressive capacity, aHSCT probably resets the immune system, including tolerance to previously self-reactive antigens (314). Follow-up studies have reported that the majority of patients did not progress clinically (298; 325), most had fewer relapses (309), contrast-enhancing MRI

activity was suppressed (297; 298; 308-310; 326; 327), and brain atrophy declined (328; 329).

Neurofilament polypeptides are released into the extracellular space and CSF upon neuronal injury, both traumatic and neurodegenerative in nature; most notably in MS (415). Neurofilaments are structural proteins that are almost exclusively, and abundantly, expressed in neurons, which makes them specifically indicative, and potential biomarkers, of neuro-axonal injury or loss. The potential for γδ T-cell-mediated ADCC in the presence of anti-Nfasc antibodies was evaluated in the context of neuro-axonal injury with the measurement of the light and heavy neurofilament subunits. NfL is considered a marker of acute inflammatory injury; its concentrations are greater during relapse and in patients with OCB, it correlates with contrast-enhancing lesions, and it relates to CSF cell count (349). On the contrary, NfH was associated with chronic CNS injury, predominates in the CSF of progressive MS patients (344), and correlates with the EDSS (332; 345-347).

RESEARCH OVERVIEW

Rationale and Hypothesis

Autologous HSCT is associated with well-documented remissions of autoimmune diseases for significant lengths of time, and can be considered a reset of the immune system, with high-dose immunosuppression used to abolish the autoimmune processes thought to contribute to the development of MS. The CSF concentration of neurofilaments is thought to indicate the degree of ongoing damage to neurons or axons.

I hypothesize that the release of neurofilament polypeptides is an indicator of active inflammatory-induced neurodegeneration in MS. If anti-Nfasc antibodies enter the CNS by diffusion across an inflamed blood-CSF barrier or via engagement of γδ T-cell-expressing FcR, a definitive treatment that completely ablates inflammation, such as immunoablation and aHSCT, should lessen the destructive injury they might mediate to neurons and axons and, thus, reduce the concentrations of measured neurofilament proteins in the CSF.

Research Questions

1. *When detectable inflammation is eliminated, is anti-Nfasc antibody reduced?*

2. *Is CNS NfH release specific to MS when compared to OND?*

3. *Does intense immunosuppressive therapy result in a decline in CSF NfH and NfL?*

4. *When inflammation is eliminated, is there a relationship between anti-Nfasc antibody titres and indicators of neurodegeneration, NfH and NfL release?*

5. *Do the CSF concentrations of NfH and NfL correlate with clinical observations?*

RESULTS

Anti-Nfasc antibody titres were measured in the sera and CSF of 19 patients that underwent aHSCT for treatment of an aggressive MS disease course. Serum and CSF were collected from each patient at baseline, prior to transplantation, and at 1, 2, and 3 years post-aHSCT.

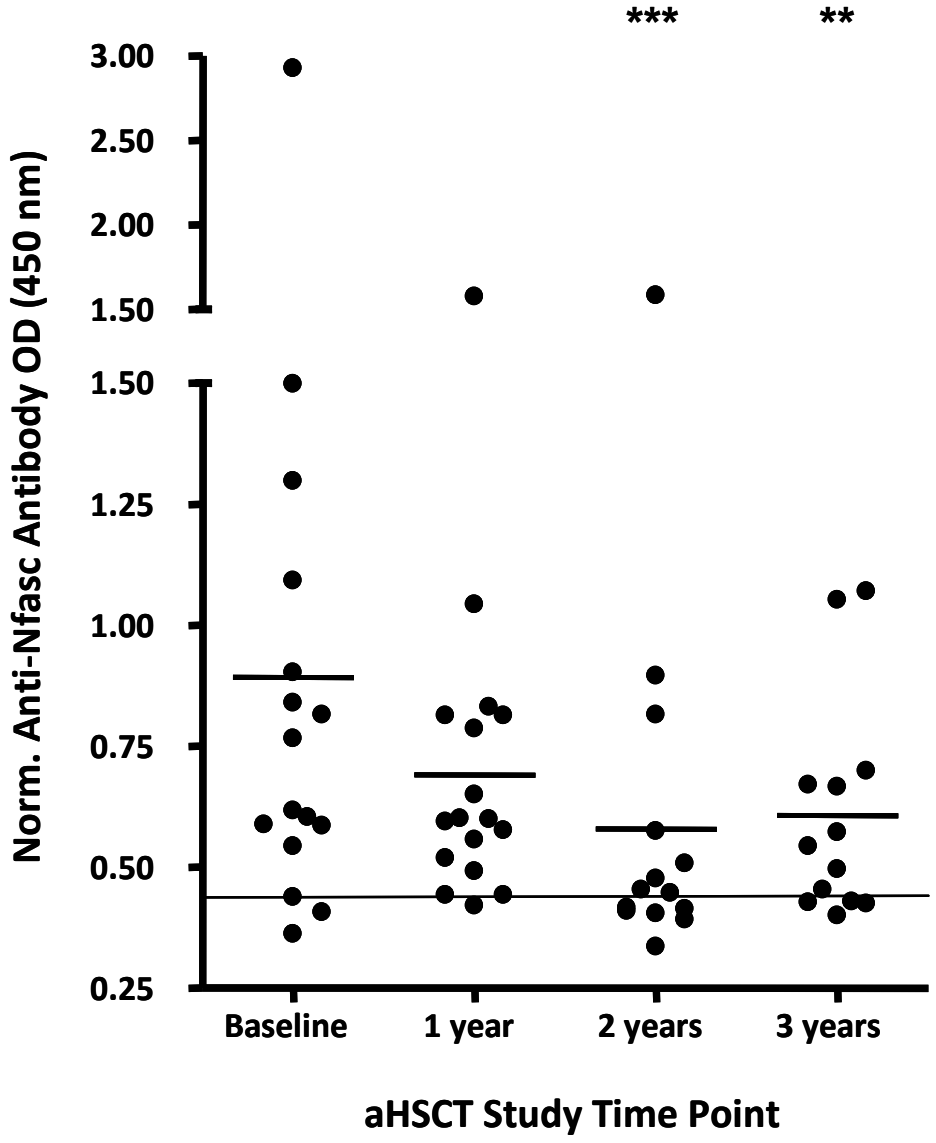
Measurement of Anti-Nfasc antibody in the serum

The OD value of 0.435 (2xSD of the mean of the serum anti-Nfasc antibody OD in the OND control group) was applied as a limit to distinguish negative from positive titres in this cohort. At baseline, positive titres were measured in 87.5% of individuals; at 1, 2 and 3 years post-aHSCT 94.1%, 53.8% and 66.7% were positive, respectively (**Fig 4.1**).

The mean serum anti-Nfasc antibody OD was 0.892 (SD, 0.629) at baseline (**Fig 4.1**). At 1 year post-aHSCT, the OD declined to 0.691 (SD, 0.284) though it failed to reach statistical significance (p=0.1516 vs. baseline). The mean anti-Nfasc antibody OD was reduced further at 2 years post-aHSCT to 0.561 (SD, 0.336, p=0.001 vs. baseline) and, while it rose slightly at 3 years post-aHSCT to 0.602 (SD, 0.235, p=0.0024 vs. baseline), the difference remained statistically significant.

Figure 4.1 Serum anti-Nfasc antibody titres are elevated in aggressive MS.

Serum anti-Nfasc antibody titres were measured by ELISA in a cohort of MS patients that underwent aHSCT for the treatment of aggressive MS. Sera were collected from each patient prior to transplantation (baseline), and at 1, 2, and 3 years post-aHSCT. The OD (450 nm) of 0.435 (2xSD of the mean of the serum anti-Nfasc OD in the OND control group) was applied as a limit to distinguish negative from positive titres in this cohort (denoted with a large horizontal bar). Positive titres were measured in 87.5% of patients at baseline, and in 94.1%, 53.8% and 66.7% of patients at 1, 2, and 3 years post-aHSCT, respectively. The mean serum anti-Nfasc antibody OD was 0.892 (SD, 0.629) at baseline and 0.691 (SD, 0.284) at 1 year. The mean anti-Nfasc antibody OD was reduced significantly at 2 and 3 years post-aHSCT, with means of 0.561 (SD 0.336) and 0.602 (SD, 0.235), respectively. Data represent the anti-Nfasc antibody OD for individual patients, and the mean anti-Nfasc antibody OD at each study time point is denoted by a short horizontal bar. n=19. ***p=0.001 and **p=0.0024 vs. baseline, obtained by the Wilcoxon signed rank test.


***
**
3.00
2.50
2.00
1.50
1.50
1.25
1.00
0.75
0.50
0.25
Norm. Anti-Nfasc Antibody OD (450 nm)
Baseline
1 year
2 years
3 years
aHSCT Study Time Point

Measurement of anti-Nfasc antibody in the CSF

The OD value of 0.154 (2xSD of the mean of the CSF anti-Nfasc antibody OD in the OND control group) was applied as a limit to distinguish negative from positive titres in this cohort. At baseline, positive titres were measured in 23.5% of patients; at 1, 2 and 3 years post-aHSCT 29.4%, 7.7% and 8.3% were positive (**Fig 4.2**).

The mean CSF anti-Nfasc antibody OD at baseline was 0.125 (SD, 0.094) (**Fig. 4.2**). At 1 year post-aHSCT the OD was slightly less at 0.111 (SD, 0.069, p=0.2513 vs. baseline), at 2 years it had decreased to 0.082 (SD, 0.067, p=0.4143 vs. baseline) and at 3 years post-aHSCT it had declined further to 0.074 (SD, 0.083, p=0.1514 vs. baseline). In spite of the serial reductions of the anti-Nfasc antibody OD at each subsequent measurement, the reductions were not statistically significant.

Correlation of anti-Nfasc antibody titres in serum and CSF

A positive correlation between the serum and CSF would indicate that peripheral antibody was gaining entry into the CNS during periods of active inflammation, marked by blood-CSF barrier breakdown. An examination of serum and CSF anti-Nfasc antibody titres revealed that they did not correlate at any time point throughout the study (p=0.7207, 0.3783, 0.4591, and 0.5137 for baseline, 1 year, 2 years, and 3 years post-aHSCT, respectively).

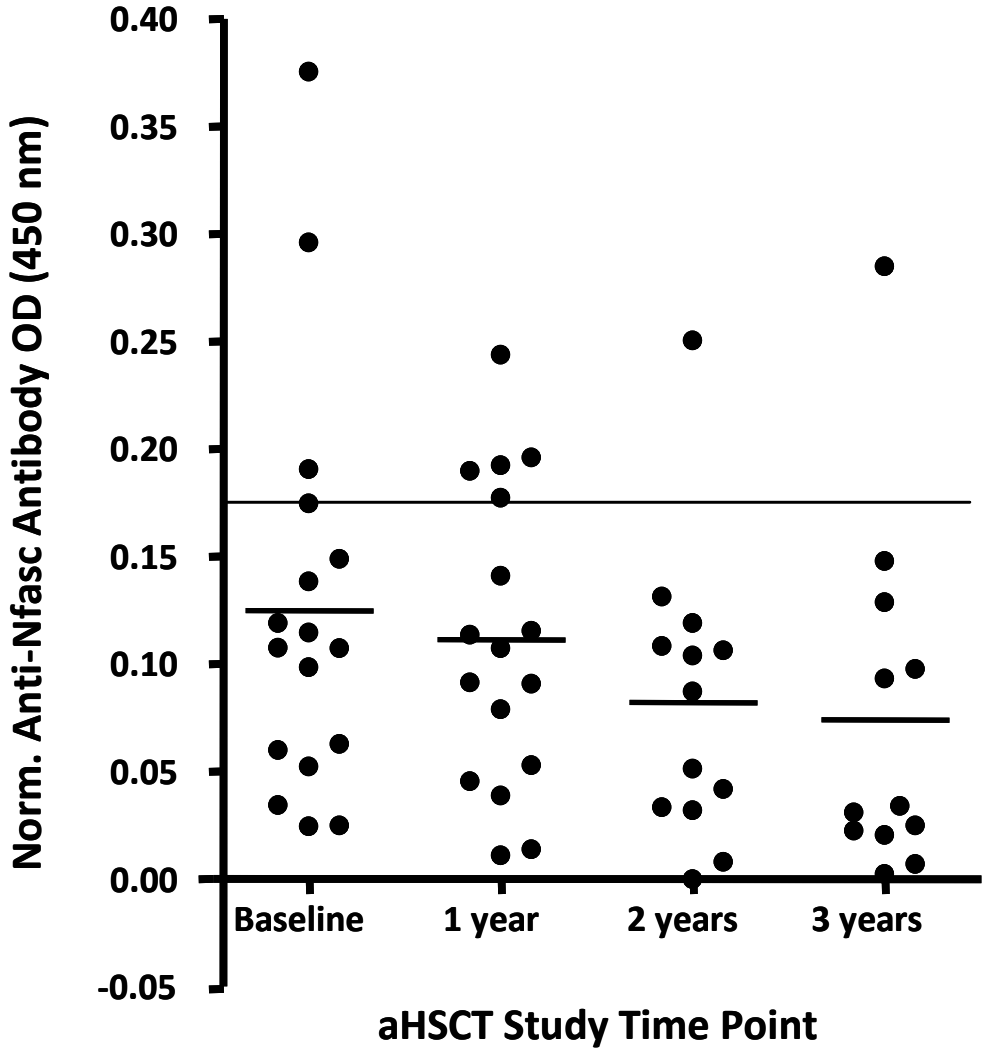
The IgG Index and albumin quotient remain stable throughout the aHSCT study

The CSF index is an indicator of the relative amount of CSF IgG compared to that of the serum, and an increase of the index reflects CNS IgG synthesis. The CSF IgG index was measured at each time point of the aHSCT study and there were no statistical differences observed between the means at any time point (p=0.1186).

The albumin quotient (Q_{alb}) is a comparison of serum and CSF albumin concentrations, and is an indicator of blood-CSF barrier permeability. The mean Q_{alb} was equal, statistically, at each time point throughout the study.

Figure 4.2 CSF anti-Nfasc antibody titres remain stable following aHSCT.

CSF anti-Nfasc antibody titres were measured by ELISA in a cohort of MS patients that underwent aHSCT for the treatment of aggressive MS. CSF samples were collected from each patient prior to transplantation (baseline), and at 1, 2, and 3 years post-aHSCT. The OD (450 nm) of 0.154 (2xSD of the mean of the serum anti-Nfasc OD in the OND control group) was applied as a limit to distinguish negative from positive titres in this cohort (denoted with a large horizontal bar). Positive titres were measured in 23.5%, 29.4%, 7.7% and 8.3% of patients at baseline and 1, 2, and 3 years, respectively. The mean CSF anti-Nfasc antibody OD was 0.125 (SD, 0.094) at baseline, and 0.111 (SD, 0.069; p=0.2513 vs. baseline), 0.082 (SD, 0.067; p=0.4143 vs. baseline), and 0.074 (SD, 0.083; p=0.1514 vs. baseline) at 1, 2, and 3 years post-aHSCT, respectively. Data represent the anti-Nfasc antibody OD for individual patients, and the mean anti-Nfasc antibody OD at each study time point is denoted by a short horizontal bar. n=19. No statistically significant differences were observed. p values were obtained by the Wilcoxon signed rank test.


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
Norm. Anti-Nfasc Antibody OD (450 nm)
Baseline
1 year
2 years
3 years
aHSCT Study Time Point

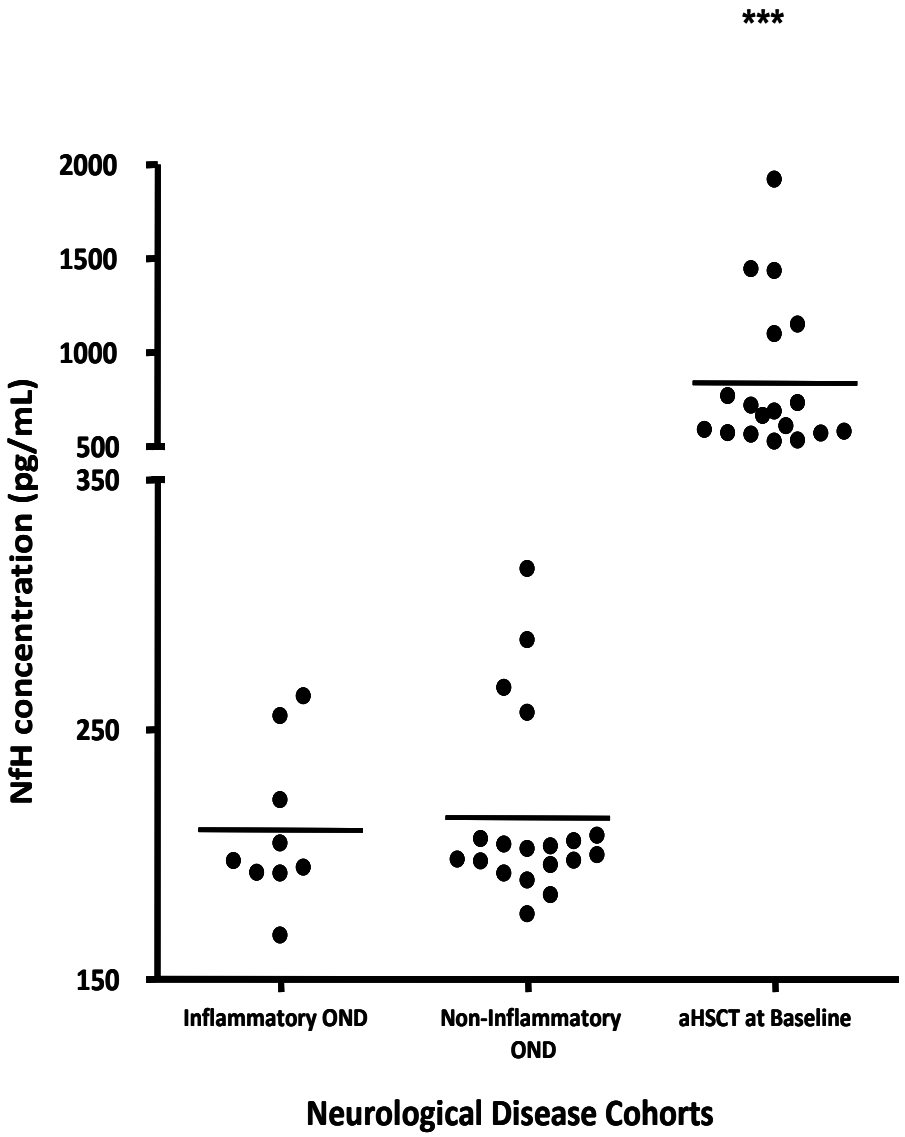
NfH is preferentially released in MS compared to other neurological diseases

NfH predominates in progressive MS and has been shown to reflect its chronic neurodegenerative component. To determine if NfH is specific to MS, it was measured in the CSF of 19 MS patients with an aggressive disease course 60 days prior to undergoing aHSCT (baseline), in addition to 28 OND control patients that were sub-categorized into inflammatory and non-inflammatory OND. Demographic information pertaining to the patients that underwent aHSCT and the OND controls is shown in Tables A2 and A3, respectively, of APPENDIX II.

The mean NfH concentration for the MS patients was 840 pg/ml (SD, 401 pg/ml), and was significantly higher than the means observed for both of the non-MS neurological disease groups, which were 217 (SD, 38 pg/ml, $p < 0.001$ vs. MS) and 210 pg/ml (SD, 34 pg/mL, $p < 0.001$ vs. MS) for the inflammatory and non-inflammatory OND controls, respectively (**Fig 4.3**).

Fig 4.3 Chronic neuro-axonal damage is specific to MS.

The CSF NfH concentration was measured by commercial ELISA in a cohort of MS patients that underwent aHSCT for the treatment of aggressive MS, and a cohort of OND controls that was divided into inflammatory and non-inflammatory OND subgroups. For the MS patients CSF was obtained 60 days prior to treatment (baseline), and at 1, 2, and 3 years post-aHSCT. For the OND controls, CSF was obtained at the time of diagnostic assessment. The MS patients had significantly higher CSF NfH concentrations ($p<0.001$) than both the inflammatory and non-inflammatory OND cohorts. Data represent the CSF NfH concentration of individual patients. The mean NfL concentration for each patient group is represented by a short horizontal bar. n=19 MS patients that underwent aHSCT and 28 OND controls. *p* values were obtained by Kruskal-Wallis ANOVA and Dunn's post-hoc test.


***
2000
1500
1000
500
350
250
150
NfH concentration (pg/mL)
Inflammatory OND
Non-Inflammatory OND
aHSCT at Baseline
Neurological Disease Cohorts

Measurement of NfH and NfL in the CSF of patients undergoing aHSCT

The neurofilament heavy and light subunits have been considered potential biomarkers for disease activity in MS. To investigate the role for aHSCT in the reduction of neuro-axonal injury, the CSF concentrations of NfH and NfL were measured at baseline, and yearly for 3 years in the MS patients following aHSCT, as a means of quantifying the degree of chronic, accumulating and early acute neuro-axonal injury that had occurred.

There was an insignificant decrease in the concentration of NfH from 840 pg/ml at baseline to 805 pg/ml (SD, 397 pg/ml) at 1 year post-aHSCT (p=0.56 vs. baseline) (**Fig 4.4**). At 2 years post-aHSCT, the mean NfH concentration had significantly decreased to 579 pg/mL (SD, 133 pg/ml, p=0.001 vs. baseline) and it declined further at 3 years to 540 pg/mL (SD, and 53 pg/ml, p=0.005 vs. baseline).

At baseline, the mean CSF NfL concentration was 2777 pg/ml (SD 3113 pg/ml); it was markedly reduced to 815 pg/ml (SD, 588 pg/ml, p=0.002 vs. baseline) at 1 year post-aHSCT and continued to decrease at 2 and 3 years post-aHSCT to 464 pg/ml (SD, 220 pg/ml, p=0.003 vs. baseline) and 305 pg/ml (SD, 194 pg/ml, p=0.002) (**Fig 4.5**), respectively.

A correlation between the concentrations of NfH and NfL would indicate that chronic and acute neurodegeneration occur concomitantly. The concentration of CSF NfH and NfL correlated at baseline (p=0.0111) and at 1 year post-aHSCT (p=0.0085), but the correlation was abolished thereafter (**Fig 4.6**).

Fig 4.4 Chronic neuro-axonal damage is reduced in the absence of inflammation.

The concentration of CSF NfH was measured by commercial ELISA in a cohort of MS patients that underwent aHSCT for the treatment of aggressive MS. CSF was obtained from each patient 60 days prior to treatment (baseline), and at 1, 2, and 3 years post-aHSCT. The mean NfH concentration was 840 pg/ml at baseline, and was 805 pg/ml (p=0.56 vs. baseline, ns), 579 pg/ml (p=0.001 vs. baseline) and 537 pg/ml (p=0.005 vs. baseline), respectively, at 1, 2, and 3 years post-aHSCT. Data represent the NfH concentration of each individual at each study time point. SPMS patient data are represented with (•) red circles and RRMS patients with (•) black circles. The mean NfH concentration at each study time point is denoted with a short horizontal bar. n=19. p values were obtained by the Wilcoxon signed rank test.

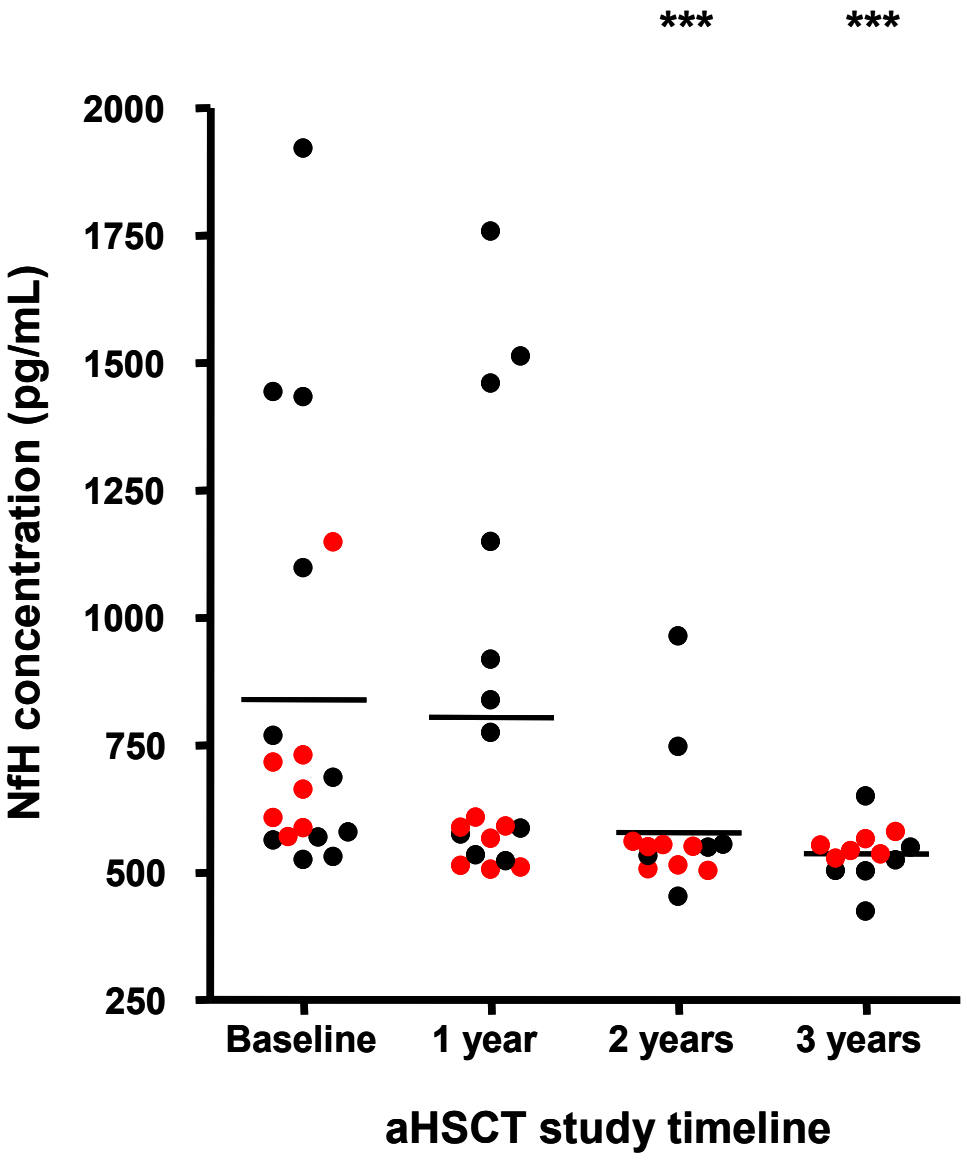

***
***
2000
1750
1500
1250
1000
750
500
250
NfH concentration (pg/mL)
Baseline
1 year
2 years
3 years
aHSCT study timeline

Fig 4.5 Acute neuro-axonal damage is reduced in the absence of inflammation.

The concentration of CSF NfL was measured by commercial ELISA in a cohort of MS patients that underwent aHSCT for the treatment of aggressive MS. CSF was obtained from each patient 60 days prior to treatment (baseline), and at 1, 2, and 3 years post-aHSCT. The mean NfL concentration was 2777 pg/ml at baseline, and was markedly reduced to 815 pg/ml (p=0.002 vs. baseline), 464 pg/ml (p=0.003 vs. baseline) and 305 pg/ml (p=0.002 vs. baseline), respectively, at 1, 2, and 3 years post-aHSCT. Data represent the NfL concentration of each individual at each study time point. SPMS patient data are represented with (●) red circles and RRMS patients with (●) black circles. The mean NfL concentration at each study time point is denoted with a short horizontal bar. n=19. p values were obtained by the Wilcoxon signed rank test.

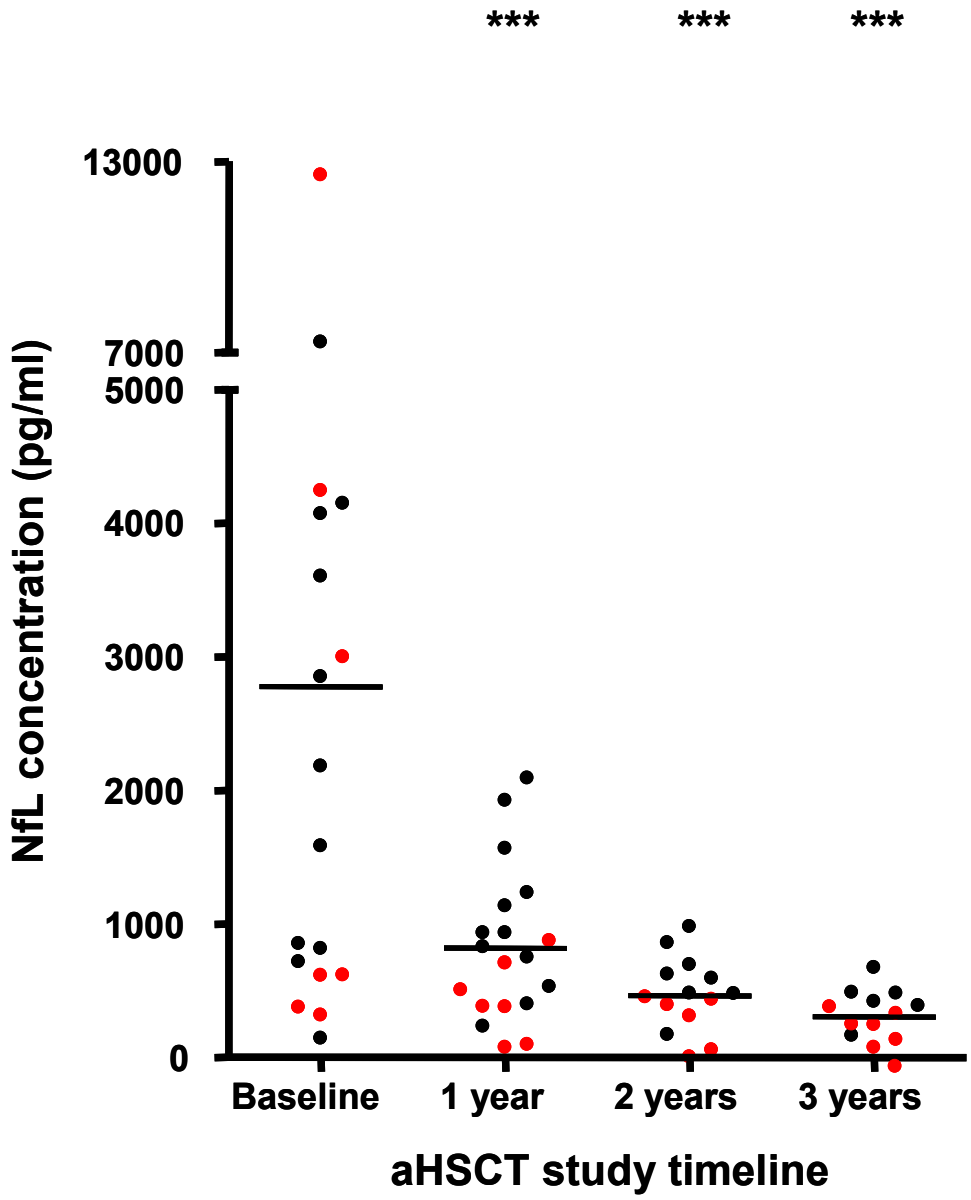
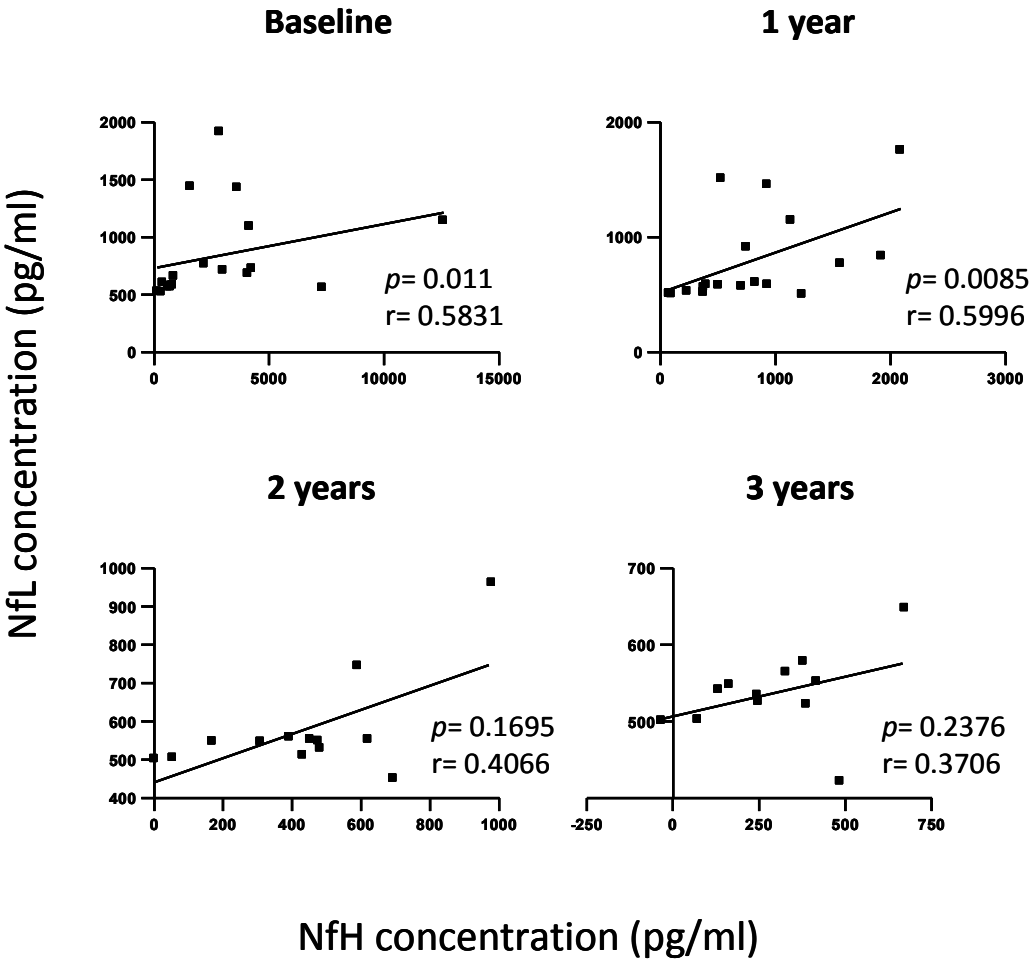

***
***
***
13000
7000
5000
4000
3000
2000
1000
0
NfL concentration (pg/ml)
Baseline
1 year
2 years
3 years
aHSCT study timeline

Fig 4.6 Acute and chronic neuro-axonal damage occur concomitantly in MS.

Positive correlations were observed between the CSF NfL and NfH concentrations at baseline (upper left panel; p=0.0111) and at 1 year (upper right panel; p=0.0085) post-aHSCT, but the relationship was abolished thereafter. Data represent the NfL and NfH concentrations for individual MS patients. n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; p values obtained by linear regression analysis.


Baseline
1 year
2 years
3 years
NfL concentration (pg/ml)
NfH concentration (pg/ml)
p= 0.011
r= 0.5831
p= 0.0085
r= 0.5996
p= 0.1695
r= 0.4066
p= 0.2376
r= 0.3706

Relationship between Anti-Nfasc antibody titre and measures of disease activity

The mean CSF anti-Nfasc antibody titre correlated with the NfH concentration at baseline (p=0.045) (**Fig 4.7**) and at 1 (p=0.031) and 2 (p=0.001) years post-aHSCT, but the correlation was abolished at the 3 year time point. In contrast, the mean CSF NfL concentration failed to correlate with the mean CSF anti-Nfasc antibody titre at baseline (p=0.823) (**Fig 4.8**), but it correlated significantly at 1 year (p=0.001), 2 years (p=0.032), and 3 years (p=0.019) post-aHSCT.

Serum-derived anti-Nfasc antibody titres failed to correlate with NfL and NfH at any time point throughout the study.

Fig 4.7 CSF anti-Nfasc antibody titres correlate with chronic neuro-axonal damage in the absence of inflammation.

The CSF anti-Nfasc antibody titres and NfH concentrations were obtained by ELISA for a cohort of MS patients who underwent aHSCT for the treatment of aggressive MS. CSF was obtained from each patient 60 days prior to treatment (baseline), and at 1, 2, and 3 years post-aHSCT. Positive correlations were observed at baseline (p=0.045), and at 1 (p=0.031) and 2 years (p=0.001) post-aHSCT. A lack of positive correlation was observed at 3 years post-aHSCT. Data represent the anti-Nfasc antibody OD and the NfH concentration for individual patients. n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; p values obtained by linear regression analysis.

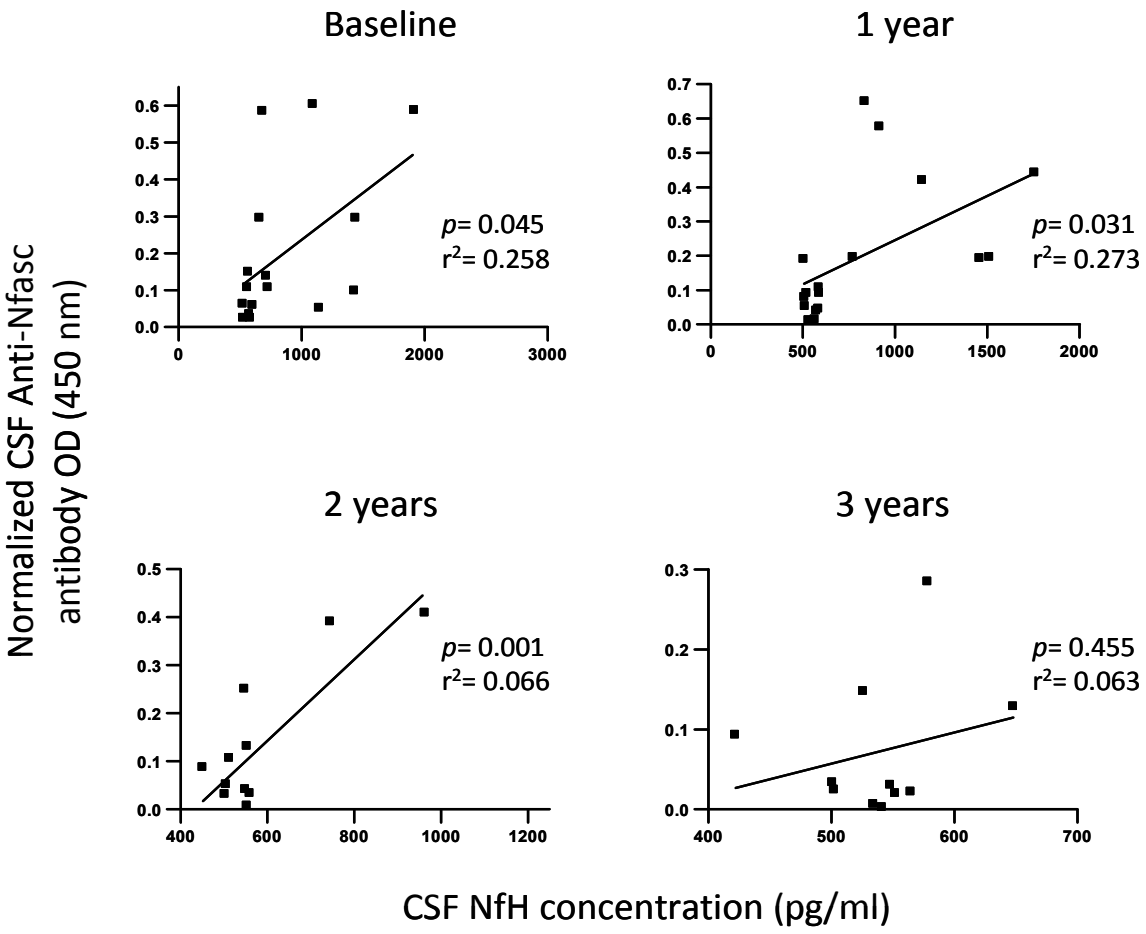
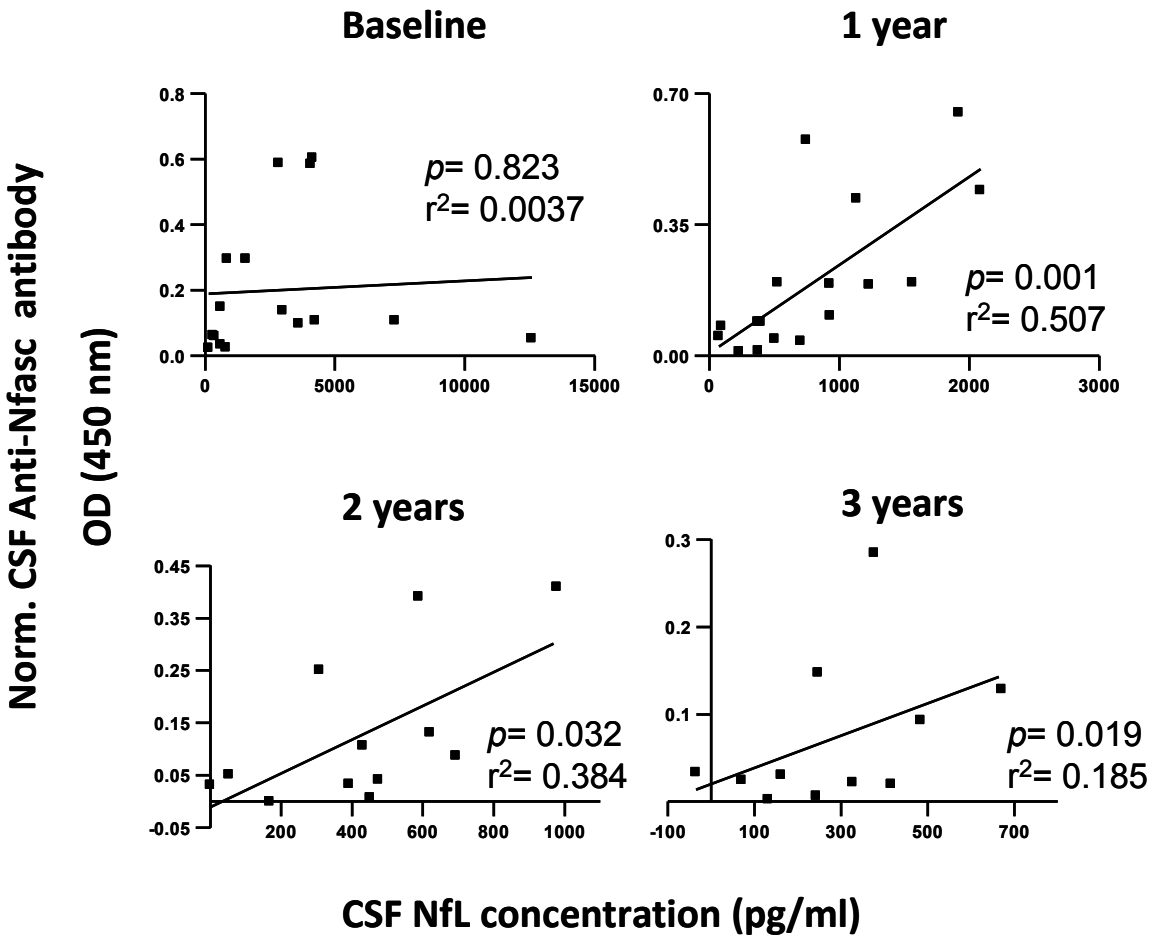

Baseline
1 year
2 years
3 years
p= 0.045
r2= 0.258
p= 0.031
r2= 0.273
p= 0.001
r2= 0.066
p= 0.455
r2= 0.063
Normalized CSF Anti-Nfasc antibody OD (450 nm)
CSF NfH concentration (pg/ml)

Fig 4.8 CSF anti-Nfasc antibody titres correlate with acute neuro-axonal damage in the absence of inflammation.

The CSF anti-Nfasc antibody titres and NfL concentrations were obtained by ELISA a cohort of MS patients who underwent aHSCT for the treatment of aggressive MS. CSF was obtained from each patient 60 days prior to treatment (baseline), and at 1, 2, and 3 years post-aHSCT. A lack of positive correlation was observed at baseline (p=0.823) in the presence of inflammation, and positive correlations were observed at 1 (p=0.001), 2 (p=0.032), and 3 years (p=0.019) post-aHSCT. Data represent the anti-Nfasc antibody titre and the NfL concentration for individual patients. n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; p values obtained by linear regression analysis.


Baseline
1 year
2 years
3 years
p= 0.823
r2= 0.0037
p= 0.001
r2= 0.507
p= 0.032
r2= 0.384
p= 0.019
r2= 0.185
Norm. CSF Anti-Nfasc antibody
OD (450 nm)
CSF NfL concentration (pg/ml)

Relationship between neurofilament concentration and measures of CNS destruction and biochemical changes in the brain

MS patients underwent MR imaging to assess the presence and volume of contrast-enhancing lesions, which indicate focal lesion areas with blood-CSF barrier involvement (416), in addition to the T1W and T2W lesion volumes, which reflect the severity of tissue injury in terms of axonal density and the inflammatory aspects of MS by differentiating between lesions and NAWM, respectively (417). Patients also underwent MR spectroscopy to assess biochemical changes in the brain as measured by the ratio of *N*AA/Cr, which is considered a metabolic marker of the integrity of neurons and axons (418) as lower concentrations of *N*AA are associated with axonal loss and dysfunction (418; 419).

No statistically significant correlations were observed between CSF NfH or NfL concentrations and contrast-enhancing, T1W and T2W lesion volume or *N*AA/Cr at any time point throughout the study when all of the patients were considered.

Active inflammation is associated more strongly with RRMS, and chronic neurodegeneration with SPMS, thus the data were stratified according to disease course to ascertain if disease activity is associated with neuro-axonal injury differently in RRMS and SPMS.

In RRMS, the NfL concentration correlated positively with the volume of contrast-enhancing (p=0.017) (**Fig 4.9**), T1W (p=0.0006) (**Fig 4.10**) and T2W (p=0.007) lesions (**Fig 4.11**), and negatively with the ratio of *N*AA/Cr (p=0.003) (**Fig 4.12**), only at baseline.

In SPMS, the T2W lesion volume correlated modestly with NfL concentrations at 1 (p=0.042) and 3 years (p=0.037) post-aHSCT (**Fig 4.13**) as well as with NfH concentrations at 1 (p=0.032) and 3 years (p=0.006) (**Fig 4.14**).

The NfH concentration failed to correlate with the *N*AA/Cr ratio at any time point for either disease course. In addition, the concentrations of NfL and NfH did not correlate with the EDSS at any time point throughout the study.

Progression-free survival was achieved for 73% of the individuals at the 3 year time point when the collection of biological samples was completed. No additional patient in this cohort has experienced MS disease progression since that time, and some have been free of progression for more than 10 years.

Fig 4.9 Acute neuro-axonal damage correlates with contrast-enhancing lesions in the presence of inflammation in RRMS.

A positive correlation was observed between the CSF concentration of NfL and contrast-enhancing lesions at baseline. No new lesions were observed in any patient following aHSCT. Data represent the NfL concentration and the T1W lesion volume for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.

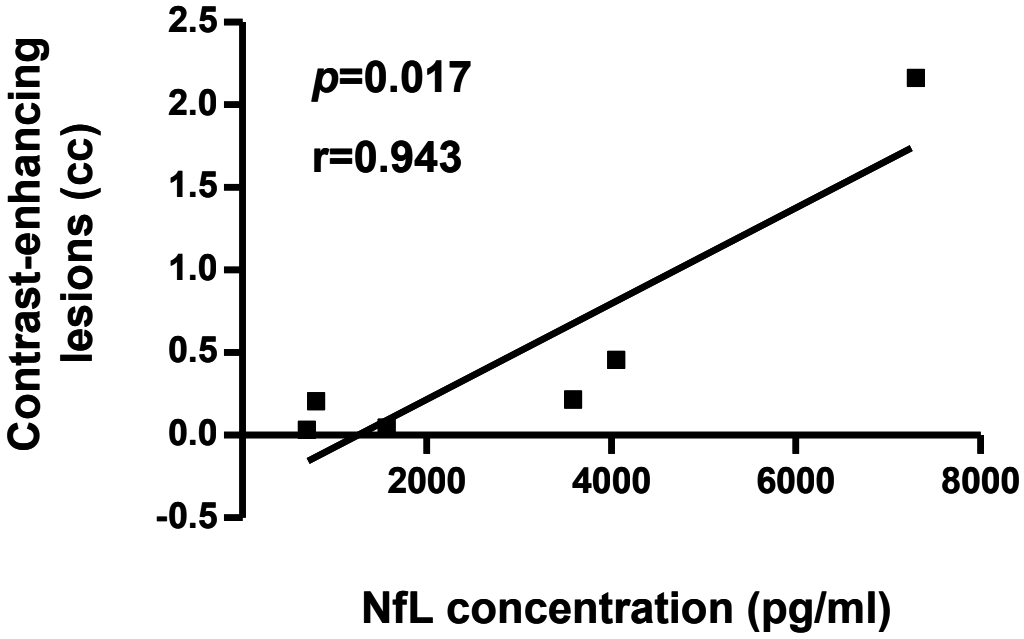

Contrast-enhancing lesions (cc)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
p=0.017
r=0.943
2000
4000
6000
8000
NfL concentration (pg/ml)

Fig 4.10 Acute neuro-axonal damage correlates with the T1W lesion volume in the presence of inflammation in RRMS.

A positive correlation was observed between the CSF concentration of NfL and the T1W lesion volume at baseline (upper left panel). A lack of significant correlation was observed at 1, 2, and 3 years post-aHSCT (upper right, bottom left and bottom right panels, respectively). Data represent the NfL concentration and the T1W lesion volume for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.

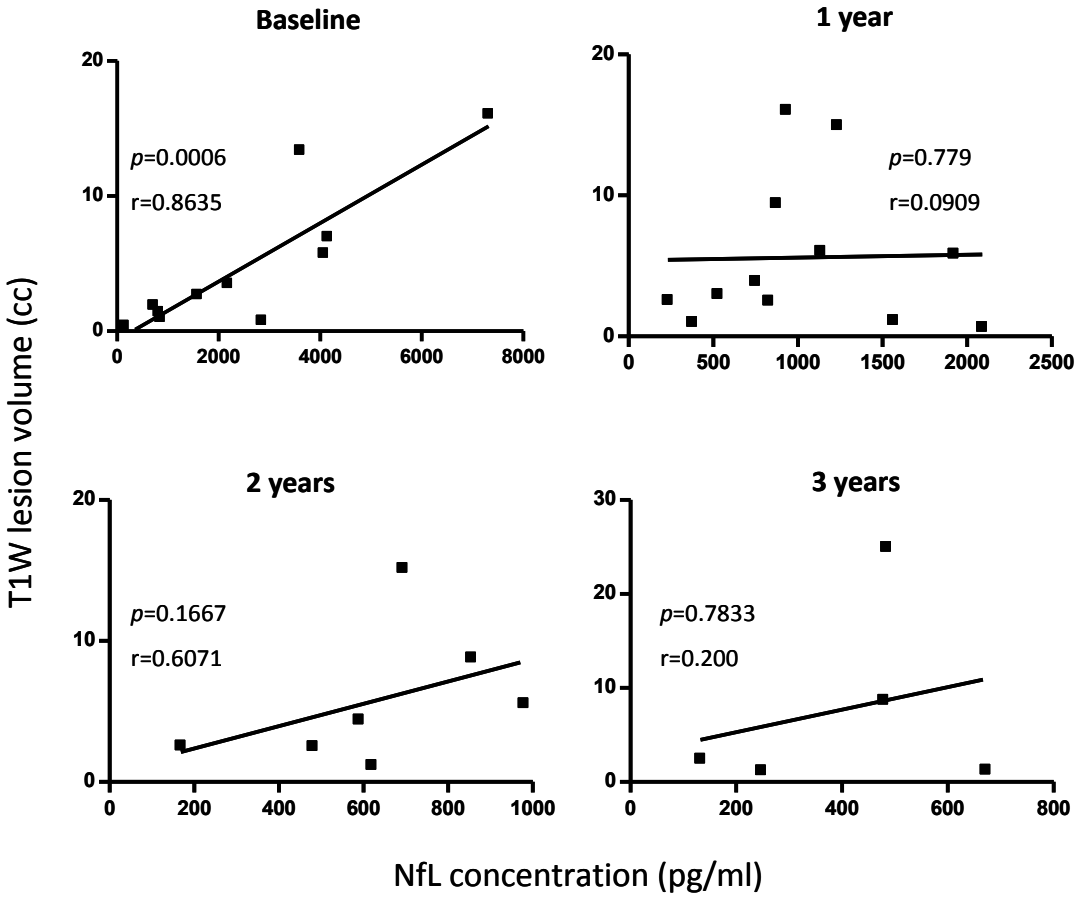

Baseline
p=0.0006
r=0.8635
1 year
p=0.779
r=0.0909
2 years
p=0.1667
r=0.6071
3 years
p=0.7833
r=0.200
T1W lesion volume (cc)
NfL concentration (pg/ml)

Fig 4.11 Acute neuro-axonal damage correlates with the T2W lesion volume in the presence of inflammation in RRMS.

A positive correlation was observed between the CSF concentration of NfL and the T2W lesion volume at baseline (upper left panel). A lack of significant correlation was observed at 1, 2, and 3 years post-aHSCT (upper right, bottom left and bottom right panels, respectively). Data represent the NfL concentration and the T2W lesion volume for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.

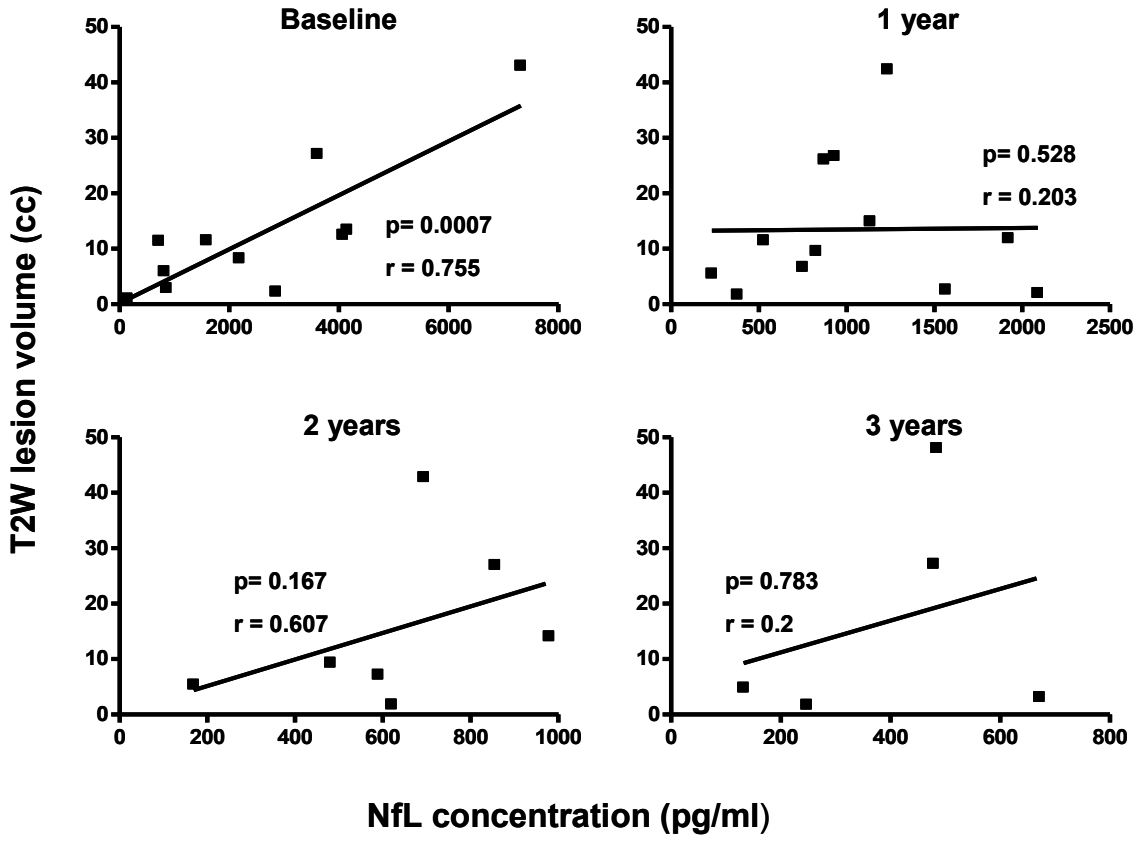

Baseline
p= 0.0007
r = 0.755
1 year
p= 0.528
r = 0.203
2 years
p= 0.167
r = 0.607
3 years
p= 0.783
r = 0.2
T2W lesion volume (cc)
NfL concentration (pg/ml)

Fig 4.12 Acute neuro-axonal damage correlates with neuronal integrity in the presence of inflammation in RRMS.

A negative correlation was observed between the CSF concentration of NfL and the *N*AA/Cr ratio at baseline (upper left panel). A lack of significant correlation was observed at 1, 2, and 3 years post-aHSCT (upper right, bottom left and bottom right panels, respectively). Data represent the NfL concentration and the *N*AA/Cr ratio for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.

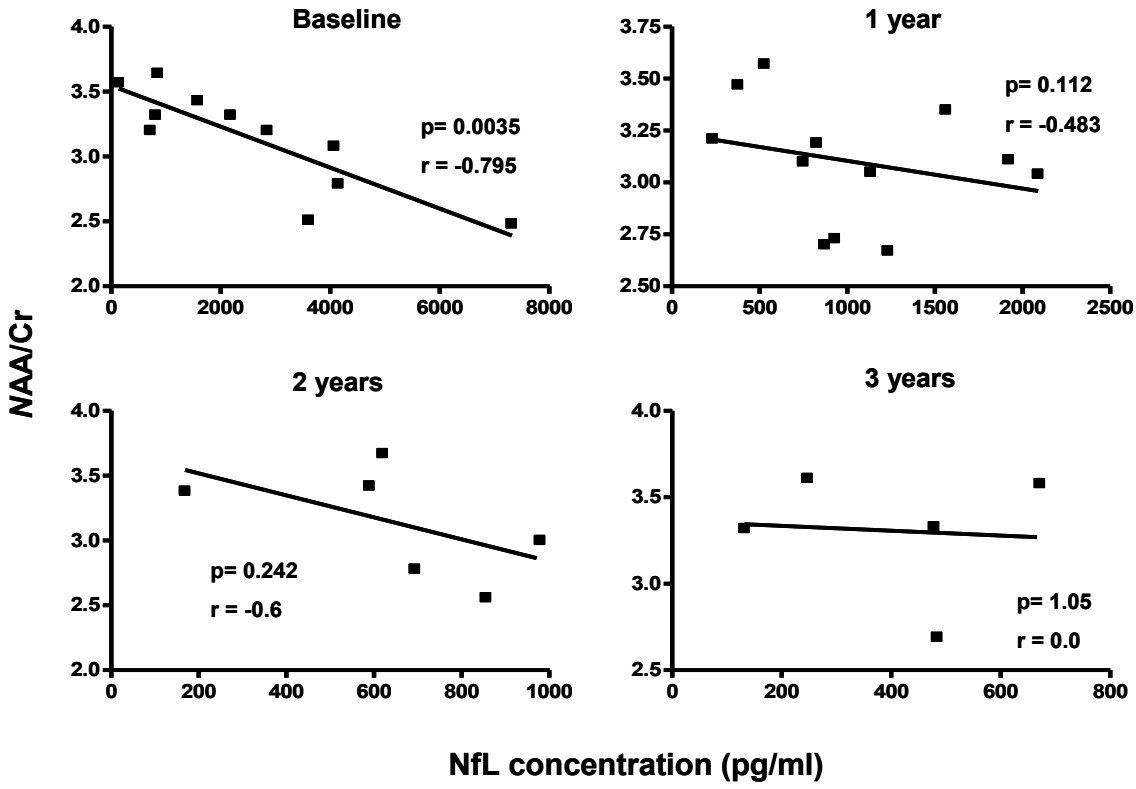

Baseline
p= 0.0035
r = -0.795
1 year
p= 0.112
r = -0.483
2 years
p= 0.242
r = -0.6
3 years
p= 1.05
r = 0.0
NAA/Cr
NfL concentration (pg/ml)

Fig 4.13 Acute neuro-axonal damage correlates with the T2W lesion volume in the absence of inflammation in SPMS.

Positive correlations were observed between the CSF NfL concentration and the T2W lesion volume at 1 (upper right panel) and 3 years (bottom right panel) post-aHSCT. A lack of significant correlation was observed at baseline (upper left panel) and at 2 years (bottom left panel) post-aHSCT. Data represent the NfL concentration and the T2W lesion volume for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.

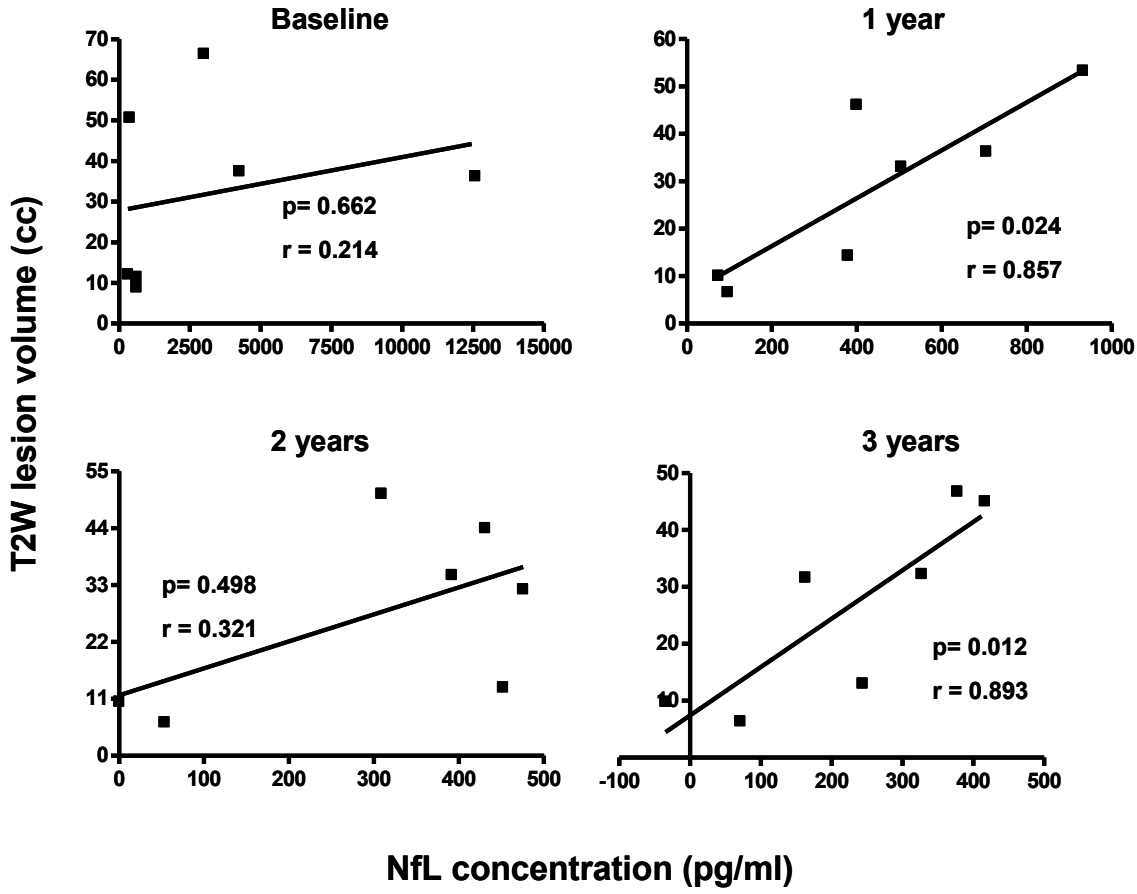
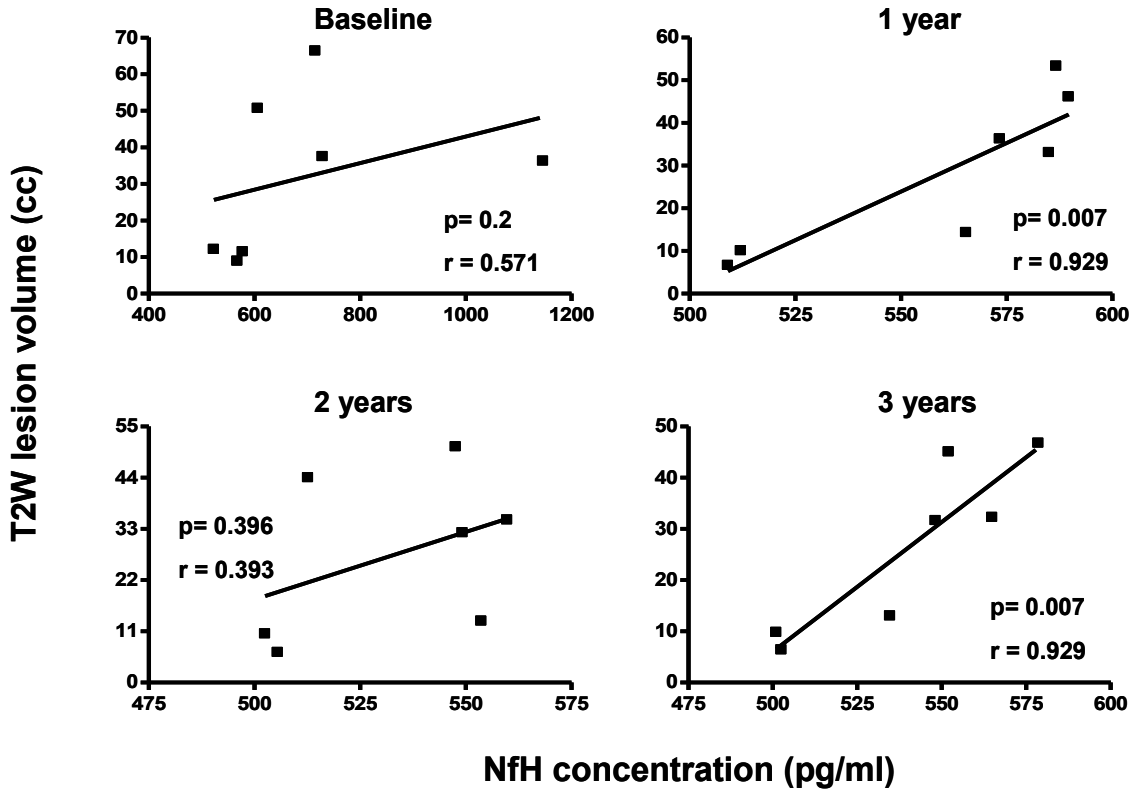

Baseline
p= 0.662
r = 0.214
1 year
p= 0.024
r = 0.857
2 years
p= 0.498
r = 0.321
3 years
p= 0.012
r = 0.893
T2W lesion volume (cc)
NfL concentration (pg/ml)

Fig 4.14 Chronic neuro-axonal damage correlates with the T2W lesion volume in the absence of inflammation in SPMS.

Positive correlations were observed between the CSF NfH concentration and the T2W lesion volume at 1 (upper right panel) and 3 years (bottom right panel) post-aHSCT. A lack of significant correlation was observed at baseline (upper left panel) and at 2 years (bottom left panel) post-aHSCT. Data represent the NfH concentration and the T2W lesion volume for individual MS patients; n=19. Correlation coefficients obtained using Spearman's rank correlation coefficient; *p* values obtained by linear regression analysis.


Baseline
p= 0.2
r = 0.571
1 year
p= 0.007
r = 0.929
2 years
p= 0.396
r = 0.393
3 years
p= 0.007
r = 0.929
T2W lesion volume (cc)
NfH concentration (pg/ml)

DISCUSSION

Anti-Nfasc antibody titres and aHSCT

In the previous Chapter, the presence of anti-Nfasc antibodies in the sera and CSF of MS patients was discussed. The CSF titres were greatest in the RRMS cohort, suggesting that the antibodies gained access to the CNS by diffusion through an inflamed blood-CSF barrier or potentially via engagement of FcR-expressing T-cells. If more anti-Nfasc antibodies gain entry into the CNS under conditions of active inflammation, then the antibody titres should be reduced upon the elimination of inflammation. To test this hypothesis, anti-Nfasc antibody titres were measured in the serum and CSF of a cohort of MS patients with an aggressive course who underwent aHSCT, in essence, to reset their immune systems. What is particularly special about this cohort is that it was possible to evaluate the serum and CSF anti-Nfasc antibody concentrations before the procedure, when the individuals were plagued by active disease, and for a minimum of 3 years thereafter to observe if and how the antibody concentrations persisted in a state that was largely free of immune-mediated disease activity. The procedure eliminated the autoreactive and, presumably, pathogenic T-cells that are thought to be responsible for the attack on CNS elements. Complete hematologic and, therefore, immunologic reconstitution with autologous progenitor HSC followed.

The majority of these subjects, approximately 87%, had a positive serum titre for anti-Nfasc antibody at baseline. The proportion was 94% at 1 year post-aHSCT, but was markedly reduced at 2 and 3 years to 54% and 67%, respectively. In the CSF compartment the baseline titres paralleled what was observed in the greater MS population studied here where approximately 24% MS patients had detectable antibodies to Nfasc. The proportion increased insignificantly to 29% of patients 1 year later, but was then reduced to less than

10% at both 2 and 3 years post-aHSCT, which supports antibody production as a component of the acute inflammatory process.

Despite a reduction in the proportion and magnitude of positive CSF titres at 2 and 3 years post-aHSCT, the decreases were not statistically significant. The opposite, however, held true for the anti-Nfasc antibody titres in the serum, where although a high proportion of positive titres persisted throughout the study, there was a substantial and significant titre reduction at 2 years post-aHSCT, and it remained at that level at least until the 3 year time point when sample collection was completed.

The increased serum titres compared to those in the CSF provide further support that the antibodies are synthesized and compartmentalized in the periphery, and the reduction of peripheral anti-Nfasc antibody following the elimination of T-cells suggests not only that anti-Nfasc antibody is being cleared but that its synthesis is an element of an inflammatory immune response that might be T-cell dependent and; indeed, the generation of memory B-cells is a T-cell dependent mechanism (420).

The flow of serum components into the CNS is a consequence of acute inflammatory blood-CSF barrier injury. It is not completely understood if blood-CSF barrier disruption precedes immune cell entry, but migration modifies permeability and is accompanied by the secretion of inflammatory cytokines and ROS, which facilitate their entry into the CNS (88). It is, thus, likely that T-cell elimination may have prevented further attack and, therefore, permitted some degree of blood-CSF barrier repair. The CSF anti-Nfasc antibody titres decreased with each subsequent yearly measurement, probably as the result of a less permeable blood-CSF barrier that would prevent serum components, including antibody, from diffusing freely. Furthermore, if anti-Nfasc antibodies enter the CNS via engagement of FcR-bearing autoreactive immune cells, such as γδ T-cells, then the removal of the T-cells

would result in concomitant reductions in CNS anti-Nfasc antibody titres. The reductions in the CSF, however, were not statistically significant and this might be because antibodies are known to persist for long periods of time, which is known to occur in the case of OCB (421; 422), but also because it is possible that antibody-secreting plasma cells became trapped in the CNS compartment behind a less permeable blood-CSF barrier following aHSCT.

Neurofilament release is reduced following aHSCT

Clinical disease progression is characterized by irreversible disability in MS (56) and is currently challenging to predict (352). There is potential in body fluid markers, or biomarkers, to predict disease progression and monitor responsiveness to therapy. In MS, the CSF has been investigated for potential biomarkers since CSF is in direct contact with brain tissues, and might contain indicators of disease pathogenesis that could be useful for diagnostic and prognostic assessment. Axonal loss is an irreversible process and is the cause of disease progression in MS (56; 423). The neurodegenerative process begins during the early stages of disease (424), and neurofilament protein subunits are promising candidate biomarkers for MS disease progression.

Neurofilaments are the major structural components of the axon cytoskeleton and are composed of three chains that differ in size based on the length of their C-terminal tails; the light, medium and heavy chains. To date, the medium chain subunit has not been investigated with respect to neurological disease. As the result of axonal damage, neurofilaments are released into the extracellular fluid where they can be measured. While the light and heavy chains share a common parent protein, and how they relate to axonal disease shares considerable overlap, there is evidence that they relate differently to biological and pathological functions in MS (352). NfL is associated with early, more subtle acute inflammatory disease activity that might be modulated with treatment. In contrast, NfH is

associated with later stages of neuronal damage and is thought to reflect the more severe ongoing irreversible injury that reflects disease progression. Nevertheless, the simultaneous measurement of NfH and NfL might provide a more comprehensive picture of the key steps that occur in the progression of MS. In the context of aHSCT, a unique opportunity is provided to investigate both the acute and chronic neuro-axonal injury that occurs in an aggressive disease state, separated from the potent inflammatory response. Validated commercial sandwich ELISAs were used for the quantitative measurement the CSF concentrations of the (phosphorylated) NfH (Biovendor) and NfL (UmanDiagnostics) chains in this cohort of MS patients with highly discriminatory antibodies to rod region epitopes.

CSF NfH was measured in a cohort of patients with aggressive MS and compared to patients with confirmed diagnoses of other neurological conditions, either classically inflammatory (such as transverse myelitis, [TM]) or non-inflammatory (such as migraines) in nature (patient information is shown in **Table A3** of APPENDIX II). The NfH concentrations were nearly identical for both of the OND cohorts and highly significantly less than for the MS patients, suggesting that neuronal injury or death that results in the release of NfH into the CSF is specific to MS, at least when compared to the other conditions that were investigated. The reduced NfH concentrations might have been expected in the cohort of non-inflammatory OND controls as these conditions are not classically associated with neurodegeneration. The comparatively reduced NfH concentration in the inflammatory OND cohort, however, is particularly interesting. More than half of the patients examined in this cohort (6/10) had confirmed diagnoses of TM. TM is similar to MS in that it is a CNS disorder marked by inflammation, perivascular monocytic and lymphocytic infiltration, demyelination and neuro-axonal injury (425). Lesions, however, are restricted to the spinal cord and could be the result of infection, demyelinating inflammation or a systemic

autoimmune reaction (426). The release of neurofilament has not yet been investigated in TM though it is generally considered an acute condition, thus, investigating NfL in this context would probably be more relevant. If neuro-axonal injury occurs in the absence of NfH release, it might reflect neuro-axonal damage that can recover, and indeed, the majority of patients do (425). The patients in this cohort demonstrated clinical evidence of MRI activity, recurrent relapses and deterioration of EDSS scores, which all point to disease progression. The elevated mean concentration of CSF NfH in this patient cohort appears to act as a biological marker for clinical disease manifestation, and suggests that NfH release is specific to MS due to the chronic, smoldering nature of the condition, which has been demonstrated previously (332).

The individuals in this MS cohort were experiencing a highly aggressive disease course leading up to aHSCT. NfL concentrations were significantly reduced at 1 year post-aHSCT, particularly in those whose levels were most elevated at baseline. The concentration was reduced to less than the baseline mean for all individuals at 1 year post-aHSCT, and the mean concentration continued to decline to at least 3 years when the follow-up collection of CSF was completed. The reductions in NfL and NfH concentrations, however, failed to correlate with anti-Nfasc antibody titres, and this might pertain to disease pathogenesis that relates to disease course. Almost 60% of this cohort was classified as RRMS while the remainder were SPMS and, despite the common aggressive disease course experienced by all, the disease characteristics that resulted in similar clinical manifestations likely varied between the subtypes. The RRMS patients were more likely to experience aggressive immune-mediated inflammation, whereas the SPMS group may have experienced more neurodegenerative processes. It might not be surprising then, that despite noticeable reductions in the mean NfL and NfH concentrations, the correlations with anti-Nfasc

antibody titres within some individuals might not have been as strong because NfL might have been a more meaningful marker for RRMS and NfH for SPMS. More importantly, however, if γδ T-cells can act as effectors in a mechanism of ADCC in concert with anti-Nfasc antibodies, the failure to correlate with indicators of neuro-axonal injury might pertain less to the actual antibody levels in the CNS, but more to the fact that the T-cells are no longer present to engage the neuro-axonal autoantibodies; thereby reducing axonal injury.

Neurofilaments as potential biomarkers

These findings provide support for NfL as an indicator of acute axonal injury, and NfH as a marker for chronic, accumulating injury as have been previously reported (340). The NfL concentrations were dramatically reduced following aHSCT, which supports a lessening of immunopathogenic disease processes that might be associated with the elimination of T-cells. The reductions of NfH, while significant, were more modest in comparison. These patients continue to have MS, and if neurodegenerative processes occur independently of inflammatory immune-mediated disease processes, particularly in the SPMS cohort, then chronic axonopathy might persist. The NfL reductions in this progressive cohort are supported by parallel observations from a recent study that demonstrated significant reductions in CSF NfL concentrations in a cohort of progressive MS patients following mitoxantrone or rituximab therapy (427), which suggests that immunosuppressive therapy reduces ongoing neuro-axonal destruction. In addition, significant reductions of NfL have been observed following natalizumab therapy in RRMS patients (356; 361).

Clinical responses to treatment require time to evolve, and what is observed in terms of tissue destruction at any given time point is likely more a reflection of what damaging inflammation occurred previously. Considering the marked NfL reduction at 1 year post-aHSCT, this indicates that, in the absence of inflammation, acute neuro-axonal injury might

lessen or recover relatively quickly. Surprisingly, though not reaching statistical significance, the mean NfL concentration at 3 years post-aHSCT was nearly half of that measured in a group of 10 OND controls. Thus, immunoablation was effective with respect to the removal of the inflammatory components in these patients, and this was associated with a reduction, perhaps cessation, of acute immune-mediated neuro-axonal tissue destruction post-aHSCT. The continued presence of measurable NfL suggests that, even in non-inflammatory conditions or potentially "normal" individuals, some degree of neurodegeneration or neuro-axonal turnover occurs. A recent report suggested that it might, at least in part, be an aspect of normal ageing (345).

The mechanisms underlying chronic neuro-axonal injury were not as swift to respond to aHSCT as the mean concentration was not significantly reduced as early as that of NfL, which likely reflects the severe damage that was ongoing leading up to treatment. If, however, a greater reduction in NfH release did occur in the year following aHSCT, it is possible that it was masked by ongoing CNS atrophy as the result of the toxic conditioning regimen undergone by the patients that crosses the blood-CSF barrier and causes tissue damage. Furthermore, some degree of neurodegeneration may occur independently of inflammatory processes, particularly in progressive MS. It is likely that once a certain level of neurodegeneration occurs, it becomes unstoppable; and treatment with immune modulators is unlikely to alter the neurodegenerative process. The natural history of MS demonstrates that once an EDSS score of 4 is reached, relapse activity is less relevant to neurodegeneration (55), and the majority of the patients in this cohort had scores beyond that threshold. A significant reduction in the concentration of NfH was observed at 2 years post-aHSCT, and indicates that active inflammation probably contributes to neurodegeneration in

MS, and that elimination of this inflammation leads to a marked reduction of axonal loss and, ultimately, disease progression (355; 428).

If NfL reflects early, acute injury and NfH reflects chronic, non-inflammatory neurodegeneration, then aHSCT might be expected to stop the release of NfL, but not NfH. Greater reductions in NfL concentrations were observed, that declined beyond the levels of the OND controls, suggesting that its release was stopped. Though NfH declined significantly, it remained elevated compared to OND controls, suggesting that neurodegeneration, not unexpectedly, continues. Only a very modest NfH reduction was observed between 2 and 3 years post-aHSCT, and the concentration was nearly identical in all of the patients at the 3 year time point. CSF was not obtained thereafter, thus it is not possible to continue the biological assessment of neuro-axonal injury but this might be an indication that the concentrations had plateaued and that a new baseline level of ongoing neurodegeneration had been reached, that was independent of inflammation.

Correlations with clinical observations

Following aHSCT no new lesions were observed, neuronal integrity improved (as measured by the *N*AA/Cr ratio), and most patients remained free of progression, indicating that disease activity was lessened in the absence of inflammation. When the entire cohort was examined, however, no statistically significant correlations were detected between the clinical measures of disease activity or the EDSS and the CSF concentrations of either neurofilament subunit. The heterogeneity of MS might be the cause of this and, in spite of a common aggressive phenotype, the disease processes could be highly variable between patients, both mechanistically and temporally. Biological responses, such as changes in neurofilament release, are likely to be observed more rapidly due to the high rate of CSF turnover (352) than clinical responses that require time for development, such as changes in

lesion volume and metabolite concentrations, which may or may not later be reflected in the EDSS score. The release of NfL has rarely been associated with the EDSS (356), probably because it is more associated with acute injury that might be recoverable and might not manifest clinically as physical disability. On the other hand, several studies have found a correlation between NfH release and the EDSS in the progressive phase of disease (332; 345-347), which supports this type of injury as irreversible and leading to progression. The failure of NfH release to correlate with the EDSS score in the current study not only pertains to the time lag that might be expected between biological improvement and the potential for subsequent clinical manifestation of that improvement, and that some clinical deficit might result from demyelination and conduction block, but also to the fact that NfH release is associated with axonal transection that cannot be repaired, thus improvement in the EDSS score might not be expected. Furthermore, in the short-term, clinical measures might not be adequately sensitive to reflect ongoing disease activity (365). Interestingly, at the 3 year time point, almost half of the individuals in the cohort had experienced improvement of the EDSS score, which might relate more to the reduction in NfL release as this type of damage is more likely to recover. Regardless, the failure of neurofilament release to correlate with the EDSS is not unexpected and indicates that even upon a reduction in neuro-axonal injury, clinical manifestation of the reduction may or may not occur, likely depending on the degree of damage and how that relates to physical disability. This suggests that the EDSS is probably not a sensitive enough tool for assessing neuro-axonal injury in MS.

The majority of this cohort experienced clinical stabilization (progression-free survival), some for as long as 10 years following aHSCT. The accumulation of disability in the progressive phase of MS is thought to result from irreversible neuro-axonal damage (419), and the remarkable reductions in CSF NfL and NfH concentrations following aHSCT

suggest that, in the absence of inflammatory stimuli that cause injury to neuro-axonal elements, disease progression is slowed or even halted. Nevertheless, some patients progressed clinically during the 3 year follow-up. Interestingly, four of the five who progressed were RRMS patients, which might suggest that they were already in transition to a progressive course at the time of the procedure. There is the possibility that surviving autoreactive T-cells were re-introduced with the autologous graft and could have continued an assault on CNS components, however, the complete absence of new inflammatory lesions post-aHSCT suggests this possibility is highly unlikely. All of the patients demonstrated the re-emergence of myelin-autoreactive T-cells as early as 6 months post-aHSCT (429). It is known, however, that these can be obtained from non-MS patients as well (430), as OGD are considered an immunodominant cell type. It is most likely that progression in these patients is the result of the neurodegenerative component of MS that occurs independently of inflammation.

Disease Course

All of the MS patients in the cohort shared an aggressive disease course but clinical disease activity is often more pronounced in the relapsing/remitting phase of MS. The cohort consisted of both RRMS (12/19) and SPMS (7/19) patients, thus the data were examined accordingly to assess any differences that might be attributed to disease course. With respect to NfL, the baseline concentrations were nearly identical between the RRMS and SPMS patients, however, following aHSCT, the NfL concentrations for the SPMS patients were half to one third of the concentrations of the RRMS patients, which suggests that the inflammatory component might remain more active in the relapsing cohort. The procedure eliminates T-cells but patients experience proportionally higher numbers of potentially autoreactive B-cells, therefore, it is possible that injurious immune-mediated mechanisms are

ongoing, particularly in the RRMS cohort. In contrast, the mean NfH concentration was elevated by 25% in the RRMS cohort at baseline, with much more pronounced serial reductions in post-aHSCT concentrations in comparison to the SPMS cohort, which demonstrated a comparatively modest reduction by the end of the follow-up period. This provides support for the occurrence of chronic neurodegeneration in the earlier phases of disease, which was previously established by Trapp et al., in the seminal publication that indicated that the majority of axonal transection occurred in early active MS lesions (68).

The disease course is also likely to play a role in disease pathogenesis, thus the relationship between clinical measures of disease activity and the release of neurofilament subunits was examined in the context of RRMS and SPMS patients within the larger cohort. In the RRMS subjects, the NfL concentration correlated positively with the contrast-enhancing lesion volume, which was similarly reported in a recent study (431), both the T1W and T2W lesion volumes, and negatively with the ratio of *N*AA/Cr at baseline, supporting the relationship between acute axonal destruction and active lesion formation and reduced neuronal integrity in the presence of inflammation. Indeed, the majority of transected axons in MS are observed in active lesions (68). Post-aHSCT, the relationships between acute neuro-axonal injury and disease activity were abolished, indicating a cessation of destructive axonal injury and suggesting that, in the absence of active inflammation, NfL might not be a good indicator of ongoing neurodegeneration. This is the first study to assess the release of NfL following immunoablation and aHSCT in a cohort of patients with a tendency for disease progression and, indeed, NfL as a biomarker has not proven as reliable as NfH with respect to the progressive phase of MS (344) where the active immune system is different than that of the relapsing/remitting phase.

With respect to the SPMS patients, both the NfL and NfH concentrations correlated with the T2W lesion volume, which differentiates between lesions and NAWM, at two of the three time points following aHST. While it is possible that both acute and chronic disease mechanisms are functioning independently, if NfL release is caused by active inflammation, it is more likely that when lesion formation continues in the progressive phase of MS, that it might be of a chronic, burning nature that concomitantly releases NfL from focal lesion areas or damages adjacent tissue. In contrast, the concentration of NfH remained elevated until after NfL was reduced, which might suggest that active inflammation directly affects both acute and chronic neurodegeneration in MS, and that, perhaps, upon the alleviation of acute injury, chronic neurodegeneration can begin to subside.

These observations emphasize heterogeneity between RRMS and SPMS, even within aggressive disease courses that might blur the clinical distinctions between them, and with a relapsing contingent that could have already been in transition to the progressive phase. In agreement with a previous study (356), the neuro-axonal destruction that results in the release of NfL appears to be congruent with measures of inflammatory disease activity preferentially in the relapsing/remitting phase of disease, as it was not indicative of destructive activity in the progressive patients, perhaps because the threshold of axonal loss was already reached, and continuing axonal loss was then unstoppable.

Interpretation

The results presented herein demonstrate that aHSCT is an effective alternative treatment for aggressive MS. Immunoablation and autologous hematopoietic and, therefore, immunological reconstitution reduces axonal destruction that is thought to be responsible for the irreversible disability that is associated with MS. This is demonstrated by marked post-treatment declines in the release of both NfL and NfH subunits into the CSF that are

accompanied clinically by a lack of new lesions, improvement in neuronal integrity and progression-free survival for the majority of the individuals.

All of the patients in this cohort experienced aggressive disease courses, and though NfL has historically been considered a potential biomarker for "early" or "acute" axonal destruction, it can be argued that it is more appropriately an indicator of active inflammation in MS. This is evidenced by marked and significant CSF NfL reductions in the absence of inflammation. Moreover, it correlated positively with contrast-enhancing, T1W and T2W lesion volumes and negatively with the ratio of *N*AA/Cr in RRMS patients at baseline in the presence of inflammation, but not following aHSCT. Furthermore, it failed to correlate with measures of active inflammation at baseline in SPMS patients where inflammatory activity might be of a different nature.

The data suggest that chronic neurodegeneration might consist of components that are both dependent upon and independent of inflammation. NfH failed to correlate with the majority of clinical measures of disease activity in both RRMS and SPMS cohorts, both in the presence and absence of inflammation. This implies that it might be a distinct pathogenic entity that might be associated with progression, which has been suggested previously (432-434), and is supported here by significant elevations in MS patients in comparison to inflammatory and non-inflammatory OND controls. In the absence of inflammation, however, NfH was significantly reduced, though not as rapidly as the reduction of NfL. This might suggest that chronic neurodegeneration could be influenced by mechanisms of acute axonal damage. It is possible that modulating or suppressing the active inflammatory component of the MS disease course might prevent some degree of the chronic destruction that commonly defines the progressive phase of MS, and that this might help to prevent

irreversible injury, which could, therefore, prolong the transition to the progressive phase where DMT have not been efficacious.

Caveats

These findings must be cautiously accepted as the sample size is small with only 19 patients, and data are missing for 1 or more time points for 7 subjects. Clinically, the individuals in this cohort were declining rapidly as aHSCT approached but that does not necessarily imply that immune-mediated tissue injury was the greatest factor in disease pathogenesis as neurodegenerative factors might be of more importance in this cohort. Of the 19 followed in the study 7 were classified as SPMS, a subtype of MS not generally defined by active inflammation. Indeed, in the SPMS sub-group of this cohort anti-Nfasc antibody titres did not correlate with the contrast-enhancing lesions and the T1W lesion volume, measures of lesion area that consists of neuro-axonal destruction and loss, at any time throughout the study. Thus, blood-CSF barrier disruption and flow of antibody into the CNS might have been a less important factor at baseline for this cohort, and it might not be surprising then that CSF anti-Nfasc antibody titres were not significantly reduced throughout the study. Nevertheless, MS is a highly heterogeneous CNS disease, yet it is classified into distinct subtypes, based on several clinical parameters that cannot provide a comprehensive representation of disease activity. While the majority of the subjects were classified as RRMS, it is likely that some individuals had already begun the transition to SPMS and it is possible that disease activity during this period fails to accurately represent what would commonly be observed in either course, and this might further confound data interpretation.

NfL and NfH release are associated more strongly with acute and chronic disease activity, respectively, but this cohort of patients is not composed of truly acute (RRMS) and chronic (SPMS) patients, as they are all actively relapsing and progressing. To better gauge

how neurofilament release relates to disease activity in MS, a more structured definition of RRMS and SPMS would be required. Perhaps then, a more comprehensive picture of how neurofilament release relates to disease course could be developed.

NfH appears to be specific to MS in comparison to OND. The CSF from the OND cohort, particularly the subjects with inflammatory OND, were derived from individuals with mainly acute conditions, such as TM, thus it is perhaps not unanticipated that NfH was not highly concentrated in those CSF samples as it has been more closely associated with chronic neurodegeneration. An assessment of NfL in these cohorts might provide a more comprehensive representation of the neurodegenerative processes that might be occurring in these subjects, especially for the inflammatory conditions, as NfL is likely to be a superior indicator of acute axonal injury.

Finally, concentrations of NfL and NfH were not investigated in the sera. An understanding of how the CSF and serum neurofilament concentrations correlate in MS patients would be extremely beneficial for patients and practitioners, as serum is much less invasive to obtain.

Conclusions and Future Directions

In the absence of inflammation following aHSCT, serum anti-Nfasc antibody titres were reduced, suggesting that antibody was being cleared but, more importantly, that antibody production might have been an element of an inflammatory immune response that was T-cell dependent. The decrease in the CSF of this cohort, while not statistically significant, suggests that anti-Nfasc antibodies might persist for long periods of time behind a repaired blood-CSF barrier in the absence of T-cell-induced inflammation. Furthermore, the findings presented here support that immune-mediated neuro-axonal damage occurs in MS. In the absence of inflammation, neuro-axonal injury, as evidenced by the concentrations

of NfL and NfH subunits, is markedly reduced yet still persists for at least 3 years. This supports a role for γδ T-cell-induced neuro-axonal ADCC, as even in the presence of persistent anti-Nfasc antibody, neuro-axonal injury was significantly reduced in the absence of T-cells.

γδ T-cells have long been associated with MS disease pathogenesis. Most recently, $CD16^+$ γδ T-cells have been positively correlated with MS disease progression (217) and shown to induce ADCC (130), though an MS patient-derived target autoantibody has yet to be investigated in this context. It is possible that the anti-Nfasc antibody titres alone fail to correlate with some measures of disease in MS because the mechanism requires both the antibodies and $CD16^+$ γδ T-cells in order for the injury to occur. I, therefore, propose that a mechanism of MS pathogenesis could involve a scenario whereby γδ T-cells could bind the Fc region of anti-Nfasc antibodies via CD16, and subsequently induce ADCC of Nfasc-expressing elements.

CHAPTER 5. γδ T-cells mediate cytolysis via ADCC in the presence of MS patient-derived anti-Nfasc antibodies

ABSTRACT

Background: Higher titres of anti-Nfasc antibodies are detected in the sera and CSF of MS patients. The antibody titres correlated with indicators of neuro-axonal injury in the CSF, raising the possibility that axonal injury may be immune-mediated and that it could involve interaction with anti-Nfasc antibodies. Following aHSCT, a definitive treatment shutting down all evidence of inflammation with resultant reductions in CSF neurofilament release, CSF anti-Nfasc antibody titres remained elevated. This suggested that the presence alone of anti-Nfasc antibodies is not enough to cause axonal injury. I propose a possible mechanism for anti-Nfasc-mediated axonal injury via ADCC, thus requiring the co-presence of FcR-bearing cells. This might explain the failure to correlate higher titres of anti-Nfasc antibodies with measures of axonal injury (neurofilament release) in aHSCT treated MS patients, since the treatment virtually shuts the blood-CSF barrier, thereby preventing the entry of FcR-bearing cells such as $CD16^+$ γδ T-cells into the CNS.

Methods: Anti-Nfasc antibodies were captured from sera using affinity chromatography. HeLa cells were stably transfected with a full-length tGFP-tagged human Nfasc-186 cDNA clone. An ADCC cytolysis assay was developed to assess the ability of γδ T-cells to cytolyse Nfasc-expressing target HeLa cells in the presence of MS patient-derived anti-Nfasc antibodies.

Results: Anti-Nfasc antibodies were harvested from high titre MS patient sera and were shown to consist largely of IgG_1 and IgG_2 isotypes. HeLa cells were transfected with a full-

length human Nfasc-186 clone. When co-cultured with $CD16^+$ γδ T-cells in the presence of MS patient-derived anti-Nfasc antibodies, the percent specific cytolysis of the Nfasc-transfected HeLa cells was significantly greater than that of the non-transfected control HeLa cells, at 18% and 1%, respectively, indicating cytolytic kill via ADCC.

Summary: These experiments prove that FcR-bearing γδ T-cells can cause axonal damage by lysing axonal membranes via ADCC, when armed with axon-specific antibodies such as anti-Nfasc. This is the first report of γδ T-cell-induced cytolysis by ADCC using both γδ T-cells and antibodies derived from MS patients.

INTRODUCTION

γδ T-cells are important effector elements of the innate immune system. They are capable of assessing the identity of invading micro-organisms and lysing them directly, or initiating a secondary response from the adaptive immune system. They have, however, been invoked in MS immunopathogenesis; they are more numerous in MS CSF than controls (218) and are concentrated in lesions (219-221). Additionally, brain-derived γδ TCRs demonstrate limited heterogeneity, which is indicative of a localized clonal response to antigen (218; 222-224). γδ T-cells have been known to exert cytotoxic effects on OGD (228; 230) and they recognize a variety of surface molecules that are upregulated upon cellular stress, such as HSP, which are expressed on OGD in MS (225-227).

γδ T-cells expressing CD16 were shown to correlate positively with MS disease progression (217) and were capable of inducing target-directed ADCC (130), a mechanism they are more commonly known to exert in response to infection. For example, γδ T-cells lyse Staphylococcal enterotoxin-coated target cells in the presence of serum containing

enterotoxin-specific antibodies (435). ADCC is a lytic process whereby FcγR-expressing effector cells, usually of the innate immune system, lyse target cells that are bound to specific IgG antibodies (123; 130). Target lysis is carried out by the release of inflammatory mediators such as perforin or granzymes, by the interaction of FasL and TRAIL, or by the production of cytokines (131).

In Chapter 3, the detection of anti-Nfasc antibodies in MS patient sera and CSF was discussed, and shown to be relevant to MS. Anti-Nfasc antibodies were compartmentalized in the periphery, and possibly gain entry into the CNS during periods of active inflammation, as the CSF titres were augmented in relapsing/remitting patients but not in progressive patients where disruption of the blood-CSF barrier is a less prominent feature of the disease course. CSF concentrations of NfL, a marker of axonal injury, correlated with anti-Nfasc antibody titres in relapsing/remitting patients.

Antibody may be crossing the blood-CSF barrier as a soluble factor, but may also be transported in by activated γδ T-cells via engagement of CD16, a low-affinity FcγR, where it is possible that the immune complex is capable of inducing cytolysis of Nfasc-expressing axonal elements via ADCC. In a cohort of MS patients undergoing aHSCT for the treatment of aggressive MS, CSF concentrations of NfL were dramatically reduced while a concomitant reduction was not observed in titres of anti-Nfasc antibody. This indicated that the antibodies alone were incapable of causing much damage. One possible explanation for this is that antibody persists for long periods of time behind the non-inflamed blood-CSF barrier and requires the entry of FcR-bearing cells to bind the antibody and cause axonal damage via ADCC. Once inflammation is eliminated, as in aHSCT, it is difficult for cells, such as γδ T-cells, to gain entry to the CNS, and the reduction in axonal injury may be

explained by this inability and, therefore, further ADCC cytolysis of axonal elements would be stopped.

RESEARCH OVERVIEW

Rationale and Hypothesis

Anti-Nfasc antibodies were detected in the sera and CSF of MS patients in this study and previously (174; 293), and MS patient-derived anti-Nfasc antibodies have been shown to elicit axonal transection and demyelination *in vitro* (292). In Chapter 3, CSF anti-Nfasc antibody titres were shown to correlate with markers of axonal injury (CSF neurofilaments). $CD16^+$ γδ T-cells correlated with clinical measures of MS disease progression and were shown to be capable of cytolysis via ADCC (130; 217).

I hypothesize that in MS, γδ T-cells can damage axonal membranes via ADCC when bound by CD16 to the Fc portion of anti-Nfasc antibodies that localize to the CNS. I will demonstrate the plausibility of this hypothesis by demonstrating *in vitro* the ADCC-specific lysis of Nfasc-expressing target cells with FcR-bearing γδ T-cells bound to anti-Nfasc antibodies, with both cells and antibodies derived from MS patients.

Research Questions

1. *What is the IgG subtype profile of the anti-Nfasc antibodies derived from MS patients?*

2. *Do MS patient-derived anti-Nfasc antibodies bind to Nfasc-expressing HeLa cells?*

3. *Do CD16-expressing γδ T-cells bind to the anti-Nfasc antibodies via their Fc region?*

4. *Do CD16-expressing γδ T-cells specifically cytolyse Nfasc-expressing HeLa cells in the presence of MS patient-derived anti-Nfasc antibodies?*

RESULTS

Quantification of MS patient-derived anti-Nfasc antibodies

Anti-Nfasc antibodies were harvested from the sera of MS patients by affinity chromatography. The concentrations of the eluted fractions were extrapolated by first generating a standard curve of known commercial anti-Nfasc antibody concentration. The concentrations of the eluted antibodies for the 4 serial elutions from SPMS patient 1304 were 0.6636 μg/μl, 6 ng/μl, 4.75 ng/μl, and 0.24 ng/μl, respectively. For the antibodies derived from PPMS patient 1023 the concentrations of the serially eluted antibodies were 0.1229 μg/μl, 8.77 ng/μl, 5.59 ng/μl, and 5.46 ng/μl, respectively.

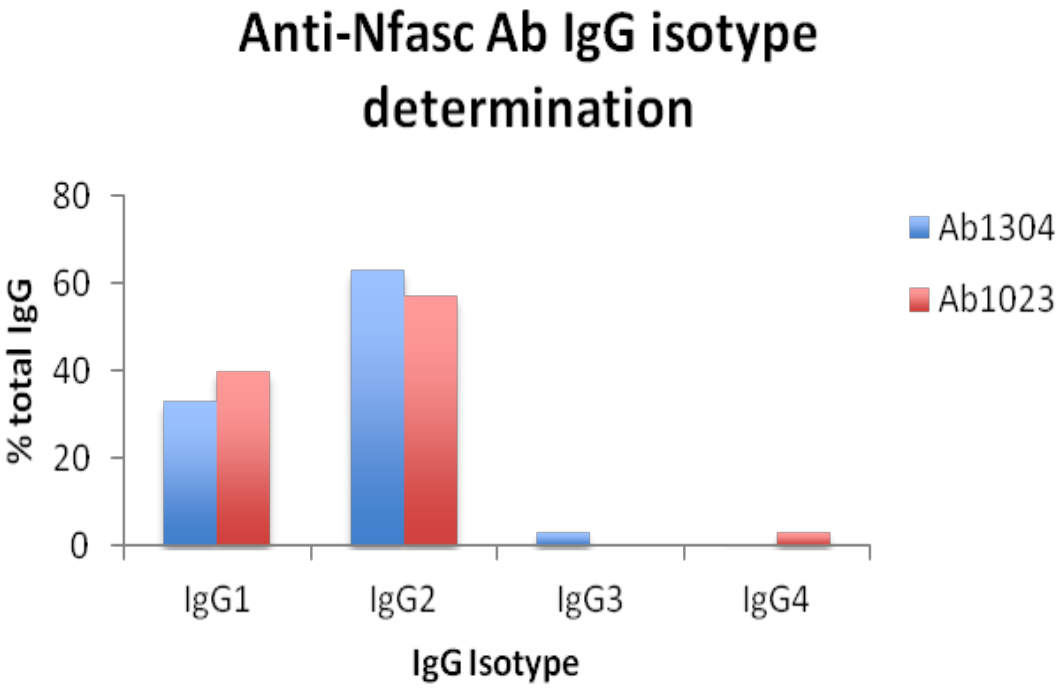
Anti-Nfasc antibody IgG isotype profile

Not all antibodies will bind to FcR, thus it was important to know whether the enriched antibodies were IgG_1 and/or IgG_2, the isotypes that will engage FcR. Some other isotypes, such as IgG_3, are superior activators of complement and might not require ADCC to lyse target cells. A human IgG subclass profile kit was utilized to determine the IgG isotype profile for the anti-Nfasc antibodies that were purified from 2 MS patients. Both anti-Nfasc fractions largely consisted of IgG_1 and IgG_2 antibodies. Proportionally, the anti-Nfasc antibodies derived from patient 1304 consisted of 33% IgG_1 and 63% IgG_2. For anti-Nfasc antibodies derived from patient 1023, 40% consisted of IgG_1 and 57% IgG_2. The anti-Nfasc antibodies derived from patient 1304 consisted of 3.1% IgG_3 and < 1% IgG_4. They were reversed in the anti-Nfasc antibodies derived from patient 1023, where the IgG_4 isotype made up 3% of the IgG antibodies, but there was no discernable IgG_3 (**Fig 5.1**). Thus, the isotype profile demonstrates that a sizable proportion of the enriched anti-Nfasc antibody is of the IgG_2 isotype, which is likely capable of binding to FcR. Only a small portion of the enriched

anti-Nfasc antibody was the IgG_3 isotype, which has superior complement-fixing capabilities (436), and could potentially lyse targets alone.

Fig 5.1 MS patient-derived anti-Nfasc antibodies are IgG_1 and IgG_2.

Anti-Nfasc antibodies were enriched from high-titre (anti-Nfasc antibody) MS patient sera by affinity chromatography using the NHS HP SpinTrap. IgG profiling of the eluted antibodies was performed using the Human IgG Subclass kit. Anti-Nfasc antibodies derived from patient 1304 (blue bars) consisted of 33% IgG_1, 63% IgG_2, and 3.1% IgG_3. Anti-Nfasc antibodies derived from patient 1023 (red bars) consisted of 40% IgG_1, 57% IgG_2, and 3% IgG_4. IgG_4 for patient 1304 and IgG_3 for patient 1023 were not discernable.


Anti-Nfasc Ab IgG isotype determination
80
60
40
20
0
% total IgG
IgG1
IgG2
IgG3
IgG4
IgG Isotype
Ab1304
Ab1023

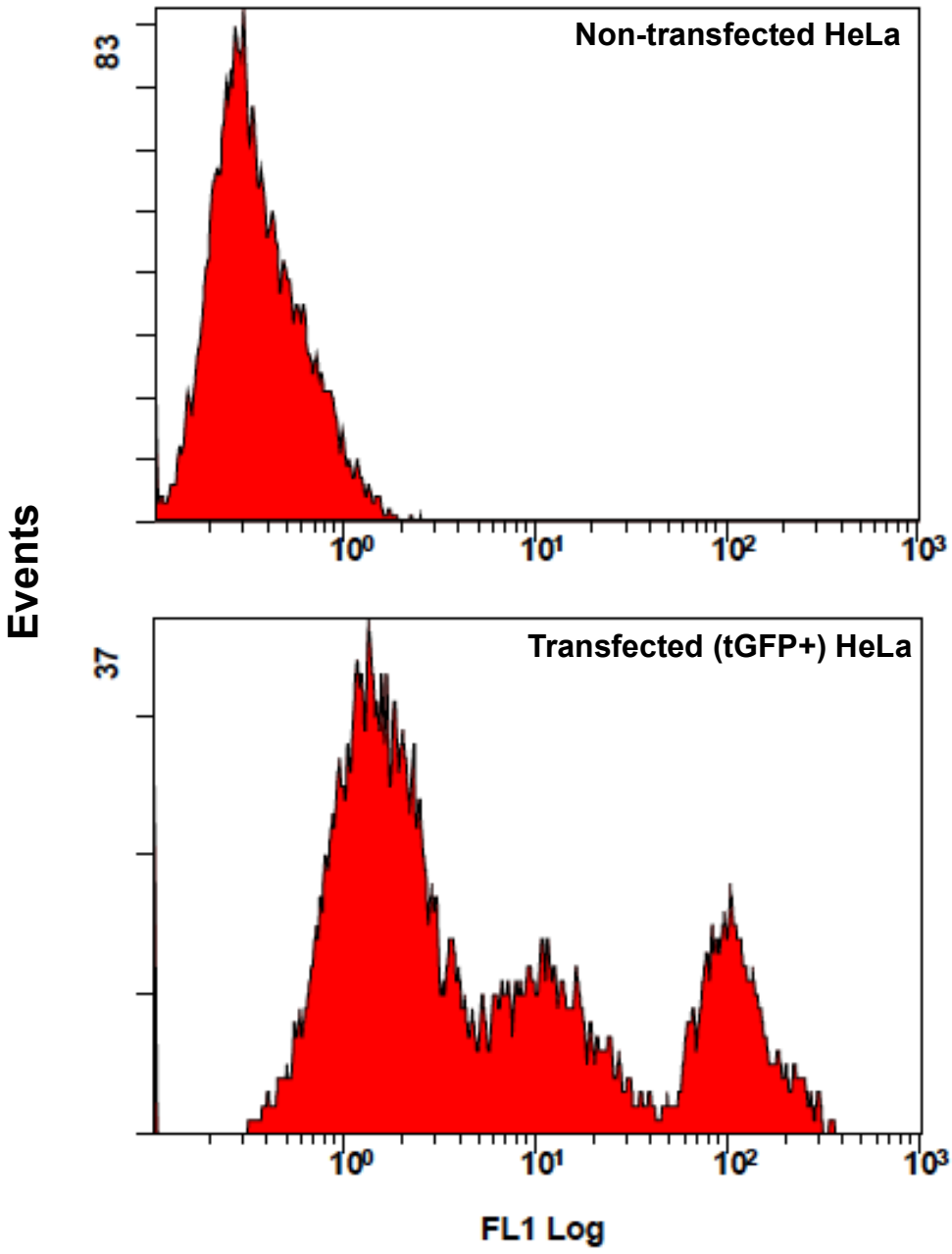
Neurofascin transfection of HeLa cells

The inability to secure a natural neuronal Nfasc-expressing target (detailed in APPENDIX I) led to the decision to transfect HeLa cells with the neuronal variant of human Nfasc (Nfasc-186). HeLa cells were chosen because they are not readily lysed by γδ T-cells. This was important because a high degree of γδ T-cell-mediated lysis could have masked increased cytolysis in the presence of antibodies.

Upon completion of the transfection procedure, the percentage of $tGFP^+$ cells was low, less than 10%, thus the cells were grown, sorted by FACS, and the $tGFP^+$ cells were allowed to expand. The percentage of $tGFP^+$ HeLa cells was confirmed by flow cytometry before each subsequent use (**Fig 5.2**), and also confirmed by IHC with confocal microscopy (**Fig 5.3**).

Figure 5.2 HeLa cell Nfasc expression detected by flow cytometry.

Representative histograms representing non-transfected control (upper panel) and Nfasc-transfected ($tGFP^+$) HeLa cells (lower panel). The emission of tGFP was redirected through the 500 nm specific band-pass optical filter and detected through FL1. The fluorescence emitted by the transfected HeLa cells was increased by 1-2 magnitudes when compared to the non-transfected cells.


Non-transfected HeLa
83
Transfected (tGFP+) HeLa
37
Events
10⁰
10¹
10²
10³
FL1 Log

Binding of anti-Nfasc antibodies to Nfasc-expressing targets and γδ T-cells

Antibody binding to its target antigen and to an effector cell is required for the induction of ADCC. MS patient-derived anti-Nfasc antibodies were added to methanol-fixed control and transfected HeLa cells. Using IHC with confocal microscopy, positive control rabbit anti-human Nfasc antibody and MS patient-derived anti-Nfasc antibodies were shown to bind to Nfasc-transfected HeLa cells (**Fig 5.3** and **Fig 5.4,** respectively). It was, however, not possible to demonstrate that the antibodies bound to the γδ T-cells via flow cytometry, using a conjugated anti-human IgG(Fab)-specific secondary antibody.

Figure 5.3 Nfasc-expression in HeLa cells.

Representative immunofluorescence image of transfected HeLa cells (white arrowheads) obtained by confocal microscopy displaying Nfasc-expression ($tGFP^+$; green; upper right panel) co-localizing with control rabbit anti-human Nfasc antibody (red; bottom left panel) in the overlay (bottom right panel). Nuclear labeling by Hoechst; bars = 20 μm.

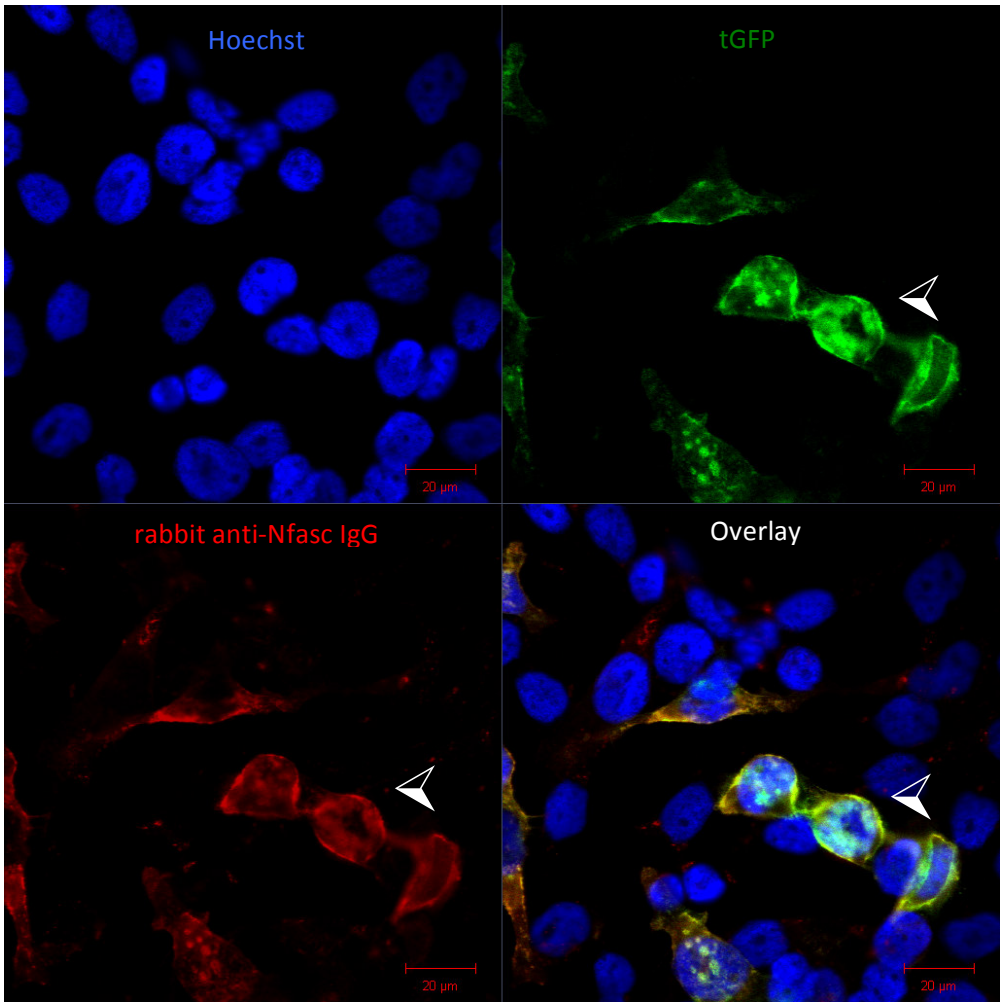
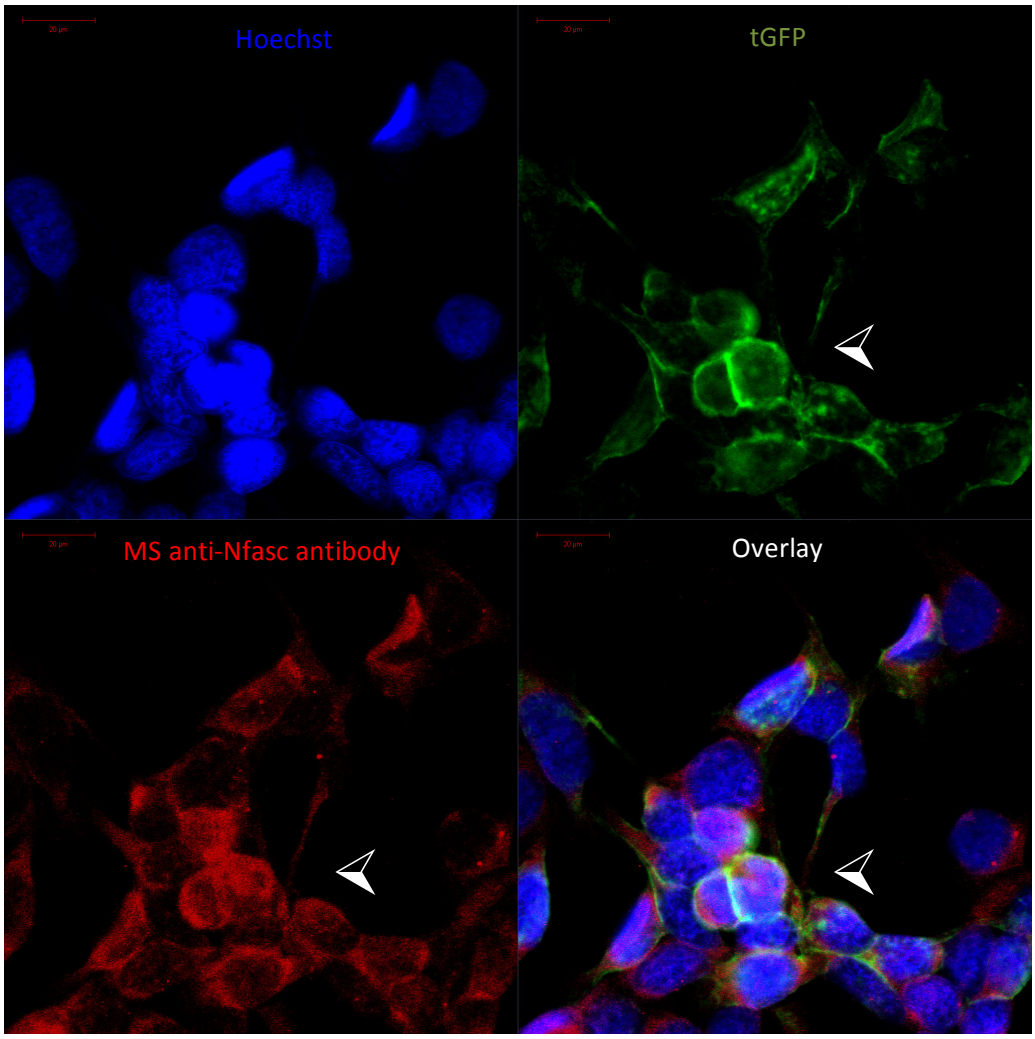

Hoechst
tGFP
rabbit anti-Nfasc IgG
Overlay
20 µm
20 µm
20 µm
20 µm

Figure 5.4 MS patient-derived anti-Nfasc antibodies bind Nfasc-expressing HeLa cells.

Representative immunofluorescence image of transfected HeLa cells obtained by confocal microscopy displaying Nfasc-expression ($tGFP^+$; green; upper right panel) co-localizing with MS patient-derived anti-Nfasc antibody (red; bottom left panel) in the overlay (bottom right panel). Nuclear labeling by Hoechst; bars = 20 μm.


Hoechst
tGFP
MS anti-Nfasc antibody
Overlay

γδ T-cells exert ADCC cytolysis on Nfasc-expressing targets

Having shown that the Nfasc-specific enriched antibody fraction contained antibody of the IgG_2 isotype, which is known to induce ADCC, and that it bound to Nfasc-expressing HeLa cells, but did not mediate lysis independently, I next needed to determine if FcR-bearing γδ T-cells could induce ADCC of Nfasc-expressing targets in the presence of anti-Nfasc antibodies. The mean percentage of spontaneous lysis observed in the control and transfected HeLa cells prior to the addition of γδ T-cells was 14.6% (SD 5.7%) and 10% (SD 9.8%), respectively. The mean additional HeLa cell cytolysis in the presence of γδ T-cells was 11% (SD 4.7%) and 10% (SD 7.1%) for the control and transfected HeLa cells, respectively, indicating that both control and transfected HeLa cells were equally susceptible to γδ T-cell cytotoxicity in the absence of binding antibodies. In the presence of MS patient-derived anti-Nfasc antibody (derived from patient 1304) specific γδ T-cell-induced cytolysis was observed in 18.3% of Nfasc-transfected HeLa cells in comparison to 1.3% of the non-transfected controls, indicating that the combination of Nfasc-specific antibodies and CD16-expressing γδ T-cells increased cytolysis of Nfasc-expressing target cells by a full order of magnitude (>10-fold).

γδ T-cell FcR engagement has been known to cause activation, thus an IgG isotype control antibody was used to determine if antibody-binding alone was capable of activating the γδ T-cells and eliciting HeLa cell lysis. In the presence of the IgG isotype control the percentage of cytolysis 4.5% and 3.7% for non-transfected control and transected HeLa cells, respectively, indicating that simple antibody binding to the FcR on γδ T-cells was insufficient to induce lysis of the HeLa cells; only the combination of Nfasc-specific antibody and the γδ T-cells was capable of significantly increasing the lysis of Nfasc expressing HeLa cells.

Not all combinations of γδ T-cells and anti-Nfasc antibodies resulted in substantial ADCC with Nfasc-expressing HeLa cells and anti-Nfasc antibody. Assays were repeated with γδ T-cells derived from five additional MS patients (expressing CD16 from 18% to 63.7%) but no significant increases in cytolysis were noted (**Table 5.1**). When experiments were conducted with the same γδ T-cell cultures, but in the presence of anti-Nfasc antibody derived from a different MS patient (patient 1023), again no substantial increases in cytolysis were noted. These observations indicate that this mechanism of ADCC via γδ T-cells binding to anti-Nfasc is highly selective for the right combination of cell and antibody. Further experiments might help to determine what particular characteristics of γδ T-cell or antibody might favour the ADCC reaction. Since γδ T-cells and anti-Nfasc antibodies are present in the CNS of many patients, it might also explain why only in certain patients we observe evidence of axonal damage.

Table 5.1: Specific γδ T-cell-mediated cytolysis of HeLa cell targets in the presence of MS patient-derived anti-Nfasc antibody (Ab1304)

MS Patient ID	MS Subtype	% γδTCR/ $CD16^+$	% Specific Cytolysis Control HeLa	% Specific Cytolysis Transfected HeLa
891	RRMS	32.1	1.3	18.3
1046	PPMS	18.9	-3.7	-1.4
1012	PPMS	28.4	2.8	-1.4
893	PPMS	63.7	-2	-3.5
1372	RRMS	18	-2.2	-0.1
1316	RRMS	59.7	-8.4	7.1

DISCUSSION

In Chapter 3, it was established that the presence of anti-Nfasc antibodies may be relevant to mechanisms of CNS injury in MS. Antibodies were detected in high titres in the CSF of relapsing/remitting patients, with higher titres correlating with neuro-axonal injury, as evidenced by the release of NfL. Despite undergoing a definitive treatment to stop all inflammation, aHSCT patients still had measurable anti-Nfasc antibodies in their CSF, but no longer with the concomitant increase in NfL release [Chapter 4]. This suggested that the simple presence of the anti-Nfasc antibodies alone was insufficient to produce axonal injury. One possible explanation is that the treatment in these patients sealed the blood-CSF barrier, thus blocking the transition of inflammatory cells, such as γδ T-cells, which are capable of binding the anti-Nfasc antibodies and causing axonal damage via ADCC.

Effector functions of antibodies

Autoantibodies are frequently detected in MS lesions and have been implicated in MS pathogenesis, not only through specific antigen recognition-associated effector functions, but also possibly by biochemical mechanisms. They are most commonly observed in conjunction with complement deposition, which leads to target destruction via the membrane attack complex. Antibody and complement deposition have been observed *in situ* in areas of active demyelination with concomitant association of myelin destruction (437), and serum antibodies have been shown to bind OGD and elicit complement-dependent IgG autoantibody-mediated demyelination of terminally differentiated OGD and their contiguous myelin sheaths (292; 438). Opsonization and phagocytosis is an alternative effector mechanism whereby antibodies bind a target antigen specifically, and the antigen-antibody complex is subsequently phagocytosed by macrophages, which leads to cell and tissue destruction. Phagocytic cells containing Ig and myelin protein have been observed in MS

lesions and EAE brain tissue (437; 439). In addition, targeted anti-MOG antibody binding has been shown to phosphorylate proteins related to cellular stress responses, with subsequent reduction in cytoskeletal integrity, which results in the retraction of OGD processes and cell membranes (126; 127).

In the context of the experiments outlined herein, anti-Nfasc antibodies alone were unable to lyse Nfasc-expressing HeLa targets. First, the majority of anti-Nfasc antibodies isolated were of the IgG_1 and IgG_2 isotypes, which are only weakly capable of fixing complement (436). Secondly, no complement was present in the cultures as culture media were inactivated with heat prior to the assays. And finally, the γδ T-cells were expanded from PBMCs through activation of the γδ TCR, and contaminating αβ T-cells were eliminated by complement lysis.

ADCC

ADCC is a lytic process whereby FcγRIIIA (CD16)-expressing effector cells, usually of the innate immune system, lyse target cells that are bound to specific IgG antibodies (123; 130). Target destruction is carried out by the release of inflammatory mediators such as perforin or granzymes, by the interaction of FasL and TRAIL, or by the production of cytokines (131).

Activation of γδ T-cells via CD16 engagement results in a highly cytotoxic cell population that can release cytokines, such as TNF-α (185; 244), and the existence of CD16-expressing γδ T-cells suggests the possibility that these cells might have cytotoxic capabilities indirectly via ADCC through CD16-engagement and subsequent activation. A role for γδ T-cells in neuro-axonal injury is particularly interesting as $CD16^+$ MS patient-derived γδ T-cells have been shown to correlate with disease progression in MS, and to exert

ADCC of rituximab-coated targets (130; 217), a humanized mAb that recognizes B-cell CD20. γδ T-cell ADCC cytolysis involving MS patient-derived antibody, however, has not been reported previously.

Anti-Nfasc antibodies were harvested from the sera of two high-titre MS patients by affinity chromatography, and the resultant IgG fractions consisted mainly of IgG_1 and IgG_2 isotypes. Though the greatest proportion of IgG in adult serum is IgG_1, and its proportion remains high in the sera of these individuals, it is remarkable that the majority of the anti-Nfasc antibodies harvested from these MS patients is of the IgG_2 isotype, given that IgG_2 is generally considered to be the most potent IgG effector in autoimmune diseases (440-442). Furthermore, IgG_{2a} has been shown to exhibit enhanced ADCC activity compared to IgG_{2b} (443), though the proportions of IgG_{2a} or IgG_{2b} were not evaluated in the harvested IgG fractions in the current study.

When studies using neuronal cultures were dismissed (see APPENDIX I), HeLa cells were chosen as a suitable target cell for transfection as they grow rapidly in culture, and findings from the Freedman laboratory demonstrated that HeLa cells are relatively resistant to γδ T-cell-mediated cytolysis. Furthermore, a previous report demonstrated successful Nfasc-transfection of HeLa cells (174).

Our interest lies in the γδ T-cell-mediated immunopathogenic mechanisms that might target neuro-axonal elements and contribute to the irreversible injury that is associated with MS. In contrast to Nfasc-155, the oligodendroglial variant, Nfasc-186, is expressed exclusively on neurons and axons. Pan-neurofascin antibodies selectively bind to Nfasc-186 in culture and *in vivo*, where they have been shown to affect axonal transport, suggesting that the axonal variant is the antigenic target (174), perhaps because it is more accessible *in vivo*

than Nfasc-155. Thus, for these reasons Nfasc-186, the neuro-axonal variant that is expressed at the nodes of Ranvier, was chosen and transfection was carried out.

With the successful Nfasc-transfection and growth of HeLa cells, an ADCC cytolysis assay was developed that involved Nfasc-transfected HeLa cells as targets and MS patient-derived γδ T-cells as effectors in the presence or absence of MS patient-derived anti-Nfasc antibodies. Various trials were conducted using combinations of E:T ratios and lengths of time for co-culture. Small E:T ratios (less than 4:1) failed to exert noticeable target lysis, while longer, such as overnight, co-culture incubations and greater E:T ratios resulted in nonspecific lysis of the majority of targets. Finally the assays were established with 4:1 E:T ratios with 4 hours of co-culture incubation. The assays demonstrated that the non-transfected control and transfected HeLa cells were equally susceptible to γδ T-cell cytolysis, and that transfection alone did not make the HeLa cells more susceptible to γδ T-cell-mediated cytolysis.

Previous work suggested that FcR-bearing γδ T-cells might be important in causing neuro-axonal injury in MS if they were to bind antibodies capable of recognizing neuro-axonal targets and inflicting damage via ADCC. This work now provides direct proof of the plausibility of this proposed mechanism of immune injury in MS, and demonstrates for the first time that γδ T-cells can exert ADCC in the presence of MS patient-derived anti-Nfasc antibody. Co-culture of MS patient-derived γδ T-cells in the presence of MS patient-derived anti-Nfasc antibody 1304 significantly augmented cytolysis of Nfasc-transfected HeLa cells 14-fold higher than the control HeLa cells.

ADCC is a highly multifactorial mechanism and there is likely to be involvement from a variety of variables. To tease them apart at a basic level would require a large number

of assays that would investigate the importance of the antibody isotype profile, the affinity of CD16 for IgG and, of course, how these relate to the specificity of disease course. I believe the γδ T-cell-induced target cell lysis in the presence of anti-Nfasc antibodies is specific because the γδ T-cell cultures were nearly pure; at least 98% of cells in all cultures expressed the γδ TCR, thus it is unlikely that other immune cells had any effect. It remains possible, however, that a small number of contaminating immune cells persisted in the γδ T-cell cultures. The procedure for γδ T-cell expansion eliminates contaminating cells that express the αβ TCR, and monocytic cells and macrophages are eliminated by attrition as factors for their survival were withheld from the culture medium. It is, therefore, improbable that Nfasc-expressing HeLa cells that were opsonized with anti-Nfasc antibody could have been recognized and destroyed by another immune cell. The enriched anti-Nfasc antibody fraction contained a large proportion of IgG_2, which is capable of mediating ADCC, and augmented lysis was not observed in the presence of the IgG isotype control. Low-affinity engagement of γδ T-cells and the anti-Nfasc antibodies alone could tickle γδ T-cell FcRs, or promote activation that might augment γδ T-cell cytotoxicity towards the HeLa cell targets. Had non-specific activation been applicable, however, enhanced cytolysis would also have been observed in the control HeLa cultures in the presence of anti-Nfasc antibodies as well since antigenic specificity would have been irrelevant, but that was not observed. Taken together, the combined data and controls indicate that cytolysis was accomplished via ADCC. Furthermore, lysis was independent of complement activation, which is an important function of IgG antibodies, due to heating of the culture medium.

Antibody heterogeneity

The PPMS patient-derived anti-Nfasc antibody (patient 1023) fraction failed to exert any effect in the ADCC cytolysis assays, regardless of the γδ T-cell culture, which suggests that the synthesis of antibodies in disease does not always relate to a biological consequence in terms of effector function (123), and diversity in disease pathogenesis provides one explanation. MS is heterogeneous in terms of histopathology, CSF phenotype, disease course, and therapeutic responsiveness. In accordance, different disease mechanisms surely play a role in tissue destruction between individuals (64). The relative proportion of IgG_2 in this antibody fraction was consistent with that of the anti-Nfasc antibody derived from patient 1304, which was shown to mediate ADCC, and helps to negate the possibility that the antibody alone could fix complement. It is unlikely that all patients have a prominent antibody response, and specific autoantibodies might characterize a distinct clinical subtype (123). While the total CSF IgG concentrations have been shown to be higher in PPMS than RRMS (444), very few of these antibody targets have been identified and, moreover, their functional capacity is unknown. Demyelinating IgG autoreactivity (containing autoantibodies to Nfasc) was a prominent feature of RRMS sera but not of PPMS, which suggests that IgG autoantibodies might play a less prominent role in the PPMS disease course (292). However while PPMS patients have been shown to display the highest prevalence of anti-MOG antibodies, a parallel increase in complement activity was not observed; it was similar for all sera, regardless of disease course (438). The presence of anti-ganglioside antibodies correlates with progressive MS (445), yet PPMS patients showed no evidence of IgM against myelin lipids compared to significant proportions of RRMS and SPMS (446). These findings highlight the heterogeneity in MS with respect to antibody production and effector function, even within disease courses.

MS subtype and cells

The number of γδ T-cell cultures assayed was small and included cells derived from 3 PPMS and 3 RRMS patients. Specific cytolysis of Nfasc-expressing HeLa cells was not observed in any one of the PPMS-derived cultures, which is consistent with the non-inflammatory nature of this disease course when compared to RRMS and SPMS. Comprehensive immunophenotyping of CSF and blood-derived leukocytes has demonstrated that though considerable overlap exists between the MS courses, SPMS patients are immunologically more analogous to RRMS than to PPMS patients. RRMS patients have more $CD4^+$ and $CD8^+$ T-cells and B-cells compared to PPMS, and RRMS and SPMS have elevated proportions of B-cells in comparison with non-inflammatory neurologic disease. Also, higher proportions of monocytes and granulocytes are observed in PPMS compared to RRMS (447).

γδ T-cell CD16

To exert ADCC the effector γδ T-cells require the expression of CD16; $CD16^+$ γδ T-cells exert potent cytotoxicity through a variety of mechanisms but produce low levels of cytokines in contrast to $CD16^-$ γδ T-cells that produce higher levels of cytokines but have less potential for cytotoxicity (185). A previous investigation in the Freedman laboratory observed that γδ T-cell-induced ADCC cytolysis increased with the increasing number of $CD16^+$ γδ T-cells (130). Each cytolysis assay performed herein was conducted with the same E:T ratio, which indicates that there were more $CD16^+$ γδ T-cells in populations that consisted of higher percentages of CD16-expressing cells. However, the percentage of $CD16^+$ cells varied greatly between the γδ T-cells assayed, and cytolysis did not relate significantly to CD16 expression. This might be because there are a minimal number of cells

required to generate a maximal signal and, therefore, increasing the number would not have an effect at the chosen E:T ratio. Furthermore, it is unlikely that all FcR engage polyclonal antibody with the same affinity.

Though γδ T-cell CD16 expression increases with disease progression in MS (217), FcR and IgG cytotoxicity have not been directly implicated in MS disease pathogenesis. FcγR, however, are important for mediating MOG-induced EAE (448). It has been suggested that allelic polymorphisms in FcR might correlate with Ig-based therapeutics by their variable affinity for Ig and, indeed, the low-affinity homozygous genotype of FcγRIIIA-158 was associated with a more favourable response to mAb TNF inhibitor therapy for inflammatory arthritis (449) and improved response to rituximab in non-Hodgkin's lymphoma (450). FcR affinity might be a considerable factor, given the significantly different affinities they display for the IgG subclasses (451). Affinity could influence the downstream effector functions of FcR-Ig engagement such as ADCC cytotoxicity, complement-dependent cytotoxicity or apoptosis, and has been shown for chimeric CD16 that bound humanized mAb with higher affinity and exerted greater cytotoxicity (452). Moreover, the R131 allele of CD32 (FcγRIIA) is over-represented in SLE compared to closely-related discoid lupus erythematosus and primary antiphospholipid syndrome. This allelic variant has lower affinity for IgG_2 and contributes to impaired clearance of circulating immune complexes, which might influence disease expression (453; 454). An investigation into the allelic polymorphisms of γδ T-cell CD16 in MS could shed some light on how this molecule might mediate ADCC in the context of MS.

In addition to FcR heterogeneity in ADCC, co-activation signals might enhance the response. Activation of CD16 in resting and IL-2-activated NK cells was enhanced by

engagement of non-activating receptors 2B4, NKG2D, DNAM-1 and CD2, and resulted in increased target ADCC cytolysis (455). These receptors and others, such as CD137, are also expressed by γδ T-cells (185; 456-458), with ligands concomitantly expressed by some HeLa cells (459-462). Given the selectivity of γδ T-cell-mediated ADCC demonstrated herein, interaction with one or more co-activating receptors could account for the increased cytolysis.

The specificity of the pathogenic response almost certainly varies between MS patients, as has been shown herein and previously (292). Defining the specificity of these autoantibodies is imperative to the elucidation of their clinical significance. The relative rarity of this result suggests that ADCC is not a primary mechanism of γδ T-cell-induced cytotoxicity in most MS patients, particularly with respect to PPMS. Of the effector functions known to be exerted by γδ T-cells, target cell lysis via the release of inflammatory immune mediators such as perforin or granzymes, or through direct effector-to-target contact via Fas/FasL have come to be the most widely reported. I now demonstrate for the first time that MS patient-derived γδ T-cells can exert ADCC of a disease-relevant target, Nfasc-expressing cells, in the presence of anti-Nfasc antibody derived from a MS patient.

Caveats

A number of technical caveats that may have had an effect on the results must be considered. Firstly, the γδ T-cells were previously expanded and frozen, often for a number of years. While observations from the Freedman laboratory indicate that the CD16 phenotype and cytotoxicity persist upon thawing, the γδ T-cells used for these experiments, nevertheless, may not be as potently cytotoxic following cryopreservation. In addition, the CD16 expression was highly variable between the γδ T-cell cultures. Given that γδ T-cell

ADCC potentially correlates with CD16 expression, future experiments should consist of cultures with more uniform CD16 expression. This can be achieved by expanding γδ T-cells and using FACS to obtain pure populations of CD16-expressing cells, which can then be used, not only for identical numbers of CD16-expressing cells in future assays, but to investigate different proportions to determine if a dose-response exists.

The search for a neuronal cell that expressed Nfasc consistently proved to be very challenging. As discussed in APPENDIX I, various cell lines were assessed. Some failed to grow efficiently, others appeared to express Nfasc only transiently, and some derivative cultures consisted mostly of astrocytes, rather than neurons. The decision to transfect HeLa cells with the neuronal variant of Nfasc introduced the caveat that the target cell was not of neuronal origin, however, it eliminated the variable and transient expression of the target antigen and allowed for consistency between assays. Moreover, it confirmed that the ADCC was indeed directed at the neuro-axonal variant of Nfasc. In the future, however, the search for a Nfasc-expressing cell could be resumed.

It was not possible to demonstrate that the MS patient-derived anti-Nfasc antibody bound via its Fc region to γδ T-cell CD16 using flow cytometry. Attempts at demonstrating binding involved the addition of the anti-Nfasc antibodies to γδ T-cells both prior to and post fixation, and incubating at 37, 21 and 4°C, followed by the addition of a conjugated anti-human IgG(Fab) antibody. Augmented extracellular staining was not observed in the presence of anti-Nfasc antibodies. CD16 is a low-affinity FcR, thus antibody may have bound, but detached during fixation or washing, which would prevent binding of the conjugated secondary antibody. Nevertheless, the MS patient-derived anti-Nfasc antibodies recognized and bound transfected HeLa cells and increased cytolysis of the transfected HeLa

cells was observed in the presence of anti-Nfasc antibodies. Furthermore, while it is possible for IgG alone to tickle the FcR and mediate nonspecific activation that could augment lethality, this phenomenon was not observed in the presence of the isotype control, which suggests against this mechanism and leaves only the γδ T-cell-FcR-bound antibody inducing cytolysis in an ADCC fashion as the only plausible explanation for the enhanced lysis of the Nfasc-expressing HeLa cells in the presence of MS patient-derived anti-Nfasc antibody.

Additionally, it was not possible to demonstrate MS patient-derived anti-Nfasc antibody binding to Nfasc-transfected HeLa cells by flow cytometry. Unsuccessful attempts were made to bind the antibody to detached HeLa cells both prior and post-fixation. HeLa cells adhere to the substrate of the culture vessel, and it is possible that upon detachment, alteration of the cell membrane resulted in the inaccessibility of surface-expressed Nfasc-186, which could, thus, prevent binding. Anti-Nfasc antibody was then added to adherent HeLa cells prior to detachment, however binding could not be observed using this method either. EDTA, rather than trypsin, was used to detach the cells for flow cytometric analysis to prevent cleavage of Nfasc-186, though it remains possible that the EDTA prohibited the antibody-antigen engagement.

Cytolysis was not accomplished in any culture in the presence of the anti-Nfasc 1023 antibody. A variety of reasons might account for this but the most evident would be that the IgG_2 in the fraction consisted of the IgG_{2b} isotype, which does not elicit downstream exertion of ADCC. Following antigenic stimulation B-cells undergo isotype switching, which enables the alteration of effector function and, thus, contributes diversity to the humoural immune response. It is possible that the preferential response of the antibodies derived from this MS patient might have been to initiate a complement cascade, though these experiments were not repeated in the presence of complement.

There are a number of factors that, if known, might have been beneficial for experimental design. It would have been of interest to know if $CD16^+$ γδ T-cells were increased in the patients with higher anti-Nfasc antibody titres, as this might provide a clue as to which patients might be expected to experience this form of pathogenesis. Additionally, it would be of interest to determine if ADCC cytolysis is specific to a subset of cells bearing a particular phenotype, as γδ T-cells exist in distinct populations and can be described by their variable region gene segments, ligand expression, such as NK receptor expression, capacity for antigen recognition and effector function (463). Furthermore, which inflammatory mediators are responsible for target cytolysis remains unknown. Assessing the presence of perforin or granzymes in the culture medium might shed some light on the lytic mechanisms elicited upon antibody engagement. Moreover, it is unknown if there is an optimal amount of target-bound IgG that is required to elicit an ADCC cytolytic response. It is known, for example, that the amount of antigen-specific IgG bound to $CD4^+$ T-cells is an important element for the initiation of robust ADCC in HIV infection (464).

Interpretation and Future Directions

The etiology of immune dysregulation in MS is not well understood. A combination of genetic factors and environmental stimuli are thought to be responsible for the development and persistence of immune dysfunction whereby elements of the CNS are targeted, causing focal and diffuse tissue injury throughout the CNS. For most patients, as the disease progresses the active inflammatory relapsing-remitting phase transitions to that of a less inflammatory but progressive neurodegenerative nature marked by fewer relapses yet clinical disability progression. The majority of DMT for MS target the immune system and the blood-CSF barrier; they have proven efficacy with respect to reducing relapse rates and

slowing disease progression though, during the latter progressive phase, efficacy declines as the neurodegenerative processes seem largely independent of immune-mediated inflammation.

Many components of the immune system, both innate and adaptive, are involved in MS pathology, which is initiated by leukocyte infiltration into the CNS, though it is unclear whether blood-CSF dysfunction might precede immune cell entry. PBMCs have been considered the major cellular effectors, producing an array of cytokines, ROS, MMPs, and other soluble factors that lead to tissue injury and recruitment of additional immune effectors. Antibody production by B-cells is now known to be a common immune mechanism in MS, though few antigenic targets have been identified, and their pathological significance remains elusive. Activated B-cells produce and secrete antibody in the periphery, but as MS progresses, they have been shown to do this in the CNS as well, in follicle-like structures in the meninges. In the current study, antibodies specific to the cell adhesion molecule, Nfasc, were detected in a proportion of MS patients. Antibody was detected in the serum but also in the CSF, thus it is possible that some of these B-cells are triggered to release localized anti-Nfasc antibody in the brain, though the intrathecal presence of antibody could also reflect diffusion through a compromised blood-CSF barrier, or transportation by penetrating FcR(CD16)-bearing cells, usually of the innate immune system, which can bind the Fc region of IgG antibodies.

CSF anti-Nfasc antibody titres were greater in the relapsing/remitting course than the progressive courses, and the serum and CSF titres correlated in MS patients, which suggests that localized antibody production in the CNS is less likely, and that peripheral antibody diffuses or is transported into the CNS through the compromised blood-CSF barrier during periods of active inflammation which occur more commonly in RRMS. In a separate cohort

of MS patients that underwent aHSCT for treatment of aggressive disease, anti-Nfasc antibody titres were reduced following the procedure in the serum, however, a concomitant reduction was not observed in the CSF. None of these individuals experienced additional lesions post-aHSCT, suggesting that antibody entry did not occur by diffusion. B-cells are not targeted by the immunoablative regimen, therefore, it is possible that antibody-producing cells became trapped post-aHSCT behind a less permeable blood-CSF barrier in the absence of inflammation and continued to produce anti-Nfasc antibodies.

In this study, it was shown for the first time that some γδ T-cells are capable of inducing ADCC cytolysis of Nfasc-186-expressing cells in the presence of MS patient-derived anti-Nfasc antibodies, which supports a role for both γδ T-cells and antibodies in MS pathogenesis, particularly with respect to neuro-axonal injury or transection, which are thought to drive disease progression and permanent disability. In MS patients anti-Nfasc antibody titres correlated with acute neuro-axonal injury. The significant correlation was driven by the RRMS cohort, which suggests that anti-Nfasc antibodies are contributing to injury during periods of active inflammation. Perhaps then, it is not a coincidence that γδ T-cells are more numerous in the CSF (218), more concentrated in lesions (219-221) thought to be responsible for some of the early damage and clinical symptoms observed in EAE (235). It might, therefore, be possible that during periods of active inflammation in MS γδ T-cells, in the presence of anti-Nfasc antibodies, could injure neuro-axonal elements via ADCC. Furthermore, CSF NfL and NfH were greatly reduced following aHSCT, despite the intrathecal persistence of anti-Nfasc antibody, which supports a role for an additional component, such as T-cells, perhaps γδ T-cells, in antibody-mediated axonal injury in MS. If γδ T-cells can act as effectors in a mechanism of ADCC in concert with anti-Nfasc

antibodies, then the reduction of injury in the presence of antibody alone might be because the T-cells were no longer present to engage the autoantibodies and subsequently mediate ADCC, thereby reducing axonal injury.

The data demonstrate that γδ T-cell-mediated Nfasc-specific ADCC is an important potential mechanism early in the disease course. Anti-Nfasc antibodies are synthesized early in MS; this was demonstrated by already higher CSF titres in RRMS and by lower titres in the patients who were classified as probably having MS, particularly in the periphery where they are probably synthesized. Correlations were observed between the serum and CSF of MS patients, which indicates that peripheral antibody is gaining entry to the CNS through the blood-CSF barrier, which displays evidence of compromise during periods of active inflammation that are more common in RRMS. CSF anti-Nfasc antibody concentrations correlated positively with NfL, a marker of acute axonal injury, and the significance was driven by the RRMS cohort. In addition, CSF antibody titres were reduced in patients over the age of 50 years, suggesting that blood-CSF permeability declines with with age.

Furthermore, γδ T-cell-induced ADCC of Nfasc-expressing cells in the presence of MS patient-derived anti-Nfasc antibodies was shown to be possible. More experiments are needed to understand how this might relate to disease course and progression, and while neuro-axonal injury and degeneration occur in all stages of MS, it is probable that this mechanism has more impact in the earlier stages as immune-mediated mechanisms are less prominent in the progressive phase, and seminal findings by Trapp et al. demonstrated that the majority of axonal transection occurs in active lesions in RRMS (68). With respect to the ADCC mechanism proposed herein, firstly, Nfasc must be accessible to become a target, therefore, immune system antigen-presenting cells must have access to the CNS and this

occurs predominantly in the relapsing/remitting phase during periods of active inflammation and blood-CSF barrier permeability. Upon peripheral antibody production, to injure CNS tissues by ADCC, both the antibody and γδ T-cells must gain access to the CNS and the simplest way for this to occur is through the inflamed and, therefore, permeable blood-CSF barrier in RRMS, though the antibody could also gain entry by binding to γδ T-cell FcRs.

Despite the neurodegenerative processes that occur in the later phases of MS, the data indicate that immune-mediated mechanisms, such as ADCC, may be less important in PPMS and SPMS. While the serum antibody titres were elevated in the progressive patients compared to RRMS, they did not correlate with neuro-axonal injury, likely because the blood-CSF barrier becomes less permeable as the autoimmune component becomes less prominent as the disease progresses. Indeed, the intrathecal anti-Nfasc titres were lower in progressive patients. Long-lived plasma cells might continue to produce anti-Nfasc antibody in the periphery that could persist for long periods of time, though it might be irrelevant if its target antigen is inaccessible. In contrast, it is likely that some immune cells can cross the blood-CSF barrier via normal trafficking, independently of permeability. Whether or not $CD16^+$ or $CD16^-$ γδ T-cells have such capabilities, however, remains unknown.

A previous study demonstrated that $CD16^+$ γδ T-cells are capable of mediating ADCC, and that specific lysis correlates with increasing CD16 expression (130; 217). It might have been expected then, that the γδ T-cells derived from progressive MS patients would have been more likely to mediate ADCC in the cytolysis assays described herein. Not one of the three assays that used γδ T-cells derived from PPMS patients demonstrated enhanced cytolysis in the presence of anti-Nfasc antibodies, and the γδ T-cells that were shown to mediate ADCC were derived from an RRMS patient. Furthermore, the γδ T-cell

CD16 expression did not appear to relate to ADCC capability in the assays described. Additional experiments are, therefore, required to determine if disease course or CD16 expression are associated with γδ T-cell-mediated ADCC. The study that demonstrated a dose response for $CD16^+$ γδ T-cell-mediated ADCC utilized a commercially purified mAb, whereas the antibody fraction used for these experiments was polyclonal and enriched from patient sera, thus making qualitative observations more complicated. It is unlikely, for example, that all $CD16^+$ γδ T-cells engage polyclonal antibodies with the same affinity and the antibody fraction both within and between patients is likely heterogeneous. Moreover it is possible that the mechanism was saturated and that the experimental approach requires manipulation to optimize the conditions for ADCC.

The data presented herein provide evidence that γδ T-cell-mediated ADCC is possible in the presence of MS patient-derived anti-Nfasc antibody. The enhanced CSF anti-Nfasc antibody titres in the RRMS cohort that correlated with acute neuro-axonal injury strongly suggest that this mechanism may be more relevant in the earlier relapsing/remitting course of MS in comparison to the progressive courses. It would, therefore, be beneficial to target this mechanism early in the disease course as DMT are effective almost exclusively during periods of disease with a pronounced inflammatory component. DMT have been shown to reduce relapse rates and slow disease progression. As patients transition to a more progressive phase, however, neurodegeneration proceeds in the absence of inflammatory mechanisms, therefore, early treatment to modify the immune component is imperative to prolonging the time to the development of progressive MS. The importance of immune system modification is evident in a cohort of MS patients that underwent aHSCT as a treatment for aggressive MS. Serum anti-Nfasc antibody titres in this cohort declined

following treatment, which suggests that B-cell activation and antibody production are at least somewhat T-cell dependent. In contrast, however, the CSF titres were not significantly reduced, likely because antibody-secreting B-cells became trapped behind a repaired blood-CSF barrier. Interestingly, both acute and chronic neuro-axonal injury were markedly reduced following treatment and this might because, even in the continued presence of antibody, the elimination of nearly all T-cells, including γδ T-cells, prevents the initiation of ADCC and preserves neuro-axonal elements. Furthermore, the majority of individuals in this cohort have remained free of disease progression, which is likely attributed to the preservation of neuro-axonal elements in the absence of some autoreactive immune cells and mechanisms. There is evidence that aHSCT elicits profound qualitative changes in the immunological cell repertoire which suggests that aHSCT might reset the immune system and could promote tolerance to formerly self-reactive antigens (314).

In conclusion, anti-Nfasc antibodies can be detected in a proportion of MS patients. The data suggest that they are produced in the periphery and that they enter the CNS during periods of active inflammation, likely during the relapsing/remitting phase, where they correlate with neuro-axonal injury. It is shown here for the first time, that these cells can mediate ADCC of Nfasc-expressing cells in the presence of MS patient-derived anti-Nfasc antibodies, and suggests that γδ T-cell-mediated ADCC of neuro-axonal elements could be a mechanism in the pathogenesis of MS. Prevention of neuro-axonal injury is particularly important in MS as it is thought to be the cause of the irreversible disability associated with MS.

SUMMARY AND CONCLUSIONS

There are many purported mechanisms of immune-mediated CNS injury, but the exact cause for neuro-axonal injury is unknown and may be different among patients. Both innate and adaptive immune mechanisms have been considered but we have been interested in mainly those of the innate immune system, in particular, the γδ T-cell component. We previously identified a subset of γδ T-cells that bear FcR ($CD16^{+}$) that seemed to be more prominent as the disease progressed, raising suspicion that they might be involved in disease progression. Typically progression is thought to arise from increasing neuro-axonal injury so we considered how FcR-bearing γδ T-cells might be involved in causing this type of damage. Since we also demonstrated that the FcR-bearing γδ T-cells were capable of mediating ADCC, we postulated that if they were to encounter antibodies that were directed against axonal elements then this might represent a mechanism underlying disease progression.

I considered several different antibodies to neuro-axonal elements but identified antibodies to Nfasc as a good potential candidate for binding to γδ T-cell FcR, as they had already been detected to be elevated in MS patients, and were shown to be involved in axonal injury both *in vitro* and *in vivo* in EAE. An ELISA was developed to detect and quantify Nfasc-specific antibodies in MS patient sera and CSF. Anti-Nfasc antibody titres were found to be elevated in MS patients, in both relapsing and chronic disease, compared to OND controls. Antibody titres were higher in the serum than the CSF, indicating that they were synthesized in the periphery and probably accessed the CNS by diffusing through an inflamed blood-CSF barrier, which is more associated with the active relapsing/remitting phase of disease.

I next wanted to determine if these anti-Nfasc antibodies correlated with known markers for axonal injury, so as not to rely simply on the clinical definition of "progressive disease", as MS is clinically highly heterogeneous. The release of neurofilaments into the CSF has been strongly associated with neuro-axonal injury, so these were quantified in the CSF of the patients that were also evaluated for the presence of anti-Nfasc antibodies. A positive correlation was observed between the presence of higher anti-Nfasc antibody titres and the amount of NfL release, especially in RRMS.

Having identified antibodies that correlated with axonal injury, it was important to know if this indicated a direct effect of anti-Nfasc antibody on causing the injury or an indirect effect that might require additional elements such as FcR-bearing γδ T-cells, which would be part of an inflammatory infiltrate in MS. To investigate this, I took advantage of a cohort of patients that underwent aHSCT, a procedure that completely abrogates inflammation. I found that despite the treatment, these patients still had measurable quantities of anti-Nfasc antibodies in the CSF. However, despite the presence of high levels of anti-Nfasc antibody, there was a significant reduction in NfL and NfH, suggesting that the presence of anti-Nfasc antibodies alone was insufficient to produce axonal injury. The missing component may well have been the presence of an FcR-bearing γδ T-cell that was no longer present as a result of the aHSCT.

In order to provide direct evidence that γδ T-cells bearing FcR could bind to anti-Nfasc antibodies and injure Nfasc-expressing targets I transfected HeLa cells with the axonal variant of Nfasc (-186), then used anti-Nfasc antibodies that were enriched from high-titre MS patient sera and put them in co-culture with MS patient-derived γδ T-cells. I observed γδ T-cell-induced cytolysis of Nfasc-transfected HeLa cells that was augmented by more than a

full order of magnitude in the presence of anti-Nfasc antibodies, which is indicative of cytolytic kill via ADCC.

I demonstrated here for the first time a mechanism by which the innate immune system (γδ T-cells) can cause injury to axons, the type of damage which is believed to underlie disease progression in MS. Therapeutics aimed at, perhaps, the reduction of Nfasc antibodies, the expression of FcR on γδ T-cells, or the cytolytic function of γδ T-cells might offer new avenues for treating MS disease progression, given that there is currently no effective treatment.

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STATEMENT OF CONTRIBUTION FROM COLLABORATORS

Dr. Harry Atkins, *Senior Scientist, University of Ottawa/OHRI.* Co-Principal Investigator of the MS aHSCT program (Chapter 5).

Mrs. Iva Stonebridge, *Research Technician, Freedman Laboratory*. Participated in the PBMC isolation, γδ T-cell expansion and purification, and flow cytometry acquisition (Chapter 4).

Dr. Alexander Strom, *Postdoctoral Fellow, Scott Laboratory*. Provided technical assistance for Western blot and immunohistochemistry experiments (Chapter 3 and APPENDIX I).

Dr. Christopher Patrick, *Graduate Student*, Scott Laboratory. Provided technical assistance for immunohistochemistry experiments (Chapter 4) and statistical guidance.

APPENDIX 1

The hunt for Nfasc-expressing cells

To test the ability of γδ T-cells to exert ADCC on Nfasc-expressing targets, a target first had to be obtained. The first cell line investigated was a murine N2a neuroblastoma (ATCC CCL-131), which was shown to weakly express Nfasc via Western blot (**Fig 1**). Subsequently, commercial and MS patient-derived anti-Nfasc antibodies were shown to bind N2a cells via flow cytometry (**Fig 2**). Two MS patient derived anti-Nfasc antibodies were assessed; 77.2% of N2a cells bound antibody derived from a PPMS patient (**Fig 2**) while only 39% of N2a cells bound antibody derived from an RRMS patient (not shown), which supports the heterogeneity in MS pathogenesis. While both the Western blot and flow cytometric assessments demonstrated that the patent-derived antibodies could bind, experiments with these cells were abandoned in favour of discovering a more relevant human cell line expressing Nfasc.

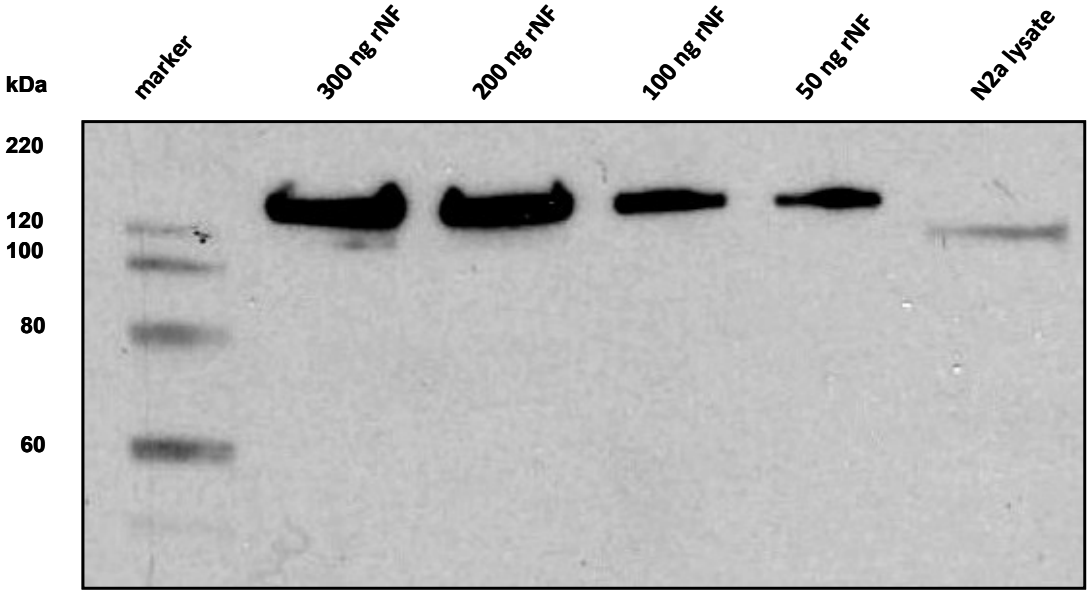

Figure 1: Murine N2a neuroblastoma expresses Nfasc-155. A Western blot demonstrating Nfasc expression of murine N2a neuroblastoma cells. Various concentrations of recombinant rat Nfasc-155 (rNF) and a lysate of N2a cells were electrophoresed by SDS-PAGE and transferred to a PVDF membrane. The membrane was probed with a 1:500 commercial goat anti-human Nfasc primary antibody and a 1:14,000 rabbit anti-goat-HRP secondary antibody. Binding was visualized with the addition of ECL solution containing H_2O_2. A single band of approximately 120 kD was observed.

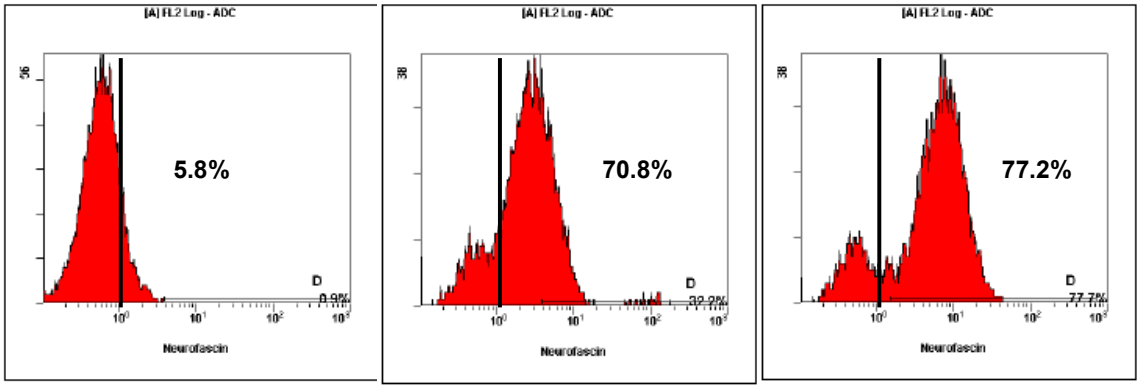

Figure 2: Commercial and MS patient derived anti-Nfasc antibodies bind murine N2a neuroblastoma. Murine neuroblastoma cells were fixed and primary antibodies consisting of a human IgG isotype control, a commercial anti-human Nfasc antibody, and a MS patient-derived anti-Nfasc antibody were bound. PE-conjugated secondary antibodies consisted of goat anti-human IgG or rabbit anti-goat IgG. Flow cytometry data was acquired using a Coulter FC500 flow cytometer and 10,000 events were acquired for each sample. The emission of PE was redirected through the 575 nm specific band-pass optical filters and detected through FL2. The fluorescence emitted by the commercial and MS patient-derived antibodies was increased by nearly a magnitude, compared to the isotype control.

A human BE(2)-M17 neuroblastoma (ATCC CRL-2267) cell line was next assessed by Western blot, however, it could not be shown to express Nfasc (not shown). Subsequently HCN-1A human cortical neuron (ATCC CRL-10442) cultures were established, but these did not grow efficiently in culture and studies using these cells were discontinued.

Human NT2/D1 teratocarcinoma cell cultures were developed and differentiated into neurospheres and neurons. A rabbit anti-human Nfasc antibody raised against an intracellular domain of Nfasc, recognizing Nfasc-155 and Nfasc-186, was kindly provided by Dr. Peter Brophy at the Centre for Neuroregeneration at the University of Edinburgh, and was utilized to determine Nfasc expression via immunohistochemistry and confocal microscopy. The rabbit anti-Nfasc antibody bound to CD1 mouse brain (**Fig 3A**), and while cells of NT2/D1-derived neurospheres initially expressed Nfasc (**Fig 3B**), expression was lost after one week of subsequent differentiation on matrigel (**Fig 4**). Furthermore, differentiating neurospheres expressed both neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) (**Fig 5D**), which indicated that the cultures contained both neuronal and astocytic cells, respectively.

When derivative neuron cultures were assessed for the expression of NSE and GFAP to distinguish neurons from glia (**Fig 5**), these cultures consisted of cells that only weakly expressed Nfasc in addition to NSE (**Figs 5A** and **5B**) or GFAP alone (**Fig 5C**). $GFAP^+$ cells, however, were greatly enriched in these cultures, indicating that the derivative cells were most likely astrocytes.

The failure of any of the neuronal derivatives to express Nfasc consistently and the large number of GFAP-expressing cells in the neurosphere and neuro-derivative cultures resulted in the decision to abandon these experiments in favour of transfecting a human cell line with the full-length neuronal variant, Nfasc-186.

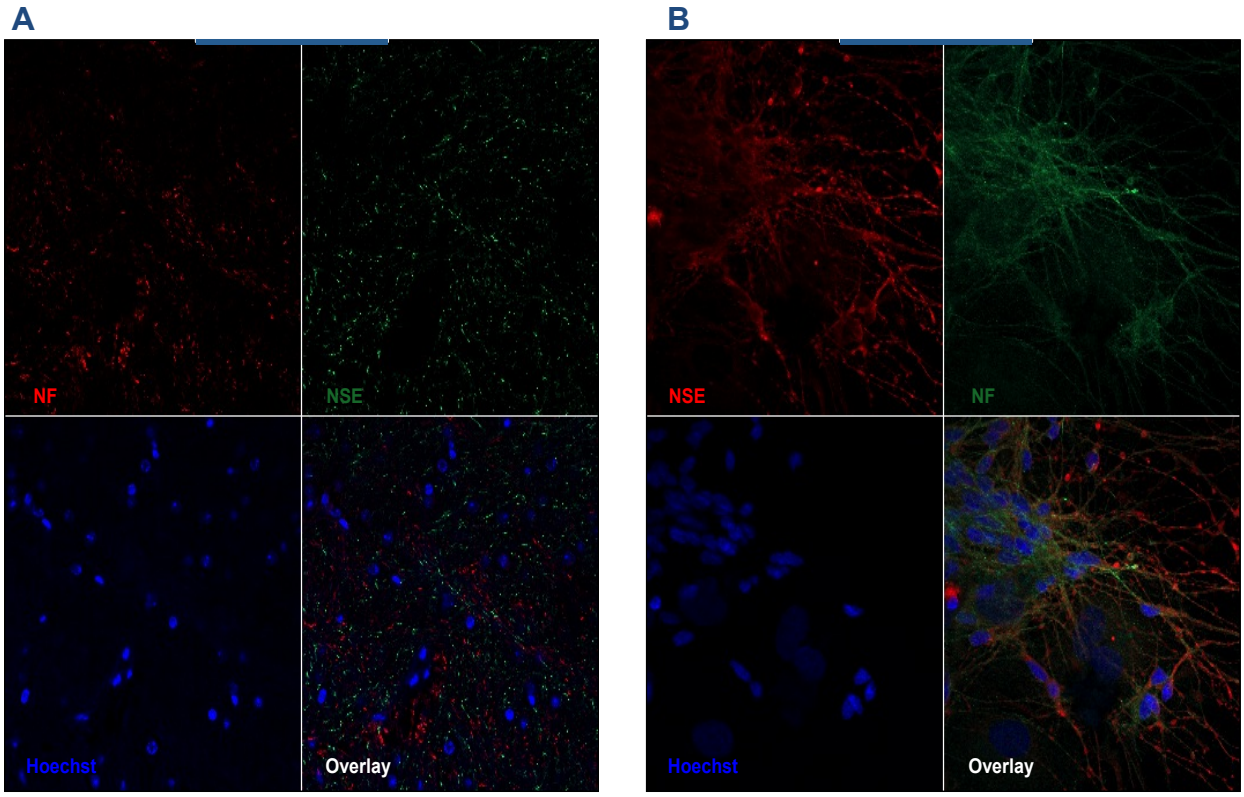

Figure 3: CD1 mouse brain and human NT2/D1-derived neurospheres express Nfasc. Representative immunofluorescence images obtained by confocal microscopy demonstrating (**A**) expression of Nfasc in CD1 mouse brain tissue and, (**B**) Nfasc co-localizing with NSE on NT2/D1-derived neurospheres that were differentiated on matrigel. Primary antibodies consisted of 1:500 rabbit anti-human Nfasc (green) and 1:500 mouse anti-human NSE (red). Secondary antibodies consisted of 1:600 goat anti-rabbit biotin and streptavidin-PC7 or donkey anti-mouse biotin and streptavidin cy-5. Nuclear labeling by 1:200 Hoechst (blue).

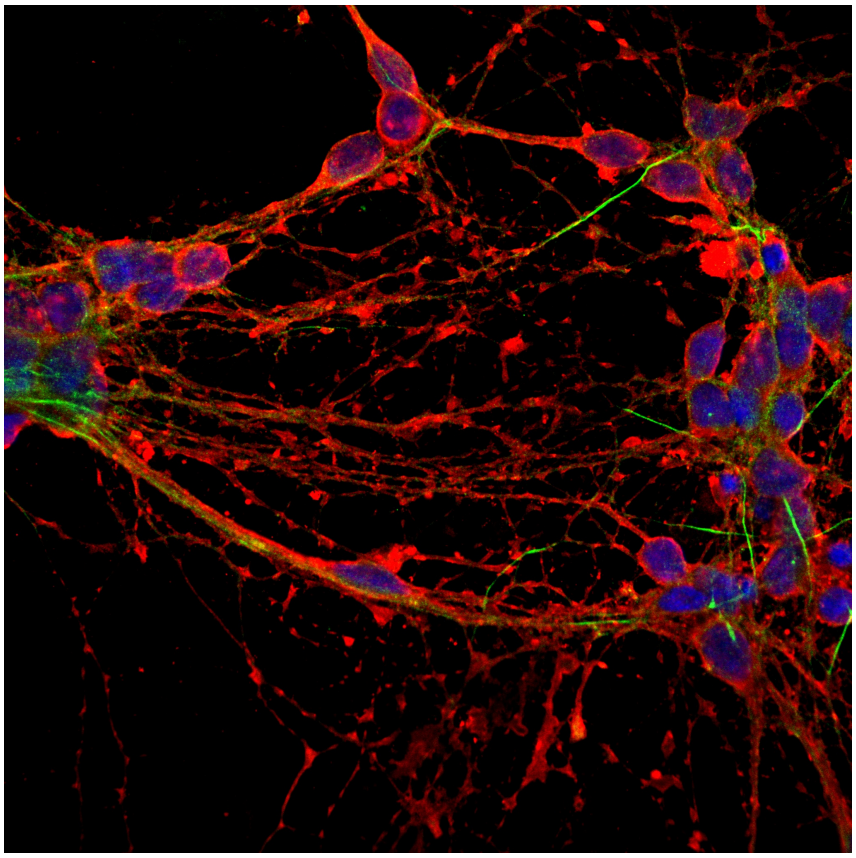

Figure 4: Nfasc expression decreases as neurosphere-derived neurons differentiate. Representative immunofluorescence image obtained by confocal microscopy demonstrating loss of Nfasc (green) expression in NT2/D1 neurosphere-derived neurons after 1 week of differentiation on matrigel. Nuclear labeling by 1:200 Hoechst (blue); neuron-specific enolase (NSE) (red).

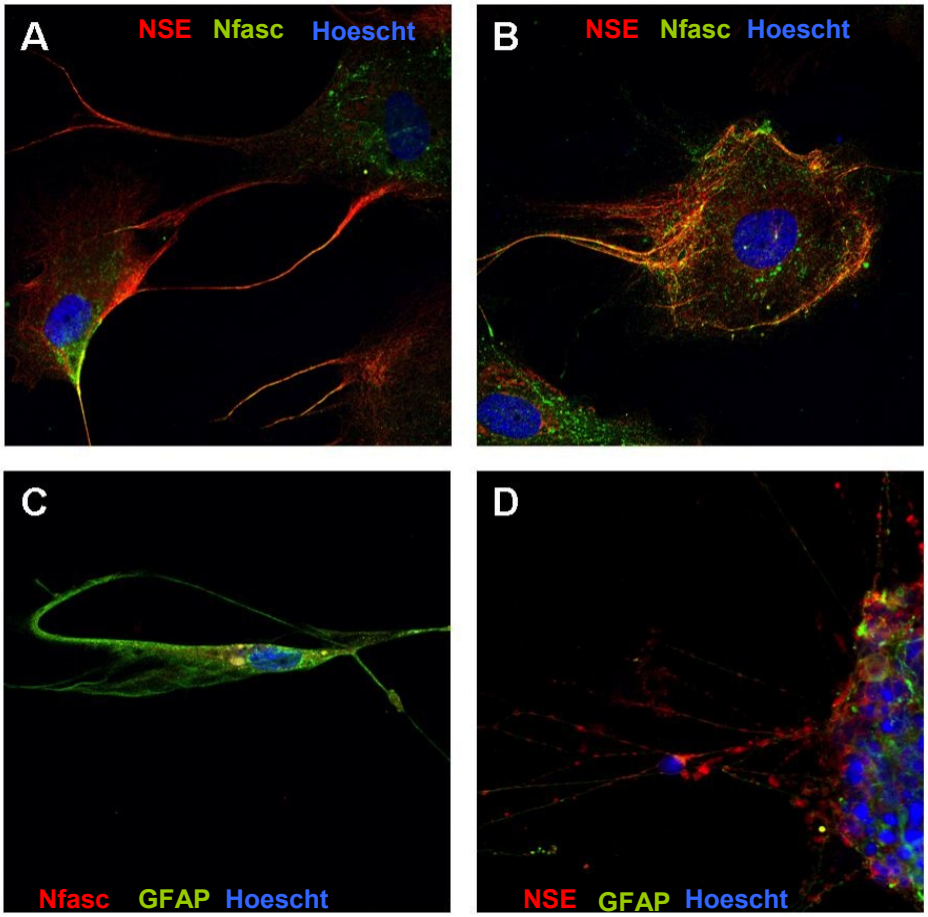

Figure 5: NT2/D1-derived neurons express Nfasc. Representative immunofluorescence images obtained by confocal microscopy demonstrating expression of Nfasc in NT2/D1 teratocarcinoma cells that were cultured in conditions that favoured neuronal differentiation. (**A** and **B**), Neuronal cells derived from a "classical neuron culture" expressing colocalized NSE (red) and Nfasc (green). **C**, An astrocyte expressing GFAP (green), but not Nfasc (red). **D**, A differentiating neurosphere expressing both NSE^{+} (red) and $GFAP^{+}$ (green) cells. Nulear labeling by 1:200 Hoescht.

Materials and Methods: Culture of murine and human cells of neuronal lineage

N2a neuroblastoma cells

Culture conditions: Murine N2a cells were kindly provided by Dr. Fraser W. Scott. Aliquots containing 2 x 10^6 cryopreserved N2a cells (CCL-131; originally obtained from ATCC, Cedarlane Corporation, Burlington, ON) were thawed quickly, washed with PBS and cultured in a T_{75} flask in 25 ml in cRPMI (Life Technologies), supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin G and 100 μg/ml streptomycin. Medium was replaced every 2 to 3 days. At confluency, the medium was removed and cells were scraped from the plate, resuspended in 4 ml cRPMI with gentle pipetting and passaged.

Cell lysis for Western blots: N2a cells were trypsinized with 0.15% trypsin (Life Technologies) for 2 min at 37°C and suspended in 10 ml PBS in a 15 ml tube. 10^6 cells were transferred to a 1.5 ml eppendorf tube, centrifuged at 4000 rpm for 4 min, and the supernatant was removed. The pellet was resuspended in 0.5 ml lysis buffer (0.5% CHAPS in 10 mM Hepes buffer), and incubated on ice for 45 min. The contents were then sonicated, and the lysed sample was centrifuged at 20,800 rpm for 15 min at 4°C and the supernatant removed. To the pellet 100 μl ddH_20 and 50 μl of 4x LDS sample buffer were added. The tubes were incubated for 10 min at 70°C and then stored at 4°C°C until use.

NT2/D1 cell culture

Culture conditions: NT2 neurons were derived from the human embryonic teratocarcinoma NTera2/cl.D1 (NT2/D1; ATCC CRL-1973, Cedarlane) cell line using adaptations of previously published protocols (Horrocks et al., 2003; Sandhu et al., 2003; Pon et al., 2007). NT2/D1 cells were seeded in a T_{25} polypropylene flask in 8 ml of complete high-glucose Dulbecco's Modified Eagle Medium (cDMEM-HG) (Life Technologies) containing 2 mM

L-glutamine, and supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. After 24 hours the medium was replaced and the cells were expanded to confluency. The culture was then trypsinized with 0.15% bovine pancreatic trypsin split into two T_{75} flasks and expanded to confluency. The protocol was then modified to accommodate the differentiation of neurospheres or classical neurons.

Growth of Neurospheres: To promote neurosphere growth NT2/D1 cells were trypsinized as previously described (465; 466), and 5 x 10^5 cells were placed into a petri plate in 10 ml of cDMEM-HG to prevent adherence, and cultured for 1 to 2 days. The DMEM-HG was then replaced with differentiation medium (cDMEM-HG supplemented with 10 μM *trans*-retinoic acid [RA]) for 2 weeks, with medium replacement every 2-3 days. Neurospheres larger than 100 μm were then harvested by passing the culture medium through a 100 μm nylon cell strainer (Falcon, BD Biosciences). For maturation, the neurospheres were backwashed into 90 mm tissue-culture-grade petri plates freshly coated with poly-D-lysine (10 μg/ml; overnight absorption and dessication) (Sigma-Aldrich) and BD Matrigel™ (0.3 mg/ml in serum-free DMEM-HG) in maturation medium, which lacked RA but contained the DNA synthesis inhibitors cytosine β-D-arabinofuroside (AraC) (1 μM; first seven days only), uridine (10 μM) and 5-fluoro-2'deoxyuridine (FDU) (10 μM) (Sigma-Aldrich).

Growth of classical neurons: Classical neuron cultures were established by seeding 2 x 10^6 NT2/D1 precursor cells in differentiation medium in a T_{75} flask, with medium replacement 3 times per week for 4 weeks. The conditioned medium was removed and saved, and the cells were trypsinized and cultured in 2 to 6 T_{75} flasks containing cDMEM-HG supplemented with DNA synthesis inhibitors as described above for 9 to 11 days, changing medium twice per week. The cells were trypsinized with 0.015% bovine pancreatic trypsin and the flasks were

gently swirled so that only the neuron layer detached. The action of trypsin was stopped with the addition of 10 ml of cDMEM-HG.

Dissociation of Neurospheres: The NeuroCult® Chemical Dissociation Kit was obtained from StemCell Technologies (Vancouver, British Columbia). All steps were performed at room temperature. Culture medium with suspended neurospheres (< 100 μm) was placed in a 15 ml tube and centrifuged at 400 rpm for 5 min. The supernatant was carefully removed, leaving less than 50 μl above the pelleted neurospheres. To the pellet, 0.5 ml of NeuroCult® Chemical Dissociation Solution A was added and the neurospheres were resuspended and a timer was set for 8 minutes. Next, 0.125 μl of NeuroCult® Chemical Dissociation Solution B was added to the cell suspension, the tube tapped gently to mix, and the timer was started. At the 3 min time point, the cell suspension was mixed by pipetting up and down 8 times with a P200 micropipettor set at 180 μl, then incubated until the 7 min time point. The cell suspension was again mixed by up and down pipetting 8 times. At 8 min, 40 μl of NeuroCult® Chemical Dissociation Solution C was added and mixed by pipetting up and down 8 times. Next, 350 μl of "Complete" NeuroCult® NSC Proliferation Medium was added to bring up the volume to approximately 1 ml, and the suspension was pipetted up and down. The cells in the single cell suspension were counted and 5 x 10^4 cells were seeded in each well of an 8-well Lab-Tek II chamber slide in maturation medium.

Immunohistochemistry

Culture medium was removed from the chamber slides and the chambers were washed with PBS. The chamber was removed from each slide and adherent cells were fixed in ice-cold methanol for 5-10 min. Forty μl universal protein block (Dako) was applied and allowed to incubate for 30 min at room temperature. Primary antibodies were diluted

accordingly in blocking buffer (1:5 universal protein block in 5% BSA buffer) and 35 µl were added to the appropriate chambers on the slide and incubated for over night at 4°C in the dark. Fifty µl of wash buffer (0.01 M Tween-20 in PBS) was applied to each section and allowed to incubate for 5 min at room temperature. The buffer was pipetted off and the step repeated. Secondary antibodies were diluted 1:600 in 0.01 M PBS, and 35 µl were added to appropriate sections and incubated for 30 min at room temperature in the dark. Washes were repeated and cover slips applied. Immunofluorescence was performed using a Zeiss LSM 510 Meta laser scanning confocal microscope (Zeiss Canada).

APPENDIX II

Additional Subject Information and demographics

Table A1: Disease duration and age of MS patients that were evaluated for serum and CSF anti-Nfasc antibody titres

Serum							
		Disease Duration (yrs)		Age at sample collection (yrs)		Anti-Nfasc antibody OD (450 nm)	
MS Subtype	n	Mean	Median	Mean	Median	Mean	Median
RRMS	49	5.1	3.4	38.2	42.5	0.275	0.162
PPMS	31	5.9	4.5	47.6	50.5	0.367	0.201
SPMS	24	9.4	7.25	38.4	46	0.423	0.325
Possible MS	15	4.4	1.2	40.5	44.5	0.173	0.149
OND	6	4.2	5.5	43.6	46.5	0.221	0.157
CSF							
RRMS	33	4.6	3	36.1	42.5	.199	0.1116
PPMS	14	5.9	4.5	46.2	47	0.074	0.0563
SPMS	14	12.2	9	38.5	42.5	0.143	0.0859
Possible MS	11	4.7	1.2	40	44	0.076	0.0758
OND	2	6.5	6.5	42.6	42.5	0.044	0.0251

Table includes data only for patients for whom disease duration history was available.

Table A2: Demographics of MS patients that underwent aHSCT

Patient ID	Sex	MS disease course	Age at aHSCT	Onset to BMT (months)
MS01	F	SPMS	30	74
MS02	M	SPMS	33	95
MS03	F	SPMS	26	121
MS04	M	SPMS	41	134
MS06	F	SPMS	27	99
MS08	M	SPMS	33	254
MS10	F	SPMS	38	68
MS13	F	SPMS	37	81
MS15	F	RRMS	43	65
MS16	M	RRMS	32	90
MS20	F	RRMS	30	67
MS24	F	RRMS	35	87
MS25	F	RRMS	24	64
MS27	F	RRMS	30	129
MS28	M	RRMS	25	21
MS29	F	RRMS	42	88
MS30	F	RRMS	33	50
MS31	M	RRMS	35	144
MS32	M	RRMS	39	62

Table A3: Demographics and CSF NfH concentration in OND controls

Inflammatory OND				
Patient ID	**Sex**	**Age**	**Diagnosis**	**NfH (pg/ml)**
934	F	42	Transverse myelitis	204.3
981	F	26	Transverse myelitis	263.18
1047	M	34	Transverse myelitis	221.52
1064	F	33	Transverse myelitis	194.5
1123	F	34	Transverse myelitis	167.34
1216	F	49	Transverse myelitis	197.26
1291	M	54	Optic neuropathy	255.28
1386	F	42	Virus	192.24
1468	F	33	Bilateral uveitis	192.54
1640	F	47	Neurosarcoidosis	285.62
Non-Inflammatory OND				
1212	F	36	Post-traumatic syndrome	206.06
1278	F	53	Hypertension	197.1
1281	F	41	Fibromyalgia	199.58
1306	F	54	Glioma	205.08
1310	F	36	Chronic Fatigue	195.56
1344	F	38	Headaches	175.9
1345	M	46	Pontine Disease	266.48
1452	M	43	Vertigo	207.34
1545	F	42	Migraine	256.5
1614	F	24	Migraine	203.82
1625	F	51	Migraine	197.86
1628	M	25	Stress, anxiety	183.6
1667	F	35	Stroke lesion	203.02
1688	F	47	Benign positional vertigo	197.4
1696	F	49	Idiopathic intracranial hypertension	314.12
1714	F	50	Migraine	202.08
1718	F	32	Migraine	189.42
1739	F	38	Graves' Disease	192.24

CURRICULUM VITAE

EDUCATION

University of Ottawa
PhD Biochemistry, Microbiology & Immunology **2005-2014**
Thesis: Discerning the mechanism of γδ T-cell mediated damage in MS: the potential role of antibodies in disease pathogenesis.

University of Guelph
MSc Pathobiology **2002-2005**
Thesis: A proteomic analysis of bovine neutrophils treated with Dexamethasone.

McMaster University
BSc Life Sciences **1998-2002**
Thesis: A comparison of altered defensin and collectin gene expression in healthy and pneumonic lungs of calves.

PEER-REVIEWED PUBLICATIONS

Beveridge JD, Mitchell GB and Caswell JL: Altered protein expression in neutrophils of calves treated with dexamethasone. Canadian Journal of Veterinary Research 2008, 72(3): 249-52.

Mitchell GB, Al-Haddawi MH, Clark ME, **Beveridge JD**, and Caswell JL: Effect of corticosteroids and neuropeptides on the expression of defensins in bovine tracheal epithelial cells. Infection and Immunity 2007, 75(3): 1325-34.

INVITED PRESENTATIONS

Freedman MS and **Black JD.** Anti-axonal damage in MS may be mediated by interaction of innate immunity and anti-axonal antibodies. Joint ACTRIMS-ECTRIMS Meeting. Inflammation and Neurodegeneration Session. Boston, USA, 2014.

Black JD, Arnold DL, Chen J, Bowman M, Atkins HL and Freedman MS. Axonal loss or damage is reversed by autologous hematopoietic stem cell transplantation, as evidenced by a reduction in cerebrospinal fluid neurofilament release. Neuroimmune Networks Platform Presentation. endMS Research and Training Network conference. Whistler, Canada, 2010

Beveridge JD, Mitchell GB, Clark ME and Caswell JL. A Proteomic Analysis of Bovine Neutrophils Treated With Dexamethasone. Proceedings of the 7th International Veterinary Immunology Symposium. Innate Immunology Workshop. Quebec City, Canada, 2004

INTERNAL PRESENTATIONS

Black JD and Freedman MS. The role of anti-neurofascin antibodies in γδ T-cell mediated neuro-axonal damage: a potential mechanism in the pathogenesis of multiple sclerosis. PhD Research Seminar, University of Ottawa, Ottawa, Canada, 2013

Black JD, Arnold DL, Chen J, Bowman M, Atkins HL and Freedman MS. Autologous hematopoietic stem cell transplantation (aHSCT) reverses axonal loss or damage in multiple sclerosis, as evidenced by a reduction in cerebrospinal fluid neurofilament release. Biochemistry, Microbiology & Immunology Graduate Student Seminar Symposium; Cell Death/Survival. Ottawa, Canada, 2011.

Beveridge JD and Freedman MS. The role of anti-neuronal antibodies in γδ T cell-mediated neuro-axonal damage: a potential mechanism in the pathogenesis of multiple sclerosis. Biochemistry, Microbiology & Immunology Graduate Student Seminar Symposium. Ottawa, Canada, 2009

Beveridge JD, Stys PK and Freedman MS. Immune and ionic mechanisms of white matter injury: studies using a novel *in vitro* model of MS. Biochemistry, Microbiology & Immunology Graduate Student Seminar Symposium. Ottawa, Canada, 2007

ABSTRACTS

Black JD, Arnold DL, Chen J, Bowman M, Atkins HL and Freedman MS. Axonal loss or damage is reversed by autologous hematopoietic stem cell transplantation, as evidenced by a reduction in cerebrospinal fluid neurofilament release. Ottawa Hospital Research Institute Research Day. Neuroscience. Ottawa, Canada, 2010

Black JD and Freedman MS. Neurofascin immunoreactivity in multiple sclerosis. 10th International Congress of Neuroimmunology. Immunological Mechanisms No. 356. Barcelona, Spain, 2010

Black JD and Freedman MS. Neurofascin immunoreactivity in multiple sclerosis. Biochemistry, Microbiology & Immunology Graduate Student Research Symposium. Ottawa, Canada, 2010

Black JD and Freedman MS. Neurofascin immunoreactivity in multiple sclerosis. European Committee for Treatment and Research in Multiple Sclerosis. Immunology No. 636. Düsseldorf, Germany, 2009

Beveridge JD, Stys PK and Freedman MS. The role of anti-neuronal antibodies in γδ T cell-mediated neuro-axonal damage: a potential mechanism in the pathogenesis of multiple sclerosis. Biochemistry, Microbiology & Immunology Graduate Student Research Symposium. Ottawa, Canada, 2008

Beveridge JD, Wang W, Murzenok P, Stys PK and Freedman MS. Immune and ionic mechanisms of white matter injury: studies using a novel *in vitro* model of MS. endMS Research and Training Network. Banff, Canada, 2007

Mitchell GB, Johnson R, Clark ME, **Beveridge JD** and Caswell JL. Corticosteroid-induced alterations in the bovine bronchoalveolar lavage proteome. FASEB Meeting, Experimental Biology. No. 9638. San Diego, USA, 2005

Beveridge JD, Mitchell GB, Clark ME and Caswell JL. A Proteomic Analysis of Bovine Neutrophils Treated With Dexamethasone. Proceedings of the 7th International Veterinary Immunology Symposium. No WK3.6.2. Quebec City, Canada, 2004

Mitchell GB**, Beveridge JD** and Caswell JL. Inhibition of lipopolysaccharide-induced β-defensin gene expression in corticosteroid-treated tracheal epithelial cells. FASEB Meeting, Experimental Biology. No. 9744. Washington, USA, 2004

Mitchell GB, **Beveridge JD** and Caswell JL. Inhibition of lipopolysaccharide-induced β-defensin gene expression in corticosteroid-treated tracheal epithelial cells. FASEB Meeting, Experimental Biology. Washington, USA, 2004

AWARDS

Joint ACTRIMS-ECTRIMS Meeting Educational Grant (declined)	**2014**
endMS Quebec-Ottawa Regional Research and Training Centre Travel Award	**2010**
University of Ottawa, Dept. of Microbiology & Immunology Travel Award	**2010**
International Society for Neuroimmunology Travel Award	**2010**
Biochemistry, Microbiology & Immunology Graduate Student Research Symposium (University of Ottawa) - winner in PhD category	**2010**
University of Ottawa, Dept. of Microbiology & Immunology Travel Award	**2009**
University of Ottawa, Faculty of Graduate and Postdoctoral Studies Travel Award	**2009**
Biochemistry, Microbiology & Immunology Graduate Student Research Symposium (University of Ottawa) – winner in PhD category	**2008**
University of Ottawa Excellence Scholarship	**2007, 2008, 2009, 2010**
Multiple Sclerosis Society of Canada Studentship	**2007, 2008, 2009, 2010**
University of Ottawa, Faculty of Graduate and Postdoctoral Studies Scholarship	**2006**
Lena Cook Memorial Scholarship	**2005**
D.G. Ingram Memorial Travel Scholarship	**2004**
University of Guelph Graduate Scholarship	**2003**

TEACHING ASSISTANTSHIPS

Introduction to Biochemistry **2010, 2011**
Senior Laboratory Demonstrator
Department of Biochemistry, University of Ottawa

Molecular Biology Laboratory **2009, 2010**
Teaching Assistant
Department of Biochemistry, University of Ottawa

Political and Economic Context of Healthcare **2006**
Teaching Assistant
School of Nursing, University of Ottawa

RELATED WORK EXPERIENCE

University of Guelph Animal Health Laboratory; Dr. Jan Shapiro
Post-Mortem Room Assistant **2001**
Assisted with necropsies and prepared tissues for histological examination.
Compiled a manual of Standard Operating Procedures.

CONTINUING PROFESSIONAL DEVELOPMENT

The Rising Tide of Emerging Infectious Diseases in an Immunocompromised World (symposium), The University of Ottawa Emerging Pathogens Research Centre, Ottawa, Canada, December 1, 2010

The biological basis of progressive MS, Serono Symposia International Foundation, Montreal, Canada, November 6, 2010

10th Course of the European School of Neuroimmunology, Event code 4674, Barcelona, Spain October 26, 2010

1st and 2nd Annual Brain Health Research Day, The University of Ottawa Brain and Mind Research Institute, Ottawa, Canada, June 2009, 2010

World Congress on Treatment and Research in Multiple Sclerosis, Montreal, Canada, September 17-20, 2008

Neuroscience 2007, The Society for Neuroscience, San Diego, USA, November 3-7, 2007

Ottawa Hospital Research Institute (OHRI) Research Day, Ottawa, Canada, 2006, 2007, 2008, 2009, 2010, 2011